Expression of microRNA in Alveolar Macrophages Deficient in PPARγ

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The nuclear transcription factor Peroxisome proliferator-activated receptor gamma (PPARγ) is a negative regulator of macrophage activation and inflammatory mediators. Alveolar macrophages of healthy individuals constitutively express PPARγ. Decreased activity and expression of PPARγ are observed in the alveolar macrophages from patients suffering from inflammatory conditions such as pulmonary alveolar proteinosis (PAP) and sarcoidosis. These finding suggest that PPARγ activity may have an integral role in maintaining lung homeostasis. This study tested the hypothesis that microRNA expression would be dysregulated in murine alveolar macrophages deficient in PPARγ. microRNA (miR) are small non-coding RNA molecules that post-transcriptionally regulate the expression of messenger RNA.

Evaluation of microRNA in the murine model of PAP, the GM-CSF-KO mouse, demonstrates the elevation of miR-27a and miR-27b which target PPARγ. The deficiency of PPARγ and the lipid transporters ABCA1 and ABCG1 have been shown to contribute to the pathology of PAP. The microRNA miR-33-3p and miR-33-5p, which target these lipid transporters, were also elevated in GM-CSF-KO mice. Pulmonary granulomas comparable to those observed in pulmonary sarcoidosis are induced by

instillation of multiwall carbon nanotubes (MWCNT) in C57Bl/6 mice. These animals have decreased PPARγ activity and show elevated expression of miR-27a and miR-27b. It was also observed that the expression of the transporters ABCA1 and ABCG1 were decreased in MWCNT instilled mice. Expression of miR-33-3p and miR-33-5p was elevated in MWCNT instilled animals. The expression of microRNA that affects the activity of NF-κB is also elevated in both murine models.

We next investigated the use of PPARγ agonist rosiglitazone on the expression of microRNA and messenger RNA. The use of rosiglitazone altered the expression of microRNA in both GM-CSF-KO and C57Bl/6+MWCNT mice. Rosiglitazone treatment altered the expression of the lipid transporter ABCA1and ABCG1 in C57Bl/6+MWCNT mice. The elevation of proinflammatory cytokines was also observed.

Taken together, these observations support the hypothesis that PPARγ activity effects the microRNA and gene expression in alveolar macrophages which is critical to overall lung homeostasis. Understanding the relationship between PPARγ and microRNA in alveolar macrophage biology will provide insight into the regulation of the lung environment and possible therapeutic targets.

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List of Abbreviations

ABC: ATP-binding cassette

BAL: bronchoalveolar lavage

C: Celsius

cDNA: complementary deoxyribonucleic acid

CO_2: carbon dioxide

CT: cycle threshold

DNA: deoxyribonucleic acid

FBS: fetal bovine serum

GAPDH: glyceraldehyde 3 phosphate dehydrogenase

GM-CSF: granulocyte-macrophage colony-stimulating factor

GM-CSF KO: granulocyte-macrophage colony-stimulating factor knockout

IFNγ: interferon-gamma

IP: Intraperitoneal

Kg: kilogram

KO: knockout

LPS: lipopolysaccharide

M: molar

mL: milliliter

miRNA: microRNA

mRNA: messenger ribonucleic acid

ng: nanogram

nm: nanometer

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

PAP: pulmonary alveolar proteinosis

PBS: phosphate buffered saline

PPAR: peroxisome proliferator-activated receptor

PPARγ- KO: peroxisome proliferator-activated receptor-gamma knockout

PPRE: peroxisome proliferator-activated receptor response element

Rosi: rosiglitazone

RNA: ribonucleic acid

rpm: revolutions per minute

RT-PCR: real time-polymerase chain reaction

SEM: standard error mean

Th-1: Type 1 T helper cell

μg: microgram

μL: microliter

Chapter1: Introduction

1.1 Alveolar Macrophage:

For more than 100 years macrophages have been defined by their phagocytic abilities. In his original descriptions of innate immunity, Metchnikoff credited phagocytes with influencing development, ensuring homeostasis, and protecting the host from infection [1]. Macrophages are distributed throughout the body and named according to the tissue in which they reside. Alveolar macrophages are derived from circulating monocytes or from the present self-renewing alveolar macrophage population in the lung [2,3]. The unique lung environment with the presence of oxygen and surfactant plays a prominent role in shaping the phenotype of the alveolar macrophage. These features can be reproduced when either peritoneal macrophages or bone marrow macrophages are transferred into the lung [2].

1.2 GM-CSF-Knockout Mouse

Granulocyte macrophage-colony stimulating factor (GM-CSF) was initially thought to only be a hematopoietic growth factor but surprisingly when GM-CSF is disrupted in a murine model no hematopoietic abnormalities were detected. GM-CSF knockout mice (GM-CSF-KO) have lung pathology similar to that of the human disease pulmonary alveolar proteinosis (PAP) [4,5]. In the GM-CSF-KO mouse surfactant builds up in the lung and lipid laden alveolar macrophages mirror those observed in patients afflicted with PAP [4]. When given exogenous

GMCSF therapy, the lung pathology resolves in both PAP patients and the GM-CSF-KO mice [6,7].

In an effort to determine a mechanism in which GM-CSF regulates both the overall lung environment and the alveolar macrophage, Thomassen et al. evaluated the expression of peroxisome proliferator-activated receptor gamma (PPARγ). PPAR-γ which is regulated by GM-CSF, is known to regulate lipid and glucose metabolism as well as inflammatory genes [3]. A potential pathway for GM-CSF mediated lung homeostasis through the regulation of PPARγ was proposed [8].

Evaluation of PPARγ in the alveolar macrophages from both PAP patients and the GM-CSF-KO model revealed a deficiency of both mRNA and nuclear translocation of the protein [6]. Other lipid metabolic genes found to be dysregulated in both PAP patients and GM-CSF-KO mice were lipid transporters ABCA1 and ABCG1 are upregulated and downregulated, respectively [6]. When patients were treated with exogenous GM-CSF, Bonfield et al. reported an increase in PPARγ expression to control levels [8]. Correction of ABCG1 deficiency was observed in primary alveolar macrophages from PAP patients and GM-CSF-KO mice cultured with GM-CSF, or from PAP patients receiving GM-CSF therapy [6]. Thomassen et al. verified the pivotal role of PPARγ in this system by utilizing a lenti-viral-PPARγ transduction system in primary human alveolar macrophages *in vitro*; the expression of ABCG1 was significantly up regulated compared to lenti-eGFP control cultures [6]. Human alveolar

macrophages from PAP patients cultured with the selective PPARγ ligand, Rosiglitazone, also exhibited increased gene expression of ABCG1 [6]. These data demonstrate the upstream regulation of PPARγ by extracellular GM-CSF as well as the importance of PPARγ to alveolar macrophage homeostasis.

1.3 Peroxisome Proliferator-Activated Receptor-gamma:

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear-receptor superfamily, reviewed by Smith et al [9]. There are three PPAR isoforms: PPAR-α, PPAR-β/δ, and PPAR-γ. The PPARs are transcribed from unique genes but share a common protein structure [10]. Each of the transcription factors work in a unique fashion with varying tissue distribution, reviewed by Tontonoz et al [9,11]. PPARα was first identified to induce proliferation of peroxisomes in the liver [9]. PPAR β/δ is prevalent in most tissues of the body and is involved in lipid oxidation and keratinocyte differentiation [9] [10]. PPARγ is typically associated with adipocytes, for its role in lipid and glucose metabolism, but also acts as a negative regulator of pro-inflammatory mediators [9] [10].

PPARs regulate gene expression by several different mechanisms, including the inhibition of inflammatory mediators, prevention of transcription, and regulation of PPARγ response elements [12,13]. PPAR's can modulate the inflammatory response by sequestrating the coactivators of major inflammatory regulators including NF-κB, AP-1, and STAT-1, this mechanism is termed transrepression [14-16]. In combination with other proteins, PPARγ forms

complexes which prevent transcriptional activation of inflammatory response genes in macrophages (Figure 1:b) [12]. These effects are promoted by PPARγ specific agonists which include rosiglitazone. PPARγ ligands, when bound, also promote the localization to PPARγ-response elements which promote or prevent the expression of target genes (Figure1: a) [13]. A single ligand can inhibit the transcription of particular genes while promoting the expression of others.

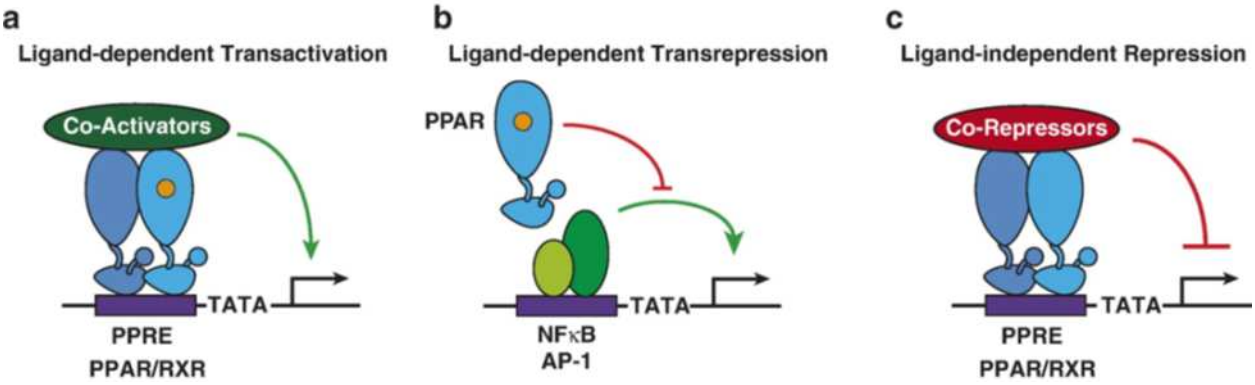

Figure 1: Transcriptional activities of the peroxisome proliferator-activated receptors. PPARs can both activate and inhibit gene expression. (a) Ligand-dependent transactivation. PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR-response elements (PPRE) in target genes as heterodimers with RXR. Binding of agonists ligand leads to the recruitment of coactivator complexes that modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter. (b) Ligand-dependent transrepression. PPARs repress transcription in a ligand-dependent manner by antagonizing the actions of other transcription factors (yellow), such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). (c) ligand-independent repression. PPARs bind to response elements in the absence of ligand and recruit corepressor complexes that mediate active repression. This complex antagonizes the actions of coactivators and maintains genes in a repressed state in the absence of ligand. (Adapted from Ricote et al 2007 [13]).

