The Role of Inflammasomes in Asbestos-Induced Mesothelial to Fibroblastic Transition

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THE ROLE OF INFLAMMASOMES IN ASBESTOS-INDUCED MESOTHELIAL TO FIBROBLASTIC TRANSITION.

A Dissertation Presented

by

Joyce Kathleen Thompson

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The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cellular, Molecular and Biomedical Sciences

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ABSTRACT

Malignant Mesothelioma (MM) is a fatal disease with a low median survival between 8 to 12 months after diagnosis. MM has a long latency period (10-60 years), is causally related to asbestos exposure, and is refractory to all available modes of therapy. Despite the causal association between asbestos exposure and MM however, the mechanisms by which asbestos induces this deadly disease remain unclear. Chronic inflammation due to the presence of asbestos fibers is believed to play an important role in all aspects of MM pathogenesis, from development to progression and resistance. Chronic inflammation has been shown to promote dysregulated wound repair, fibrosis and epithelial to mesenchymal transition (EMT). One of the inflammatory pathways that asbestos activates is the inflammasome (a multiprotein scaffold that assembles in response to various stimuli to facilitate the activation of caspase-1), which has been implicated in several chronic inflammatory diseases and disorders. The nucleotide binding oligomerization domain (NOD) - like receptor containing a pyrin domain 3 (NLRP3) inflammasome, both as a whole or via its components [NLRP3, apoptosis related speck-like protein containing a CARD (caspase activating and recruitment domain) (ASC) and caspase-1] as well as its products, IL-1β and IL-18, has been implicated in the development of EMT during chronic inflammation.

Asbestos fibers, especially the amphiboles, are non-biodegradable and thus persist in tissues of the body for years after exposure. In mesothelial cells, the squamous epithelial-like cells that line the serosal cavities of the body, from which MM originates, asbestos chronically activates the NLRP3 inflammasome. Asbestos also activates the NLRP3 inflammasome in human macrophages that can lead to the establishment of a chronic inflammation environment. We therefore hypothesized that asbestos dependent regulation of the inflammasome played a role in mesothelial to fibroblastic transition to facilitate eventual neoplastic transformation of the mesothelial cells.

Using in vitro models, siRNA knockdown approaches as well as in vivo models of asbestos exposure utilizing inflammasome component knockout mice, we demonstrate that asbestos-induced reactive oxygen species generation modulates the redox state of the endogenous antioxidant, thioredoxin, causing its dissociation from thioredoxin interacting protein to promote activation of the inflammasome. We also show that the inflammasome plays a role in asbestos-induced mesothelial to fibroblastic transition (MFT) (a form of EMT occurring in the mesothelial cells) both in vitro and in vivo with a requirement for caspase-1 in vivo to promote thickening of the submesothelium. Through our studies, we have identified tissue factor pathway inhibitor 2 (TFPI2) and fibroblast growth factor 2 (FGF2) as molecules that are upregulated in response to asbestos exposure with potential roles in the progression of asbestos-induced MFT. There is a dearth of diagnostic biomarkers that enable early detection of MM, thus with further studies these two molecules could be explored as biomarkers of asbestos exposure/disease progression. TFPI2 levels were downregulated in response to blockage of IL-1β signaling and thus could be harnessed as a potential marker for therapy efficiency with further studies.
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## TABLE OF CONTENTS

Content

ACKNOWLEDGEMENTS........................................................................................................ iii

LIST OF TABLES ......................................................................................................................... ix

LIST OF FIGURES ....................................................................................................................... x

CHAPTER ONE: COMPREHENSIVE LITERATURE REVIEW ................................................. 1

1.1 Introduction.......................................................................................................................... 1

1.2 Asbestos ............................................................................................................................... 3

1.3 Asbestos and Oxidant Generation ..................................................................................... 6

1.3.1 Effects of Asbestos-Induced Oxidant Generation ......................................................... 8

1.4 Asbestos-Related Diseases ............................................................................................... 14

1.4.1 Pleural Plaques ............................................................................................................... 14

1.4.2 Pleural Effusion .............................................................................................................. 15

1.4.3 Asbestosis ....................................................................................................................... 15

1.4.4 Lung Cancer ................................................................................................................... 16

1.4.5 Malignant Mesothelioma ............................................................................................. 17