To investigate the specific role of PPARγ in lung homeostasis, Malur et al utilized a conditional PPARγ-knockout murine model (PPARγ–KO) The strain is deficient of PPARγ in myeloid cells which include macrophages and neutrophils. In the PPARγ-KO model GM-CSF is active and its expression is unchanged from that of C57/Bl6 mice [17]. The PPARγ-KO mice exhibit lipid loaded "foamy" alveolar macrophages that are similar but have less lipid accumulation than the GM-CSF-KO alveolar macrophage. Evaluation of the lipid transporters ABCA1 and ABCG1 showed dysregulation matching that of pulmonary alveolar proteinosis patients and GM-CSF-KO mice: i.e. the upregulation of ABCA1 and downregulation of ABCG1 [18]. The use of a lenti-viral expression vector (lenti-PPARγ) to restore PPARγ in the alveolar macrophages reversed the down regulation of ABCG1 as well as other cholesterol efflux genes [18,19].

The pulmonary response in the PPARγ knockout model was characterized as a TH-1 associated inflammatory response by Malur et al [20]. It was observed that in the absence of PPARγ, Interferon-γ (IFN-γ) levels were elevated and led to an increase in inducible nitric oxide synthase (iNOS) in the alveolar macrophages of PPARγ-KO mice. Total leukocytes were increased in the PPARγ-KO mice and the percentage of lymphocytes was elevated compared to C57/Bl6 controls with a predominance of $CD8^+$ versus $CD4^+$ T cells. Evaluation of TH-1 associated cytokines in addition to IFN-γ revealed increased production of IP-10 (CXCL10), MIP-1α, and IL-12 (p40) in BAL cells and BAL fluid collected from the PPARγ-KO mice [20].

To confirm that the absence of PPARγ promoted an inflammatory phenotype in alveolar macrophages, BAL cells from C57/Bl6 wild type animals were cultured with PPARγ specific antagonist GW9662. *Ex vivo* macrophages cultured with GW9662 showed increased IFNγ and iNOS expression and, as expected, decreased PPARγ expression. IFNγ or iNOS could not be detected in control cultures. Immunocytochemistry confirmed these data showing an inverse relationship between PPARγ and IFNγ and iNOS in GW9662 positive or control cultures [20]. Interestingly when the alveolar macrophages from PPARγ-KO mice were cultured with neutralizing anti-IFNγ for 24 hours, iNOS expression was significantly decreased. Malur et al. postulated that PPARγ antagonizes the expression of IFNγ thereby preventing the activation of iNOS, thus controlling nitric oxide expression in the lung [20].

1.4 Sarcoidosis and PPARγ Deficiency

Sarcoidosis is a multisystem disease characterized by the presence non-necrotizing granulomas and more than 90 percent of afflicted individuals have pulmonary involvement [21]. Diagnosis of sarcoidosis is made by eliminating other possible causes. Clinical radiographic findings can suggest sarcoidosis but require biopsy-proven histopathology and the exclusion of infectious granulomatous diseases for a valid diagnosis of sarcoidosis [22].

Contributing to the hypothesis that PPARγ contributes to overall lung homeostasis is the observation that PPARγ expression is decreased in pulmonary sarcoidosis, and the disease can be characterized as an ongoing TH1

inflammatory phenotype [21, 22]. Culver et al collected BAL samples from eight patients with active sarcoidosis and nine healthy volunteers. It was observed that PPARγ gene expression, binding activity, and nuclear localization were decreased in patients with active sarcoidosis compared to normal controls. The group also noted an increase in NF-κB binding activity in the same patients evaluated for the PPARγ [21]. In addition to NF-κB, the expression of IFNγ was increased. [21,22].

Studying the etiology and progression of sarcoidosis has been a challenging research question. Difficulties have been noted when eliciting granuloma formation in animal models. Most of these models involve intravenous injection of antigen-bound sepharose beads [23,24]. The major drawback of these models is the rapid resolution of the granuloma structure (14-21 days). This feature makes it difficult to distinguish the effects of the initial injury from the resolution of the granuloma [25]. The observation that nanomaterials produced granuloma like lesions in the lung of animal models prompted Huizar et al. to investigate the use of multi wall carbon nanotubes (MWCNT) to produce a chronic model of pulmonary granulomatous disease [26-28]

1.5 Carbon Nanotubes

Nanomaterials represent a broad range of particles that are characterized by their extremely small size, with at least one physical dimension less than 100nm. Carbon nanomaterials can be the byproduct of combustion and can be produced from wood or gas burning stoves or products of engine exhaust [26].

Postmortem examination of the lungs of firefighters has shown the presence of granulomas [29,30]. Carbon nanotubes can be found as two types, single walled or multi walled. As the name implies, multiwall carbon nanotube have several concentric layers of carbon compared to the single walled carbon nanotubes.

Intratracheal instillation of MWCNT in C57/Bl6 mice yielded well-formed granulomas persistent up to 90 days post instillation. It was also observed through the use of immunocytochemistry that macrophages and $CD3^+$ T-cells localized to the site of the granuloma. The elevation of inflammatory cytokines was also found in MWCNT instilled animals including CCL2 and IL-10 [25]. A consecutive paper published by Huizar et al demonstrated the reduction in mRNA, nuclear localization, and binding activity of PPARγ in wild-type mice instilled with MWCNT [31]. Instillation of MWCNT in PPARγ KO mice exacerbates granuloma formation and elevation of inflammatory cytokines further, compared to C57Bl/6 mice instilled with MWCNT [31]. In another study, alveolar macrophages from MWCNT instilled C57Bl/6 mice were evaluated for gene expression characteristic of an M1 or M2 macrophage activation phenotype. Elevation of M1 associated genes was observed while M2 associated genes were not significantly different or were downregulated with the exception of CCL2 and IL-10 which had been previously reported [25,32]. The observations in the MWCNT model corresponds with the PPARγ deficiency and granulomatous histopathology observed in sarcoidosis.

1.6 microRNAs – Potential Regulators in Pulmonary Disease

The role of microRNA in chronic granulomatous disease or alveolar macrophage PPARγ deficiency has not been clarified. microRNA are small noncoding RNA that provide post transcriptional gene regulation by blocking translation or destabilizing their target messenger RNA (mRNA). microRNA are traditionally transcribed by RNA polymerase II from non-coding regions or are introns of protein coding genes [33]. Transcribed microRNA molecules fold back onto themselves forming a distinct hairpin loop termed a pri-miRNA. pri-miRNA's are cleaved by the enzyme Drosha into a shorter ~70 nucleotide hairpin and exported into the cytoplasm, the microRNA are now termed pre-miRNA. Further cleavage by the enzyme Dicer yields a shorter miRNA/miRNA duplex which consist of two microRNA [33]. The two microRNA are distinguished by their original orientation on the DNA. One side of the microRNA duplex will be incorporated into the miRNA-induced silencing complex (mRISC). The mRISC is a large protein complex which incorporates a mature microRNA sequence which provides a guide to target the appropriate mRNA for silencing or degradation. Position of Drosha and Dicer cleavage determines the mature sequence of the mature microRNA and could determine the side of the duplex to be incorporated to mRISC [33].

microRNA nomenclature consist of an identification number which are assigned in order of discovery, miR-23 was predicted or confirmed before miR-199. Some microRNA are transcribed from two locations in the genome and share a common mature sequence, these are identified by a letter following the

identification number, such as miR-27a and miR-27b. Based on the original orientation to the RNA, each mature microRNA has two sides to its stem loop designated by a 3p or 5p and are distinct microRNA. miR-27a-3p and miR-27a-5p are located beside one another in the genome but have unique seed regions and sequences, some of which overlap as a stem loop. During processing microRNA can be analyzed at varying states of maturity and have unique prefixes that were highlighted above and are written with a lower case ‘r’. In order of maturity miR-27a-3p would be listed: pri-mir-27a-3p, pre-mir-27a-3p, and finally the mature miR-27a-3p

Incorporation into an mRISC allows microRNA to become functional however the mechanism of targeting mRNA has only began to be delineated in the past several years. The mRNA targeted for degradation is determined by several factors, the first of which is the seed region. The microRNA seed region refers to base pairs 2-7 on the mature microRNA strand. Identification of complimentary sequence in the mRNA 3’ untranslated region and using those with orthologs from other species greatly increases target prediction [34]. Other factors concerning the remaining ~15 nucleotides may contribute to stability, mRNA selection or mRISC incorporation but there is not a consensus on the functions of the entire microRNA [34].

microRNA have been shown to be important effectors of the inflammatory process [35-37]. The direct regulation of microRNA by inflammatory mediators including NF-κB is involved in inhibitory loops providing possible feedback mechanisms [37]. Crouser et al conducted microarray analysis of lung tissue

and peripheral mononuclear cells from patients suffering from pulmonary sarcoidosis and observed an increase in microRNAs targeting the TGFβ pathway [35]. TGFβ is produced by alveolar macrophages and is thought to suppress T-cell activation [3]. Examination of microRNAs potentially affecting PPARγ, however, was not done, and bronchoalveolar lavage cell preparations were not studied.

1.7 Hypothesis

The hypothesis underlying this thesis is that alterations in microRNA expression are involved with alterations in alveolar macrophage PPARγ expression. To study this hypothesis, experiments will be designed to examine microRNA in mice with: a macrophage-specific PPARγ knockout, PPARγ deficiency due to GM-CSF knockout, and PPARγ deficiency due to the presence of inflammatory granulomatous disease (the MWCNT granuloma model). The results and discussion of this thesis will be presented in two parts. In chapter 3, studies characterizing the PPARγ-KO and GM-CSF-KO will be presented and discussed. In chapter 4 the studies with the model of chronic granulomatous disease will be presented and discussed.

Chapter 2: Materials and Methods

2.1 Mice*:* All studies were conducted with approval from East Carolina University Institutional Animal Care Committee (AUP#J185c and #J199a) Evaluation of basal microRNA expression from the three strains: C57Bl/6, PPARγ-KO, and GM-CSF-KO mice were conducted with animals 8-12 weeks of age with gender matched controls. C57Bl/6 wild type mice were purchased from Jackson Laboratories and set up as breeding pairs at 7 weeks of age. C57Bl/6 breeders were replaced approximately every six months. The pups of these C57Bl/6 breeding pairs were used for consecutive experiments as control animals. Macrophage specific PPARγ-KO mice are deficient of PPARγ in cells of the myeloid lineage and have been described previously [20]. Homozygous floxed PPARγ mice (flox +/+) were obtained from Dr. F.J. Gonzalez at the National Institute of Health, Bethesda, MD. These mice were crossed with transgenic animals obtained from Jackson Laboratories with the CRE gene under the control of the murine lysozyme M gene (cre+/+). Mice were genotyped and mated to produce $flox^{+/+}/cre^{+/+}$ animals. Those mice possessing either $flox^{-/-}/cre^{+/+}$ or $flox^{+/+}/cre^{-/-}$ did not show any difference from C57Bl/6 controls in initial experiments by Malur et al [20]. Mice from the $flox^{+/+}/cre^{+/+}$ lineage are used in these experiments as PPARγ-KO mice. GM-CSF-KO mice were previously attained from the lab of Dr. G Dranoff. The GMCSF-KO mouse was produced by homologous recombination in embryonic stem cells [4]. Both PPARγ-KO and GM-CSF-KO mice are on C57Bl/6 backgrounds and are compared to C57BL/6 controls as described from Jackson Labs.

2.2 Instillation of Multi Wall Carbon Nanotubes: Multi Wall Carbon Nanotubes (MWCNT) were suspended in 35% INFASURF(calfactant), in phosphate buffered saline (PBS) at a concentration of 100μg/ 50μL. 35% surfactant and MWCNT were sonicated in a water bath for 5 minutes and checked visually for any remaining large particles. Mice were sedated with isofluorane in a chamber until unresponsive. Oropharyngeal instillation of MWCNT were performed by pulling the tongue forward with forceps and instilling 50μL-volume of MWCNT suspension into the airway. The tongue was held out while the MWCNT were aspirated into the lung and released as the animal began to show signs of sensation. Retaining the tongue outside the oral cavity ensures the MWCNT's are aspirated and not swallowed. All procedures were conducted in accordance with East Carolina University Office of Environmental Health and Safety within a HEPA filtered laminar flow hood.

2.3 Bronchoalveolar Lavage (BAL): BAL cells were collected from animals euthanized with Ketamine (90mg/kg) and Xylazine (10mg/kg) injected intraperitoneal. Animals were placed in a supine position and the trachea was exposed and cannulated with plastic tubing (PlasticsOne) on a 20G needle. Whole lung lavage was conducted with warmed 20% lidocaine in PBS at 1mL aliquots X 5 for a total or 5mL and collected on ice. Collections were spun at 1800 RPM for 10 minutes at 4^{o}C to yield a cell pellet. Cell pellets were resuspended in 2mL cold PBS and cell count was performed with 10μL cell

suspension on hemocytometer and differential cell count were obtained from cytospins with 150μL cell suspension stained with modified Wright's stain. BAL cell differentials for all animals used in the following experiments had >90% macrophages. Cytospin preparations were also evaluated for any significant epithelial cell contamination that may have sloughed off during BAL collection or resulted from the insertion of the tubing into the trachea. Cell pellets were washed again in cold PBS, supernatant was removed and pellet was stored at -80^{o}C.

2.4 RNA Purification and Quantification: Total RNA was collected from BAL cell pellets using the miRNeasy Micro Kit (Qiagen) according to manufactures protocol. Total RNA (mRNA and microRNA) was eluted in 30μL water. Quantification was conducted with Nanodrop 1000 (Thermo scientific).

Reverse transcription of messenger RNA (mRNA) was performed with the RT2 First Strand kit (Qiagen) to produce cDNA for protein coding genes. mRNA quantification was conducted with Real-Time Polymerase Chain Reaction (RT-PCR) using with RT2 SYBR Green/ROX qPCR Master Mix (Qiagen). Relative quantification of mRNA cycle threshold values (CT) were compared to those of the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method [38].

microRNA were reverse transcribed utilizing the miScript II RT Kit (Qiagen). The miScript kit has two separate buffers hiFlex, which amplifies all

RNA's and hiSpec, which selectively amplifies small RNA's. Utilizing the hiSpec buffer, long RNA strands such as mRNA are repressed, and small noncoding RNA's such as miRNA and small nuclear RNA(snorRNA) are polyadenylated. After addition of the poly-A tail, small RNA's are reversed transcribed. Both messenger RNA (mRNA) and microRNA were quantified using RT-PCR. microRNA were quantified with miScript SYBR Green PCR Kit (Qiagen). Relative quantification of microRNA CT values was compared to those of SNOR68, a small nuclear RNA. dCT values were calculated as described above and used for further analysis.

The amount of total RNA was used to standardize the amount of microRNA that is needed for down-stream applications. For both mRNA and microRNA, 300-500ng of total RNA was converted with the appropriate cDNA kit and assumed that a 1:1 RNA:cDNA conversion took place. For downstream analysis, 2ng of cDNA was used to standardize the RT-PCR reactions further.

2.5 Cell Culture Assays: BAL collections were pooled from animals of the same strain to get enough cells for *in vitro* culture. BAL cells were collected as described with the exception that cells were kept at room temperature during collection, to prevent loss of cell adhesion. Medium was warmed in a 37^{o}C water bath and used to wash BAL cells two times and cell count was determined by hemocytometer. Medium consisted of RPMI medium supplemented with 10% fetal bovine serum and 1% PSG (pen-strep-glutamine). BAL cells were plated at

$1.2x10^6$ cells/well in a 12 well plate. Cells were incubated at 37°C (5% CO2) for two hours then washed twice by adding 1mL media further. Cells were then allowed to adhere for twenty-four hours prior to adding stimulus.

Stimuli were diluted as follows: Lipopolysaccharides from *salmonella enterica* serotype typhimurium (LPS) (Sigma Aldrich). LPS was suspended and diluted to a concentration of 1.0 mg/mL in sterile PBS. Prior to use, LPS was diluted to 0.1μg/mL in cell culture medium and 1mL was added in appropriate well. Rosiglitazone (Rosi) and GW9662 (GW) (Cayman Chemicals) were resuspended in 1:1 DMSO:PBS mixture to a concentration of 10mM and stored at -20°C. Prior to use Rosi and GW were diluted according to manufacturer's instructions to 10μM concentration for stimulation. Cell cultures were stimulated with medium alone, LPS, Rosi, GW, LPS+Rosi, or GW+Rosi. Stimulus were added for either 6 or 24 hours at which time total RNA was collection as previously described. RNA was reverse transcribed with the respective kits for mRNA and microRNA and RT-PCR with the appropriate reagents as described above.

2.6 Rosiglitazone I.P. Treatment: The rosiglitazone as described above was dissolved in DMSO:PBS at a 1:1 ratio at a concentration of 25mg/mL. Aliquots of 180uL was stored at -20°C and resuspended in PBS to 1.7 mL to bring the concentration to 0.08μg/μL. Mice age 8-10 weeks were weighed on day one and given 3.0mg/kg body weight ROSI via intraperitoneal (IP) injections. Injections were performed in four locations, two on each side of the animal

rotating in a clockwise manner. Rosi was administered 10 consecutive days prior to BAL collection.

2.7 Statistical Analysis: Individual BAL samples collected or those data from cell culture experiments were analyzed by student's t-test using Prizm software (GraphPad, Inc., San Diego, CA.) Values from control animals were compared to treated group and a difference of $p \leq 0.05$ was considered significant. Data are shown as mean ± SEM.

Chapter 3: Evaluation of microRNA Expression in GM-CSF and PPARγ Knockout Mice

Results:

3.1 miRNA-27b-3p is upregulated in alveolar macrophages from PPARγ-KO and GM-CSF-KO mice

microRNA miR-27a-3p and miR-27b-3p have been shown to downregulate PPARγ mRNA and may be promoted by inflammation [39]. Alveolar macrophages from C57/Bl6, PPARγ-KO, and GM-CSF-KO mice were evaluated (Figure 2). miR-27a-3p expression was not significantly different in the PPARγ-KO mice but was significantly increased 1.4 fold in the GM-CSF-KO, compared to C57/Bl6 controls. However, miR-27b-3p was significantly elevated in both models, increasing 2.9 fold in the PPARγ-KO mice and 4.2 fold in the GM-CSF-KO mice. These results suggest that miR-27b-3p may be involved in the regulation of PPARγ.

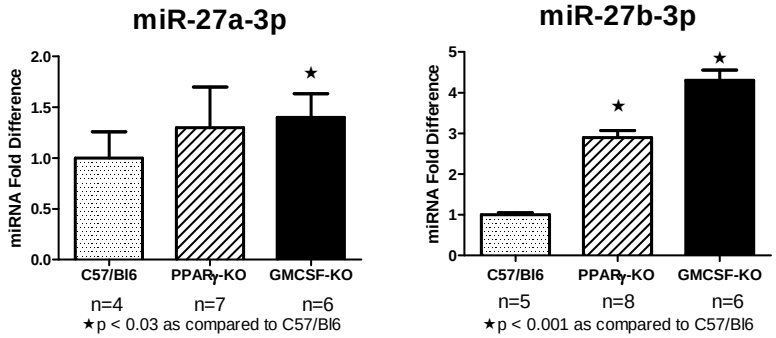

Figure 2: Expression of microRNAs targeting PPARγ are up regulated in the alveolar macrophages from PPARγ-KO and GMCSF-KO mice. Graphs represent mean fold change ±SEM compared to C57/Bl6 control animals.

3.2 miRNAs associated with NF-κB regulation are upregulated in alveolar macrophages from PPARγ-KO and GM-CSF-KO mice

Both PPARγ-KO and GM-CSF-KO models show elevated NF-κB activity in the alveolar macrophage compared to C57Bl/6 control animals (unpublished data). The expression of microRNAs which antagonize the activity of NF-κB (miR-23b-3p, miR-146a-5p, and miR-199a-3p) [39,40] were evaluated (Figure 3). The upregulation of these microRNA may provide a negative feedback loop for NF-κB [39,40]. The expression of miR-23b-3p was elevated 6.9 fold in PPARγ-KO alveolar macrophages, and 12 fold in the BAL cells of GM-CSF-KO mice. The expression of miR-146a increased 7.4 fold in the PPARγ-KO and 45.3 fold in the GM-CSF-KO BAL cells. The expression of miR-199a was elevated 3.7 fold in PPARγ-KO BAL cells and 27.9 fold in GM-CSF-KO BAL cells. Thus all of the microRNA evaluated which are associated with NF-κB regulation were upregulated in mice with deficient PPARγ.