1.5 Mesothelial cells ............................................................................................................... 18
Effect of N-acetyl-cysteine (NAC) on asbestos-induced inflammasome activation.. 75

TXNIP down-regulation attenuated asbestos-induced inflammasome activation...... 76

Discussion.................................................................................................................. 77

Conclusion................................................................................................................ 83

List of Abbreviations ............................................................................................... 84

References................................................................................................................ 86

Figures ...................................................................................................................... 90

CHAPTER THREE: ASBESTOS-INDUCED MESOTHELIAL TO FIBROBLASTIC TRANSITION IS MODULATED BY THE INFLAMMASOME.. 99

Abstract..................................................................................................................... 101

Introduction............................................................................................................. 102

Materials and Methods.......................................................................................... 103

Results...................................................................................................................... 111

Asbestos exposure induces MFT in mesothelial cells as depicted by changes in gene profile:.......................................................... 111

Asbestos exposure causes morphological changes in mesothelial cells.............. 112

Asbestos exposure causes changes in epithelial and mesenchymal markers in human mesothelial cells............................................................ 113
The NLRP3 inflammasome augments asbestos-induced MFT ........................................... 114

IL-1β signaling regulates asbestos-induced MFT .............................................................. 115

Asbestos exposure causes MFT in mice that is dependent on inflammasome activation: .......................................................................................................................................................... 117

Asbestos-induced thickening of the peritoneal wall is caspase-1 dependent .......... 118

Discussion .................................................................................................................................. 119

References .................................................................................................................................. 126

Table 3-1 .................................................................................................................................. 130

Figures .................................................................................................................................... 131

Supplemental Tables ................................................................................................................ 144

CHAPTER FOUR: INVESTIGATING THE ROLE OF IL-1 RECEPTOR ANTAGONIST AND INFLAMMASOME INHIBITOR (CRID3) IN ASBESTOS-INDUCED MFT IN VIVO AND POTENTIAL ROLES FOR TFPI2 AND FGF2 IN VITRO ......................................................................................................................... 149

Introduction ................................................................................................................................. 149

Materials and Methods .............................................................................................................. 152

Results ....................................................................................................................................... 156

Discussion ................................................................................................................................. 158

References .................................................................................................................................. 164
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3-1: EMT related gene expression changes in mesothelial cells in response to asbestos exposure</td>
<td>131</td>
</tr>
<tr>
<td>Table S3-1: Supplementary Table 1: HPM3 Up-Down Regulation (comparing to control group)</td>
<td>145</td>
</tr>
<tr>
<td>Table S3-2: Supplementary Table 2: LP9 Up-Down Regulation (comparing to control group)</td>
<td>147</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1: Scanning electron micrograph of mouse mesothelial cells cultured from mouse peritoneal wall explants</td>
<td>19</td>
</tr>
<tr>
<td>Figure 1.2: Scanning electron micrograph of mouse parietal mesothelium</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.3: Inflammation plays a role in asbestos-related diseases</td>
<td>31</td>
</tr>
<tr>
<td>Figure 2.1: Crocidolite asbestos exposure modulates Trx1 levels in mesothelial cells</td>
<td>90</td>
</tr>
<tr>
<td>Figure 2.2: Crocidolite asbestos exposure causes oxidation of Trx1 in human mesothelial cells</td>
<td>91</td>
</tr>
<tr>
<td>Figure 2.3: Inhibition of thioredoxin reductase by DNCB and pretreatment of cells with DHA rescues asbestos-induced oxidation of Trx1</td>
<td>93</td>
</tr>
<tr>
<td>Figure 2.4: Over-expression of Trx1 increases cell survival and ameliorates asbestos-induced ROS generation in LP9 cells</td>
<td>95</td>
</tr>
<tr>
<td>Figure 2.5: Asbestos-induced inflammasome priming and activation is attenuated by NAC</td>
<td>96</td>
</tr>
<tr>
<td>Figure 2.6: Knockdown of TXNIP decreases inflammasome activation</td>
<td>97</td>
</tr>
<tr>
<td>Figure 2.7: Role of ROS and antioxidants in asbestos-induced activation of the NLRP3 inflammasome</td>
<td>98</td>
</tr>
<tr>
<td>Figure 3.1: Asbestos exposure induces a morphological change in human mesothelial cells</td>
<td>132</td>
</tr>
<tr>
<td>Figure 3.2: Asbestos alters markers of a mesothelial to fibroblastic transition in HMCs</td>
<td>133</td>
</tr>
<tr>
<td>Figure 3.3: Asbestos-induced MFT is partially dependent on NLRP3</td>
<td>135</td>
</tr>
<tr>
<td>Figure 3.4: IL-1β signaling regulates asbestos-induced MFT</td>
<td>137</td>
</tr>
<tr>
<td>Figure 3.5: Asbestos exposure induces mesothelial to fibroblastic transition in the parietal peritoneal mesothelium in vivo</td>
<td>139</td>
</tr>
<tr>
<td>Figure 3.6: Caspase-1 is important for asbestos-induced mesothelium thickening in mice</td>
<td>141</td>
</tr>
<tr>
<td>Figure 3.3A-S1: Regulation of asbestos-induced MFT by IL-1β signaling in LP9 Cells</td>
<td>143</td>
</tr>
<tr>
<td>Figure 4.1A-D: Modulating IL-1β signaling does not attenuate asbestos-induced submesothelium thickening</td>
<td>170</td>
</tr>
<tr>
<td>Figure 4.1E &amp; F: Modulating IL-1β signaling does not attenuate asbestos-induced submesothelium thickening</td>
<td>171</td>
</tr>
<tr>
<td>Figure 4.2: Knockdown of TFPI2 attenuates asbestos-induced MFT</td>
<td>172</td>
</tr>
<tr>
<td>Figure 4.4 FGF2 signaling regulates IL-1β and TFPI2 levels in response to asbestos exposure</td>
<td>173</td>
</tr>
</tbody>
</table>
CHAPTER ONE: COMPREHENSIVE LITERATURE REVIEW