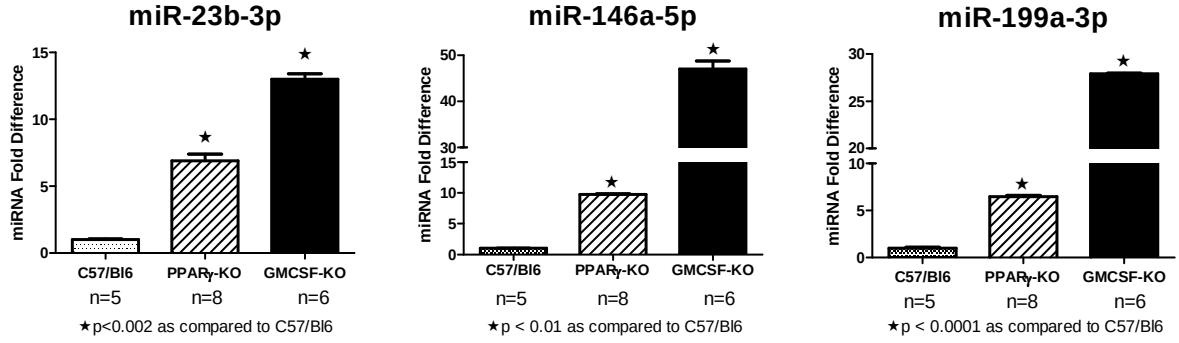

Figure 3: Expression of microRNA associated with NF-κB activity was evaluated in alveolar macrophages from C57Bl/6, PPARγ-KO and GM-CSF-KO mice. Graphs represent mean fold change compared to C57/Bl6 control animals.

3.3 miRNA targeting ABC Transporters in alveolar macrophages from PPARγ-KO and GM-CSF-KO mice are upregulated

Previous studies have shown the dysregulation of ABCA1 and ABCG1 lipid transporters contributes to lipid loading of alveolar macrophages from PPARγ-KO and GM-CSF-KO mice [6,19]. PPARγ has been shown to regulate ABCG1 expression [18]. Recently miR-33 has been associated with the regulation of ABCA1 and ABCG1 [41,42], but the status of these microRNAs in alveolar macrophages is unknown. Because of the possible involvement in lipid metabolism, miR-33-3p and miR-33-5p were evaluated (Figure 4). The alveolar macrophages from PPARγ-KO mice show upregulation of miR-33-3p (26 fold) and miR-33-5p (8.7 fold). GM-CSF-KO alveolar macrophages show an increase in miR-33-3p (2.5 fold). miR-33-3p and miR-33-5p were strongly upregulated in the alveolar macrophages from PPARγ-KO but surprisingly only miR-33-3p was upregulated in the GM-CSF-KO mice.

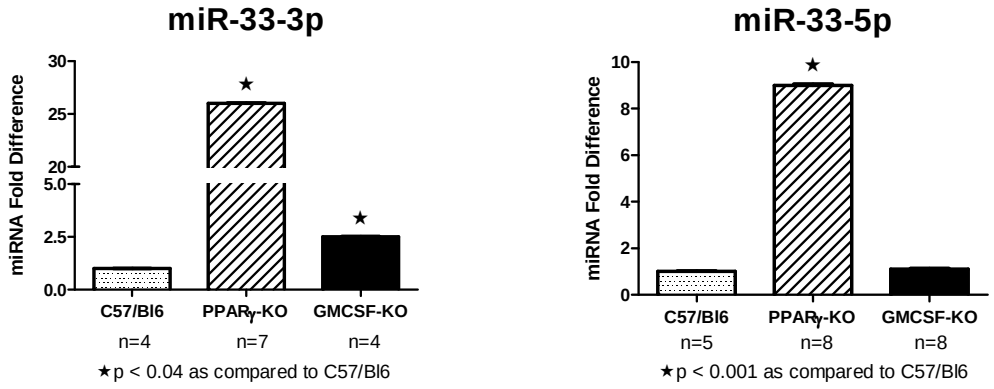

Figure 4: miR-33-3p and miR-33-5p expression in GM-CSF-KO and PPARγ-KO was evaluated by RT-PCR in alveolar macrophages. Graphs represent mean fold change compared to C57/Bl6 control animals.

3.4 *In vivo* rosiglitazone treatment effects microRNA expression in GM-CSF-KO mice.

We hypothesized that residual PPARγ may be present in the alveolar macrophages of the GM-CSF-KO mice and treatment with rosiglitazone (a synthetic ligand for PPARγ) may alter microRNA, gene expression, and enhance lipid clearance from alveolar macrophages. We evaluated the expression of miR-27a-3p and miR-27b-3p after rosiglitazone treatment (Figure 5). miR-27b-3p expression was 60% less than that of the vehicle control group. The alveolar macrophages from these animals were collected and stained with modified Wrights stain. No difference in the foamy morphology of the alveolar macrophages was observed (n=7/treatment) suggesting no decrease in lipid loading. Neither PPARγ mRNA expression nor miR-27a-3p was altered in the rosiglitazone treated mice compared to vehicle controls.

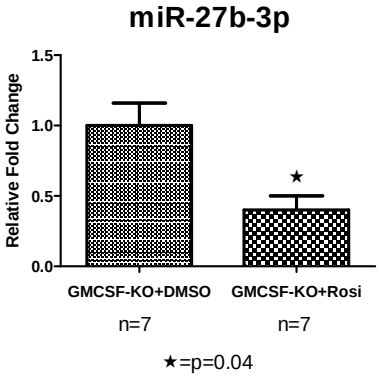

Figure 5: Expression of miR-27b-3p in rosiglitazone treated GM-CSF-KO is suppressed in alveolar macrophages. Graphs represent mean fold change compared to vehicle control animals.

3.5 *In vivo* rosiglitazone treatment does not affect lipid transporters or microRNA associated with NF-κB activity in GM-CSF-KO mice

The expression of lipid transporters ABCA1 and ABCG1 have been shown to be dysregulated in the GM-CSF-KO mouse and play a role in regulating cholesterol efflux [18]. No significant difference was observed between rosiglitazone treated mice and controls in either ABCA1 or ABCG1 expression in alveolar macrophages. The expression of miR-33-3p and miR-33-5p, which target ABCA1 and ABCG1 is not affected by rosiglitazone treatment. No differences were observed in miR-23b-3p, miR-146a-5p, or miR-199a-3p, which effect NF-κB expression.

3.6 *In vivo* rosiglitazone treatment effects microRNA expression in PPARγ-KO mice.

As a negative control for the rosiglitazone experiments conducted with GM-CSF-KO mice the PPARγ-KO mice were treated with rosiglitazone due to the lack of functional PPARγ. The expression of miR-33-5p was up regulated 24 fold ($p<0.001$) (Figure 6). The expression of miR-27a-3p, miR-27b-3p, miR-33-3p, miR-146a-5p, and 199a-3p is not statistically different. ABCA1 and ABCG1 were also evaluated in the PPARγ-KO mice treated with rosiglitazone and no difference was observed compared to the control group.

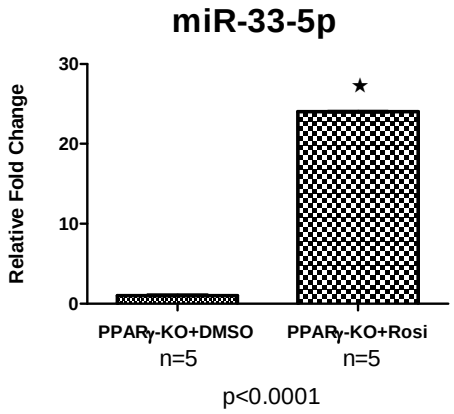

Figure 6: PPARγ-KO mice treated with rosiglitazone demonstrate a 24 fold increase in miR-33-5p expression. Graphs represent mean fold change compared to vehicle control animals.

The data collected from the PPARγ and GM-CSF knockout animals suggested that a deficiency of PPARγ expression in the alveolar macrophage effects the microRNA expression of these cells. The use of the PPARγ specific agonist rosiglitazone effects microRNA and gene expression in the alveolar macrophages from these knockout models. To further investigate the mechanism in which PPARγ effects microRNA expression in alveolar macrophages, we performed in vitro stimulation with LPS. Stimulation with LPS is known to downregulate the expression and activity of PPARγ. The costimulation with known PPARγ agonist rosiglitazone and antagonist GW9662 may provide insight into the specific regulation of microRNA in activated macrophages. We hypothesized that microRNA expression would mirror that observed in the PPARγ-KO and GM-CSF-KO mice.

3.7 Regulation of miR-146-5p in alveolar macrophages *in vitro*

The observation that elevated miR-146a-5p was associated with PPARγ deficiency in the PPARγ and GMCSF knockout mice led to the hypothesis that downregulation of PPARγ activity in wild-type C57/Bl6 alveolar macrophages would alter microRNA expression.

At 6 and 24 hour time points, neither ROSI nor GW9662 alone showed any change in miR-146a-5p compared to unstimulated (US) controls as anticipated. In contrast, at both 6 and 24 hours, LPS significantly stimulated miR-146-5p. Addition of ROSI to LPS reduced miR-146a-5p levels at 24 hours. Cultures with GW9662 and LPS had no effect on miR-146a-5p levels. These results suggest that PPARγ deficiency (as has been shown with LPS stimulation) elevated miR-146a-5p while PPARγ activation reduces expression (Figure 7). No difference was observed in the expression of miR-27a-3p, miR-27b-3p, miR-33-3p, miR-33-5p, or miR-199a-3p.

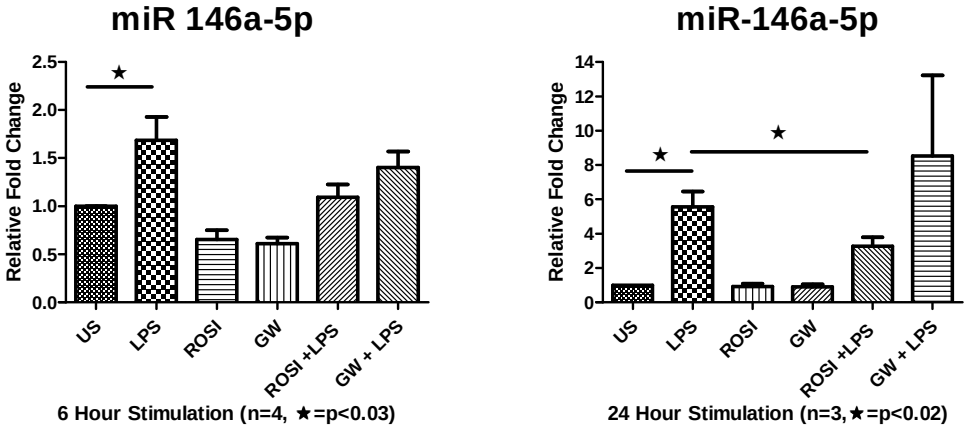

Figure 7: Expression of microRNA 146a-5p in C57Bl/6 alveolar macrophages cultured with LPS, ROSI (PPARγ agonist), or GW9662 (PPARγ antagonist) for 6 or 24 hours. Graph represents fold change compared to unstimulated controls or to LPS alone.

Summary and Discussion: Chapter 2

This study is the first to evaluate the microRNA expression in alveolar macrophages with PPARγ deficiency. microRNAs associated with inflammation, the regulation of PPARγ, and control of lipid transporters were evaluated. Alveolar macrophages from the GM-CSF-KO and PPARγ-KO mice have a pro-inflammatory phenotype which is reflected in elevated NF-κB activation. The GM-CSF-KO mice are sensitive to inflammatory stimuli and have higher NF-κB activation when compared to the PPARγ-KO mice (unpublished observation). The expression of miR-23b-3p, miR-146a-5p, and miR-199a-3p reflect this phenotype. Each of these microRNA are elevated in the PPARγ-KO and further elevated in the GM-CSF-KO alveolar macrophages (See summary, Table 1).

The microRNA miR-27a-3p and miR-27b-3p have been shown to destabilize PPARγ mRNA [39]. miR-27a-3p expression was significantly elevated only in the GMCSF-KO mice while miR-27b-3p expression had definitive increases in both strains. The fact that only miR-27b is upregulated in the PPARγ-KO suggests that the deficiency in GM-CSF (PPARγ-KO mice are not deficient in GM-CSF) may be affecting miR-27a. These data suggest that miR-27a and miR-27b upregulation may contribute to the deficiency of PPARγ in GM-CSF-KO alveolar macrophages.

The lipid transporters ABCA1 and ABCG1 have been shown to contribute to surfactant catabolism and regulation of lung homeostasis. These transporters are dysregulated in both the PPARγ-KO and GM-CSF-KO alveolar macrophages.

The microRNA miR-33-3p and miR-33-5p target ABCA1 and ABCG1 in other cell types. The microRNA-33-3p was elevated in the PPARγ-KO and GM-CSF-KO mice while miR-33-5p was elevated only in the GM-CSF-KO. The high increase in both of these microRNA in the PPARγ-KO and not the GM-CSF-KO mice may suggest that the miR-33-5p is related to the GM-CSF deficiency and not related to ABCG1 deficiency which occurs in both strains.

microRNA expression was evaluated *in vitro* cultures of alveolar macrophages from C57Bl/6 mice stimulated with LPS and PPARγ agonist and inhibitors. Only miR-146a-5p was stimulated with LPS and decreased with rosiglitazone. PPARγ expression is decreased and NF-κB activity is increased upon stimulation with LPS. The expression of miR-146a is promoted by NF-κB. Our stimulation data supports the idea that PPARγ can inhibit NF-κB activation and reduce the expression of miR-146a-5p. The fact that none of the other microRNA were stimulated may reflect one or more of the following: (a) in vitro versus in vivo settings; (b) LPS stimulation versus deletion of GM-CSF which directly reduces PPARγ; (c) or a direct deletion of PPARγ in vivo; or other factors in the lung environment participate in the regulation of these microRNA. Further studies may be carried out using GM-CSF stimulation in vitro to attempt further understanding of the GM-CSF - PPARγ - ABCG1 pathway and the involvement of microRNA. (See Appendix C, Figures A-C)

Table 1: Summary of results presented in Chapter 3

mRNA/microRNA	GM-CSF-KO*	GM-CSF-KO +Rosi**	PPARγ-KO*	PPARγ-KO +Rosi**
PPARγ mRNA	Decreased	Unchanged	Decreased	N/A
ABCA1 mRNA	Increased	Unchanged	Increased	Unchanged
ABCG1 mRNA	Decreased	Unchanged	Decreased	Unchanged
miR-27b	Increased	Decreased	Increased	Unchanged
miR-146a	Increased	Unchanged	Increased	Unchanged
miR-23b	Increased	Unchanged	Increased	Unchanged
miR-199a	Increased	Unchanged	Increased	Unchanged
mIR-33-5p	Unchanged	Unchanged	Increased	Increased

*** Compared to C57Bl/6**

**** Compared to vehicle control**

(See Appendix C, Figures A-C)

Chapter 4: microRNA Expression in Model of Chronic Granulomatous Lung Disease

As mentioned in the introduction, our laboratory interest lies in the understanding of human lung disease. One such goal is to improve the understanding of sarcoidosis pathophysiology. The relatively recent reports of carbon nanotubes in the environment favoring sarcoidosis incidence have prompted us to develop a model of MWCNT mediated lung disease that might have applicability to sarcoidosis [43]. This model has proven to be extremely interesting with pathological changes identical to those found in sarcoidosis [25,31,32]. Because of the importance of alveolar macrophages and PPARγ deficiency in sarcoidosis and this MWCNT model, microRNAs were investigated.

Results:

4.1 miRNA-27b-3p is upregulated in alveolar macrophages from C57Bl/6 mice instilled with MWCNT

The expression of PPARγ has been shown to be down regulated in C57BL/6 mice instilled with MWCNT [31]. The expression of PPARγ was monitored in MWCNT instilled mice that were collected for this study; these mice show a significant decrease in PPARγ expression, supporting previously published data. The expression of miR-27a-3p and miR-27b-3p were evaluated in the alveolar macrophage of C57Bl/6 mice instilled with MWCNT (Figure 8). The expression of miR-27b-3p increased 1.7 fold in the MWCNT mice compared to vehicle controls. No change was observed in the expression of miR-27a-3p.

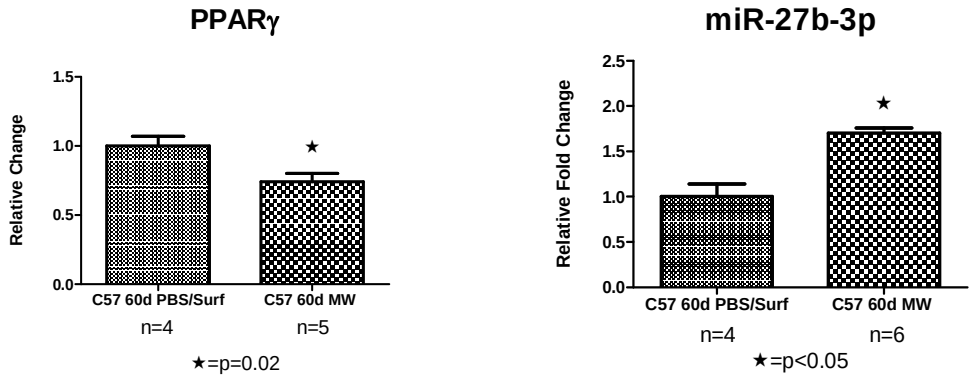

Figure 8: Expression of microRNA in C57Bl/6 mice instilled with MWCNT. MWCNT instilled mice show increased expression of miR-27b-3p. Graphs represent mean fold change compared to vehicle control.

4.2 miRNA associated with NF-κB regulation are upregulated in C57Bl/6 mice instilled with MWCNT

Activity of NF-κB and inflammatory mediators are increased in alveolar macrophages from patients with sarcoidosis [21]. Recently Huizar et al published that inflammatory mediators were also elevated in the MWCNT model [25]. microRNAs miR-23b-3p, miR-146a-5p, and miR-199a-3p were evaluated in the C57Bl/6 mice instilled with MWCNT (Figure 9). The expression of miR-23b-3p increased 1.5 fold in MWCNT instilled mice. miR-146a-5p expression increased 2.8 fold in animals instilled with MWCNT. miR-199a-3p was not altered in MWCNT instilled mice. These data demonstrate upregulation of two

microRNA that were elevated in GM-CSF and PPARγ knockout mice, however miR-199a-3p was not changed.

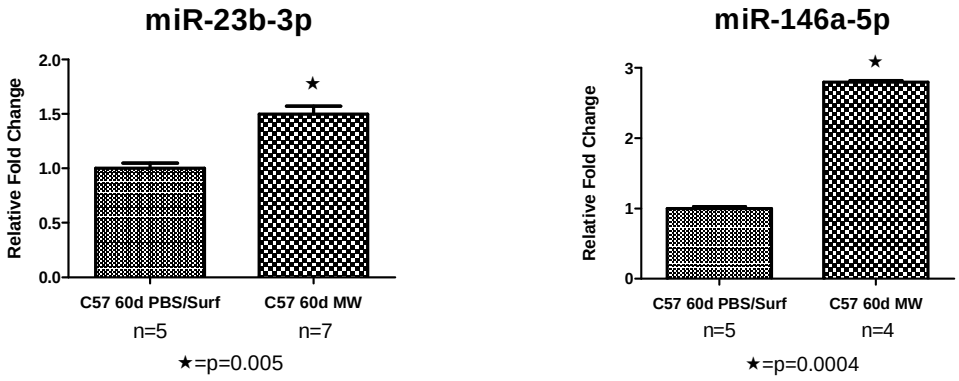

Figure 9: Expression of inflammatory microRNA in C57Bl/6 mice instilled with MWCNT for 60 days. The expression of miR-23b-3p and miR-146a-5p are elevated in alveolar macrophages from C57Bl/6 mice instilled with MWCNT. Graphs represent mean fold change compared to vehicle control.