Asbestos-Induced Inflammation in Malignant Mesothelioma and Other Lung Diseases

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1.1 Introduction

Malignant mesothelioma (MM) is a deadly disease arising from the parietal mesothelium of the pleura, peritoneum and in some rare cases, the pericardium or the tunica vaginalis of the testis (Manning et al., 2002, Neumann et al., 2013, Chekol and Sun, 2012). Malignant mesothelioma has been causally linked to asbestos exposure since the late 1950s into the early 60s (Wagner et al., 1960, Wagner, 1965). MM has a very long latency period (Thompson et al., 2014b) and the exact mechanisms involved in the development of this terrible disease in response to asbestos exposure still remains elusive. MM has a lag time between 10-60 years after initial asbestos exposure and only affects 5% of workers occupationally exposed to asbestos. As such, a genetic predisposition to the development of MM has been proposed. In fact, a small subset of MM patients have been recently shown to harbor a germline mutation in the BRCA1 associated protein (BAP1) (Testa et al., 2011,
Ohar et al., 2016) which also predisposes affected individuals to uveal and cutaneous melanoma and pancreatic cancer among others (Pilarski et al., 1993). In addition, a significant percentage of MM tumors have also been found to carry a somatic BAP1 mutation (Bott et al., 2011, Nasu et al., 2015). Recent studies have shown that loss of one copy of the BAP1 gene is enough to increase the sensitivity of mice to asbestos-induced MM when compared to their wildtype litter mates (Kadariya et al., 2016a). Other causes of MM include ionizing radiation (Li et al., 2015) and injury to the mesothelium, but the exact etiology of the disease and the potentiating factors that determine whether an asbestos exposed individual will develop MM remain unclear. The Simian virus large antigen 40 (SV40) has also been found in a subset of MM tumors giving rise to a debate that SV40 is an important etiological factor in MM development (Qi et al., 2011, Yang et al., 2008). To further complicate matters, the occult location of MM tumors makes their early detection and diagnosis difficult (Addis and Roche, 2009). Wrong diagnosis due to shared symptoms (Henderson et al., 2013a, Henderson et al., 2013b) as well the fact that symptoms usually only manifest when the tumors are large enough to compromise breathing or cause pain usually means that MM is diagnosed most frequently in its late stages (Rodriguez Panadero, 2015, van Meerbeeck et al., 2011). Unlike other cancers in which tumors from all stages are observed frequently, thereby allowing for the development of those diseases to be studied, the majority of MM cases encountered are from late stage patients. The restricted sample size as well as heterogeneity in the histological subtype also hinder an accurate assessment of tumor progression and determination of biomarkers that can point to the initial development of MM and aid in early detection. Several breakthroughs have
been made over the years in MM research but none of these have succeeded in improving the dismal overall survival of the disease (average survival 8-12 months) (van Meerbeeck et al., 2011). In order to design new therapies with improved efficacy, we need a better understanding of the mechanisms employed by asbestos to cause mesothelioma. An understanding of these mechanisms will help unlock potential targets for chemotherapeutics that are essential for recurrence thereby improving the prognosis of MM patients. Additionally, studying how asbestos causes MM will yield biomarkers that can then be followed for early diagnosis as well as treatment efficacy.