4.3 ABC Transporters are dysregulated in MWCNT instilled C57Bl/6 mice

Previous studies have shown the dysregulation of ABCA1 and ABCG1 in PPARγ deficient pathologies. ABCA1 and ABCG1 deficiencies have been linked to increased inflammatory response to antigens [43]. The expressions of ABCA1 and ABCG1 are evaluated in MWCNT instilled mice (Figure 10). ABCA1 expression in MWCNT instilled mice decreased 40% compared to vehicle control animals. ABCG1 expression decreased 30% in MWCNT instilled animals compared to vehicle controls. The downregulation of both ABC Transporters suggest they may play a role in promoting granuloma formation.

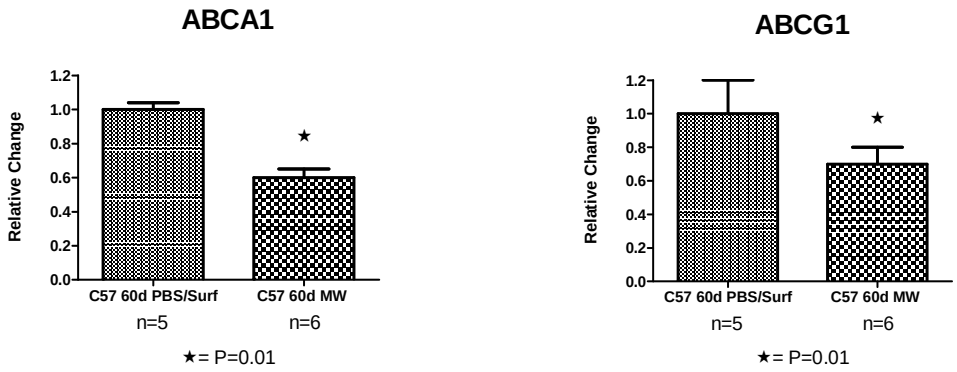

Figure 10: Expression of ABCA1 and ABCG1 is dysregulated in C57Bl/6 mice instilled with MWCNT. ABCA1 is decreased 40% in MWCNT instilled animals and ABCG1 expression is decreased 30% in MWCNT instilled animals compared to vehicle controls. Graphs represent mean fold change compared to vehicle control.

4.4 miRNA targeting ABC Transporters are elevated in C57Bl/6 mice instilled with MWCNT

The microRNA miR-33-3p and miR-33-5p have been reported to target the mRNA of ABCA1 and ABCG1. The expressions of microRNA miR-33-3p and miR-33-5p are evaluated in the MWCNT instilled mice (Figure 11). The expression of miR-33-5p was increased 7.5 fold while miR-33-3p was not affected in C57Bl/6 mice instilled with MWCNT compared to those instilled with vehicle control.

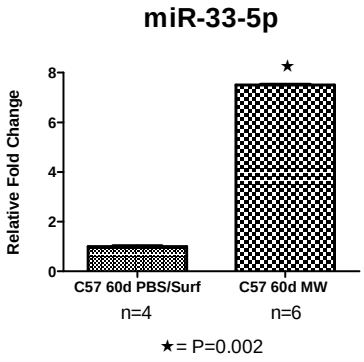

Figure 11: Expression of microRNA targeting ABC Transporters. The expression of miR-33-5p was elevated in C57Bl/6 mice instilled with MWCNT compared to control

4.5 *In vivo* rosiglitazone treatment increased miR-27b-3p in alveolar macrophages from C57Bl/6 mice instilled with MWCNT

The inflammatory phenotype observed in the MWCNT model may be due to the deficiency of PPARγ expression. The effect of rosiglitazone treatment on PPARγ and microRNA expression was evaluated in C57Bl/6 MWCNT instilled mice. The expression of miR-27b-3p increased 1.5 fold in MWCNT mice treated with rosiglitazone compared to vehicle control animals. Evaluation of PPARγ mRNA and miR-27a-3p showed no difference between rosiglitazone injected mice and vehicle controls (Figure 12).

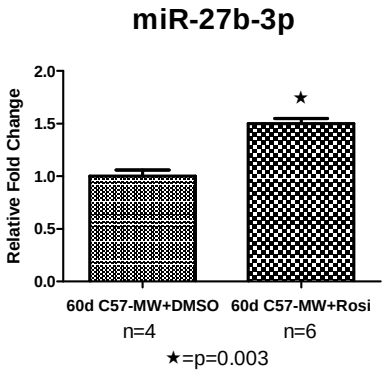

Figure 12: Expression of microRNA from C57Bl/6 mice with rosiglitazone treatment shows miR-27b-3p increased 1.5 fold after rosiglitazone treatment compared to animals receiving vehicle alone. Mean fold change is compared to vehicle control.

4.6 *In vivo* rosiglitazone treatment increased miRNA associated with NF-κB regulation in MWCNT instilled C57Bl/6 mice

An inflammatory phenotype is observed in C57Bl/6 mice instilled with MWCNT [25]. The effect of PPARγ agonist rosiglitazone was evaluated on the expression of microRNA that may regulate NF-κB (Figure 13). Rosiglitazone injections increased the expression miR-146a-5p expression 1.6 fold in MWCNT instilled mice receiving rosiglitazone injections. The expression of miR-23b-3p and miR-199a-3p was not altered in MWCNT instilled mice.

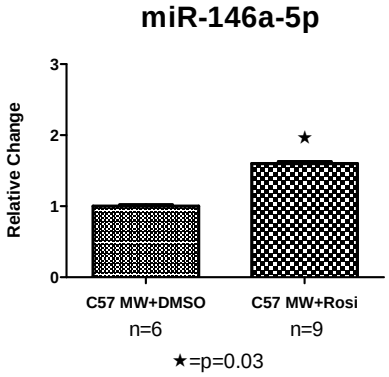

Figure 13: Expression of inflammatory microRNA inC57Bl/6 mice increase in MWCNT instilled mice treated with rosiglitazone. Graph represents mean fold change compared to vehicle control.

4.7 ABC Transporters are dysregulated in MWCNT instilled C57Bl/6 mice and can be affected with rosiglitazone treatment

The effects of rosiglitazone injections on ABC Transporter expression were evaluated in C57Bl/6 mice instilled with MWCNT. The expression of ABCA1 expression was increased 1.5 fold in C57Bl/6 mice instilled with MWCNT then given rosiglitazone injections. The expression of ABCG1 increased 1.4 fold in C57Bl/6 mice instilled with MWCNT given rosiglitazone injections. (Figure 14)

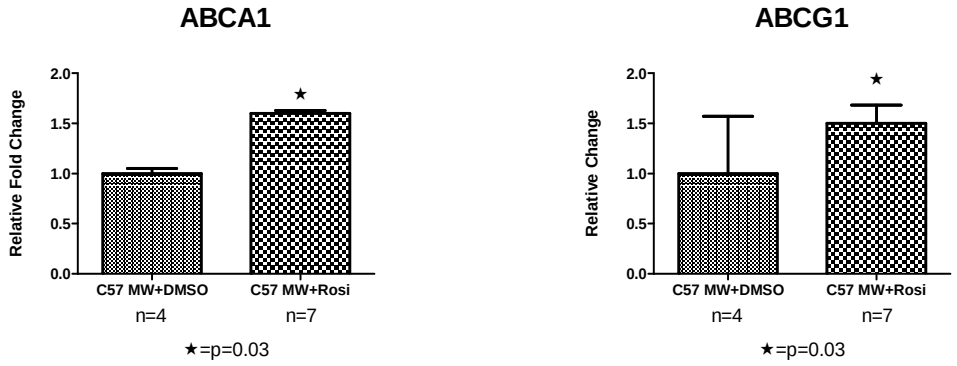

Figure 14: Rosiglitazone effects expression of ABC Transporters in MWCNT instilled mice. ABCA1 and ABCG1 expression are increased in MWCNT instilled animals receiving rosiglitazone. Graphs represent mean fold change compared to vehicle controls.

4.8 Expression of microRNA targeting lipid transporters evaluated in MWCNT model

The expression of the microRNA targeting the ABC Transporters is evaluated in MWCNT instilled C57Bl/6 mice treated with rosiglitazone (Figure 15). Rosiglitazone induced a 2.3 fold increase in miR-33-5p expression. The expression of miR-33-3p was not changed, however compared to animals receiving vehicle control.

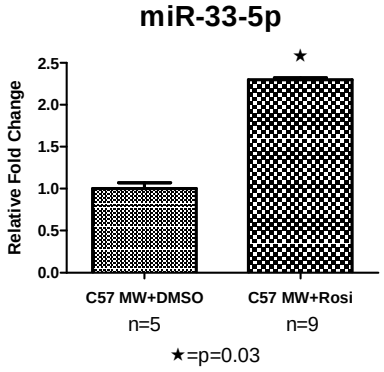

Figure 15: Expression of mR-33-5p in MWCNT instilled mice increased with rosiglitazone treatment. Graphs represent mean fold change compared to vehicle controls.

Table 2: Summary of results presented in Chapter 4

mRNA/microRNA	C57Bl/6+MWCNT*	C57Bl/6+MWCNT+Rosi**
PPARγ mRNA	Decreased	Unchanged
ABCA1 mRNA	Decreased	Increased
ABCG1 mRNA	Decreased	Increased
miR-27b	Increased	Increased
miR-146a	Increased	Increased
miR-23b	Increased	Unchanged
miR-199a	Unchanged	Unchanged
mIR-33-5p	Increased	Increased

***** **Compared to PBS/Surfactant treated mice**

****** **Compared to vehicle control treated MWCNT**

Summary/Discussion:

Sarcoidosis is a chronic granulomatous disease which primarily affects the lung. The etiology is unknown, but recent studies have suggested that environmental factors including carbon nanotubes may play a role in granuloma formation [44]. Previous animal models were limited because of the lack of chronicity. Our laboratory has developed a model of chronic granulomas using MWCNT [25]. Studies showing that the deficiency of PPARγ in knock out mice affected microRNA expression (chapter 3) prompted our investigation of miRNAs in the MWCNT model of granulomatous lung disease. Previous observations have shown deficient PPARγ in the alveolar macrophages of MWCNT instilled mice [31]. The expression of microRNA and their predicted targets were evaluated in MWCNT instilled mice. We have found that miR-27b which has been shown in other systems to downregulate PPARγ is increased [45,46]. We also determined that PPARγ regulated genes ABCA1 and ABCG1 were downregulated. Interestingly, both ABCA1 and ABCG1 are recognized as suppressors of proinflammatory cytokines in addition to their functions as facilitators of lipid metabolism [47,48]. We investigated miR-33 expression which was also elevated. miR-33 has been shown to downregulate the expression of ABCA1 and ABCG1 in other systems [42,49]. NF-κB activation is an important driver of miR-33 expression [50]. Earlier studies demonstrated the up regulation of NF-κB in alveolar macrophages of patients with sarcoidosis [51]. Based on the involvement of NF-κB in granulomatous disease and the upregulation of miR-23b, miR-146a and miR-199a in alveolar macrophages from PPARγ-KO mice, we

next investigated these miRNAs activated by NF-κB. Both miR-23b and miR-146a were upregulated, surprisingly miR-199a was not changed.

In order to begin to understand the relationship between these identified genes and microRNAs, we decided to investigate the *in vivo* effects of a PPARγ agonist, rosiglitazone. As a PPARγ ligand, rosiglitazone would not be expected to increase PPARγ expression but PPARγ activity should be enhanced. As expected PPARγ gene expression was not altered with ligand, but increased activity was triggered. The PPARγ regulated genes, ABCA1 and ABCG1 mRNA expression were enhanced with rosiglitazone treatment.

Surprisingly, miR-27b was also enhanced but miR-27b may be more involved in gene expression of PPARγ rather than activity. We expected miR-33 to be down regulated because of the enhanced expression of ABCA1 and ABCG1. This increase of miR-33-5p and increase in mRNA of the ABC transporters would suggest that in this system, miR-33-5p is not efficient in regulating the transporters. Whether increased PPARγ activity has an effect on miR-33 expression has not been previously reported. In our *in vivo* system this upregulation of miR-33 could be an indirect effect. The histopathology of rosiglitazone treated mice must be investigated before conclusions can be draw with regard to the efficacy of PPARγ ligand therapy. It may also be necessary to extend the dosing schedule for rosiglitazone beyond 10 days in order for definitive changes to be seen in granuloma formation.

Further investigations are necessary to sort out the complex inter-relationships between microRNA and altered gene expression in our MWCNT granulomatous disease model.

(See Appendix C, Figures A-E)

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Appendix A: Animal Use Protocols

East Carolina University

imal Care and
se Committee
212 Ed wards Life
Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

J185b Approval
Original

June 21, 2011

Mary Jane Thomassen, Ph.D.
Department of Medicine
Brody 3E
ECU Brody School of Medicine

Dear Dr. Thomassen:

Your Animal Use Protocol entitled, "Macrophages and Surfactant Homeostasis" (AUP #J185b) was reviewed by this institution's Animal Care and Use Committee on 6/21/11. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy 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.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

East Carolina University

...hal Care and
...l Committee
212 Life Science Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

August 2, 2011

Mary Jane Thomassen, Ph.D.
Department of Internal Medicine
Brody 3E
ECU Brody School of Medicine

Dear Dr. Thomassen:

Your Animal Use Protocol entitled, 'Carbon Nanotubes as a Tool for Generating an Experimental Model of Pulmonary Sarcoidosis' (AUP #J199a) was reviewed by this institution's Animal Care and Use Committee on 8/2/11. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy 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.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

Appendix B: DCT VALUES USED FOR DATA

Figure 2: Expression of microRNAs targeting PPARγ

miR-27a-3p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	0.12		
1479	0.14		
1480	0.78		
1481	0.16		
PPARγ-KO Control			
1501		-0.640	
1502		0.260	
1503		0.350	
1849		-0.100	
1850		-0.400	
1851		0.000	
1852		0.300	
GMKO Control			
1611			-0.24
1613			0.06
1683			-0.30
1685			-0.40
1687			-0.10
1691			0.10
miR-27b-3p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	2.72		
1479	2.63		
1480	2.38		
1481	2.13		
PPARγ-KO Control			
1501		0.44	
1502		1.13	
1503		1.68	
1849		1.20	
1850		0.60	
1851		1.20	
1852		1.60	

GMKO Control			
1611			0.13
1613			0.01
1683			0.33
1685			0.51
1687			1.14
1691			0.56

Figure 3: Expression of microRNA associated with NF-κB activity

miR-23b-3p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	2.57		
1479	2.55		
1480	2.83		
1481	2.30		
1482	2.95		
PPARγ-KO Control			
1500		-1.80	
1501		-1.74	
1502		-1.52	
1503		-1.18	
1849		1.40	
1850		0.60	
1851		1.10	
1852		1.60	
GMKO Control			
1611			-1.94
1613			-2.58
1683			0.01
1685			0.14
1687			0.77
1691			-0.48

miR-146a-5p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	6.66		
1479	6.36		
1480	5.44		

1481	5.95		
1482	5.67		
PPARγ-KO Control			
1500		2.46	
1501		2.16	
1502		3.10	
1503		3.03	
1849		2.90	
1850		3.40	
1851		4.20	
1852		4.10	
GMKO Control			
1611			-1.00
1613			-1.20
1683			0.80
1685			1.20
1687			1.30
1691			1.60

miR-199a-3p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	11.55		
1479	11.27		
1480	12.11		
1481	10.75		
1482	11.88		
PPARγ-KO Control			
1500		9.32	
1501		8.69	
1502		9.05	
1503		8.15	
1849		10.90	
1850		9.30	
1851		10.10	
1852		11.00	
GMKO Control			
1611			5.20
1613			5.20
1683			7.90
1685			7.96
1687			8.63
1691			7.03

Figure 4: miR-33-3p and miR-33-5p expression in GM-CSF-KO and PPARγ-KO

miR-33-3p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	11.61		
1479	11.50		
1480	12.28		
1481	12.29		
PPARγ-KO Control			
1501		10.21	
1502		11.30	
1503		11.14	
1849		4.20	
1850		3.60	
1851		4.60	
1852		5.60	
GMKO Control			
1683			10.47
1685			10.23
1687			10.43
1691			11.12

miR-33-5p	C57Bl/6	PPARγ-KO	GM-CSF-KO
C57 Control			
1478	9.64		
1479	10.09		
1480	10.08		
1481	8.99		
1482	9.51		
PPARγ-KO Control			
1500		7.33	
1501		7.14	
1502		7.58	
1503		7.92	
1849		5.60	
1850		5.20	
1851		5.50	
1852		5.60	

GMKO Control			
1611			8.77
1613			7.87
1612			8.01
1620			10.07
1683			9.57
1685			10.51
1687			8.16
1691			12.64

Figure 5: Expression of miR-27b-3p in rosiglitazone treated GM-CSF-KO

miR-27b-3p	GMCSF-KO+DMSO	GMCSF-KO+Rosi
GMKO+10d DMSO		
1925	0.95	
1926	0.98	
1927	0.78	
1946	1.60	
1947	0.90	
1948	1.20	
1949	2.30	
GMKO+10d Rosi		
1920		1.4
1921		1.4
1922		1.6
1923		2.1
1924		2.5
1943		1.6
1944		2.3

Figure 6: PPARγ-KO mice treated with rosiglitazone

miR-33-5p	PPARγ-KO+DMSO	PPARγ-KO+Rosi

PPARγ-KO+DMSO		
1966.	6.6	
1967	4.8	
1968	4.8	
1969	4.3	
1970	5.1	
PPARγ-KO+Rosi		
1971		9.5
1972		10.1
1973		8.5
1974		10.4
1975		9.9

Figure 7: Expression of microRNA 146a-5p

6 hour	US	LPS	Rosi	GW	Rosi+LPS	GW+LPS
1765	3.5000	3.00	4.10	4.00	3.10	3.00
1774	3.9000	2.70	4.20	4.30	4.40	3.00
1779	2.9000	2.50	4.30	3.80	2.70	2.70
1838	2.7000	2.00	3.00	3.70	2.40	2.40
24 Hour	US	LPS	Rosi	GW	Rosi+LPS	GW+LPS
1748	3.0	1.0	3.8	3.5	1.7	3.3
1756	3.4	.15	3.1	3.8	1.3	-.7
1760	3.0	.5	3.2	2.8	1.5	.9

Figure 8: Expression of microRNA in C57Bl/6 mice instilled with MWCNT

PPARγ mRNA	C57 60d PBS/Surf	C57 60d MWCNT
C57 60d PBS/Surfactant		
1703	1.7	
1704	2.1	
1889	2.4	
1890	2.1	
C57 60d MWCNT		
1713		2.6

1714		2.8
1717		3.4
1720		2.4
1726		2.5

miR-27b-3p	C57 60d MWCNT	C57 60d PBS/Surf
C57+MW		
1573	0.75	
1574	0.85	
1575	0.89	
1495	1.02	
C57 60d PBS/Surf		
1703		1.6
1704		1.5
1705		1.5
1889		0.9
1890		2.2
1892		2.6

Figure 9: Expression of inflammatory microRNA in C57Bl/6 mice

miR-23b-3p	C57 60d MWCNT	C57 60d PBS/Surf
C57+MW		
1573	1.54	
1574	1.39	
1575	1.04	
1495	1.45	
1893	0.90	
1894	1.30	
1895	2.00	
C57 60d PBS/Surf		
1703		1.7
1704		1.9
1705		2.1
1890		2.3
1892		2.2
miR-146a-5p	C57 60d MWCNT	C57 60d PBS/Surf
C57+MW		
1573	4.41	

1574	4.49	
1575	3.99	
1495	4.58	
C57 60d PBS/Surf		
1703		6.2
1704		6.6
1705		5.6
1890		5.6
1892		5.7

Figure 10: Expression of ABCA1 and ABCG1

ABCA1 mRNA	C57 60d PBS/Surf	C57 60d MWCNT
C57 60d PBS/Surfactant		
1703	2.0	
1704	2.1	
1705	2.3	
1889	2.6	
1890	2.5	
C57 60d MWCNT		
1717		2.9
1720		3.3
1726		3.4
1794		2.6
1795		2.9
1675		2.4

ABCG1 mRNA	C57 60d PBS/Surf	C57 60d MWCNT
C57 60d PBS/Surfactant		
1703	-1.0	
1704	-0.4	
1705	-0.7	
1889	-0.4	
1890	-0.4	
C57 60d MWCNT		
1717		0.60
1720		0.20
1726		0.70
1794		-0.10
1795		-0.04
1675		-0.50

Figure 11: Expression of microRNA targeting ABC Transporters

miR-33-5p	C57 60d MWCNT	C57 60d PBS/Surf
C57 60d MWCNT		
1573	5.92	
1574	5.67	
1575	5.86	
1495	6.48	
C57 60d PBS/Surf		
1703		9.1
1704		8.2
1705		8.7
1889		4.4
1890		5.0
1892		5.2

Figure 12: Expression of microRNA from C57Bl/6 mice with rosiglitazone treatment

miR-27b-3p	C57 60d MWCNT	C57 60d PBS/Surf
C57 60d MWCNT		
1573	0.75	
1574	0.85	
1575	0.89	
1495	1.02	
C57 60d PBS/Surf		
1703		1.6
1704		1.5
1705		1.5
1889		0.9
1890		2.2
1892		2.6

Figure 13: Expression of inflammatory microRNA inC57Bl/6 mice

miR-146a-5p	C57 MW+ DMSO	C57 MW+Rosi
C57 MW+DMSO		
1928	5.10	

1929	5.10	
1988	5.70	
1989	5.60	
1996	6.10	
1997	5.70	
C57 MW+Rosi		
1909		4.19
1910		4.39
1911		4.59
1933		5.50
1934		5.80
1978		5.10
1979		5.20
1980		5.00
1981		4.40

Figure 14: Rosiglitazone effects expression of ABC Transporters in MWCNT

ABCA1	C57 MW+ DMSO	C57 MW+Rosi
C57 MW+DMSO		
1988	2.9	
1989	3.3	
1996	3.4	
1997	2.6	
C57 MW+Rosi		
1908		2.3
1909		2.8
1910		3.1
1978		2.3
1979		2.6
1980		2.5
1981		2.4

ABCG1	C57 MW+ DMSO	C57 MW+Rosi
C57 MW+DMSO		
1988	0.01	
1989	-0.40	
1996	-0.10	
1997	-0.10	
C57 MW+Rosi		
1908		-1.10
1909		-0.60

1910		-0.40
1978		-0.90
1979		-0.70
1980		-0.60
1981		-0.60

Figure 15: Expression of mR-33-5p in MWCNT instilled mice

ABCG1	C57 MW+ DMSO	C57 MW+Rosi
C57 MW+DMSO		
1928	6.50	
1929	6.50	
1988	8.80	
1996	8.90	
1997	6.60	
C57 60d MW+10d Rosi Inj		
1909		6.71
1910		6.56
1911		6.93
1933		6.20
1934		6.50
1978		5.90
1979		5.90
1980		6.40
1981		5.50

APPENDIX C: PROPOSED MECHANISIMS

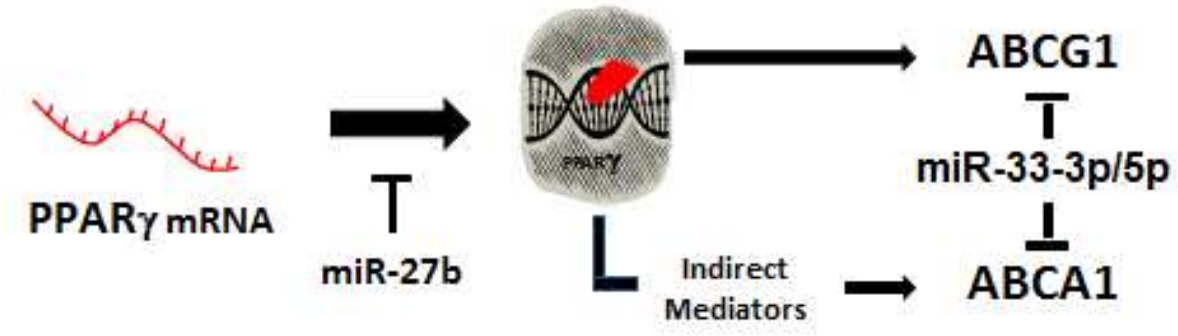

Figure A: Proposed interaction of miR-27b and miR-33-3p/5p with PPARγ and ABC Transporters. miR-27b targets the messenger RNA of PPARγ while miR-33-3p and miR-33-5p targets that of ABCA1 and ABCG1.

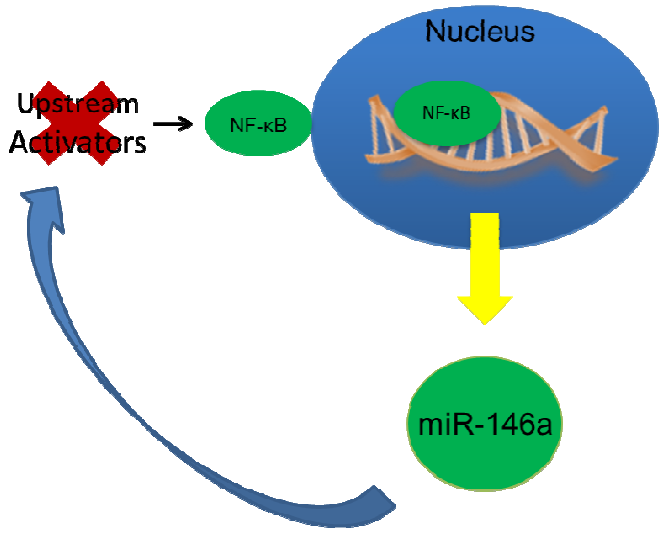

Figure B: Regulation of NF-κB by miR-146a. NF-κB acts as a promoter for miR-146a expression. miR-146a targets upstream activators of NF-κB and has been shown to decrease NF-κB activity [38].

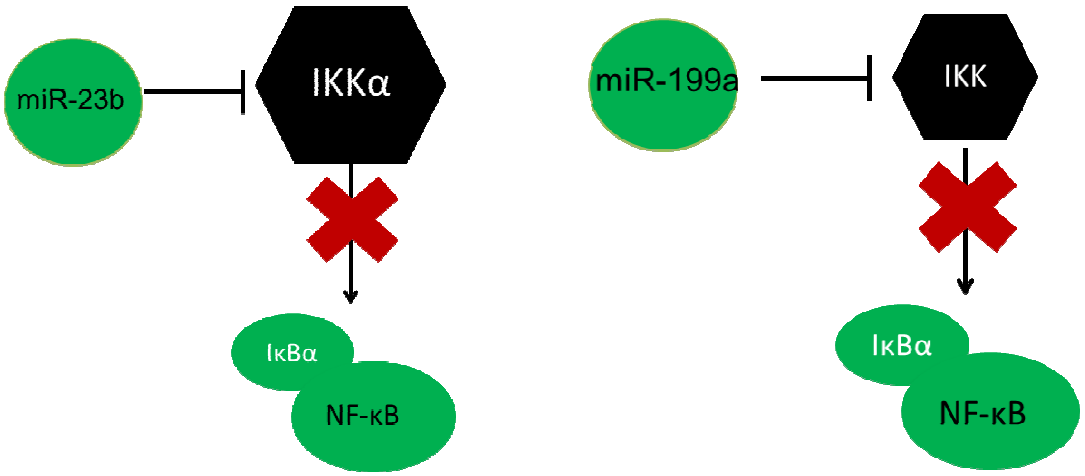

Figure C: Proposed effect of miR-23b and miR-199a on NF-κB activity. miR-23b and miR-199a have been reported to target the mRNA of IKK Kinase subunits [38,39]. Inhibition of IKK Kinase may decrease NF-κB activity.

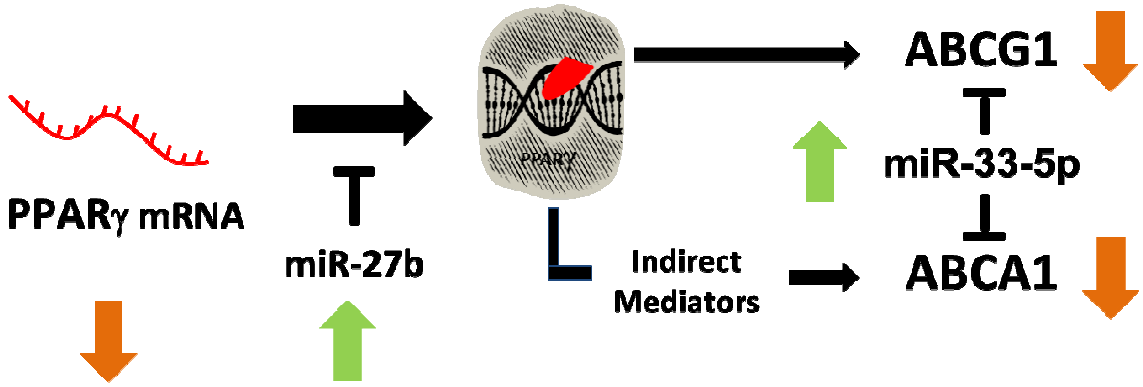

Figure D: Effects of MWCNT exposure on gene and microRNA expression of the alveolar macrophage.

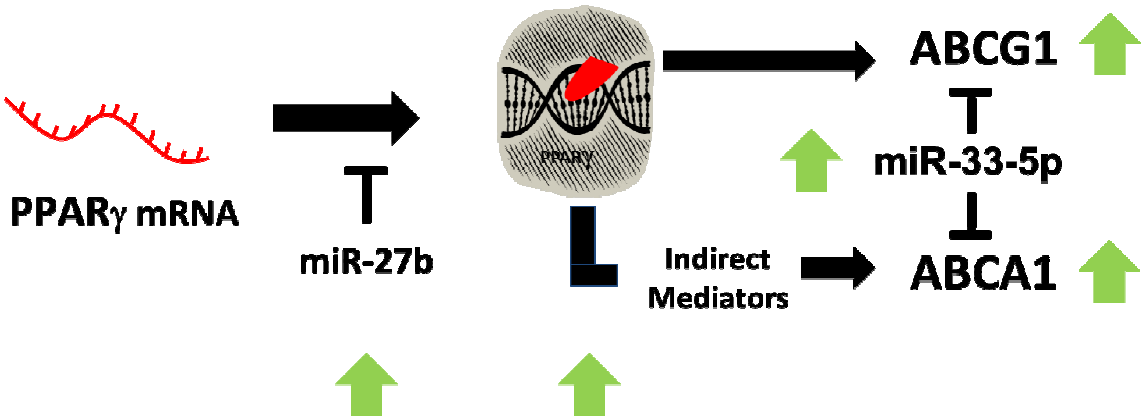

Figure E: Effect of rosiglitazone treatment on gene and microRNA expression in MWCNT instilled animals.