In this body of work, we investigate the role of the inflammasome and its regulation in response to asbestos in asbestos-induced mesothelial to fibroblastic transition which we believe may serve as a point of initiation for MM.

1.2 Asbestos

The group of hydrated silica fibers, collectively referred to as asbestos fibers, was used extensively during the industrial age due to their high tensile strength, thermal and fire resistance. As such, they were employed in construction work for insulation and fireproofing as well as in shipbuilding. Any endeavor that required insulation from high temperatures and provided great fire risks relied on asbestos. The name asbestos, originating from the Greek and meaning “unquenchable”, is actually a commercial name referring to six hydrated silica fibers of economic importance. These fibers can further be divided in two groups- serpentine asbestos which refers to the curly asbestiform fibers, the most economically relevant member being chrysotile asbestos. Chrysotile asbestos accounts for 95% of all mined and commercially available asbestos presently in use. The
second group is referred to as the amphibole asbestos fibers which possess a needle-like morphology. The remaining five asbestos fibers (amosite, crocidolite, tremolite, actinolite and anthophyllite) are found in this group. While the serpentine asbestos fibers have Mg$^{2+}$ ions in their chemical structure ($\text{Mg}_3\text{Si}_2\text{O}_5\text{(OH)}_4$) (Gaze, 1965) (which leaches out of the fiber in the presence of biological fluids (Morgan et al., 1977), the amphiboles vary in their iron content (both in the valency state and location of the iron ions in their crystal structure (Ghio et al., 2008, Gaze, 1965, Srivastava et al., 2010, Turci et al., 2011). Due to the organization of the asbestos crystals, serpentine fibers occur as rolled up sheets that form hollow fibers while the amphiboles form solid alternating tetrahedral sheets of silicate and octahedral cation-oxides that result in their needle-like morphology (Gaze, 1965).

Now that asbestos use has been reduced and banned in the construction industry, we are left with large numbers of buildings and ships that contain copious amounts of asbestos. Members of the workforce that were involved in the mining, weaving into textile, application of and plumbing with asbestos fibers were exposed to asbestos fibers from their work environment. Unfortunately, these workers also carried the fibers home with them in the form of the dust on their work clothes, which were handled and laundered by family members, exposing them to asbestos fibers in the process (Rom and Palmer, 1974). Towns that surrounded asbestos mines and textile mills were not spared this exposure either, as their air was filled with dust from these activities (Lemen et al., 1980). So long as asbestos sheets and other products are intact, they are relatively safe. Should they break or fracture in any way, however, they then pose a health risk to those exposed. One might ask, why is this the case? The answer lies in the friable nature of the asbestos fiber. Once broken,
the fiber and any product made from it continues to break into ever smaller pieces like fabric that is unraveling and fraying. This produces fiber particles that are small enough to become airborne and, thus, respirable. It was noted well before the twentieth century that people who worked with asbestos died earlier than others and presented with symptoms that were in the early twentieth century referred to as pneumoconiosis (Seiler, 1928). The only common thread between these cases was the occupation of the individuals and the role asbestos played in those occupations.

Reports indicate that miners of asbestos who happened to smoke have a 95% increase in their risk of developing lung cancer (Norbet et al., 2015). Cases referred to as pneumoconiosis, and later on determined to be asbestos-induced lung or pleural fibrosis, seemed to be most common in individuals with a history of occupational exposure (Manning et al., 2002). In some cases, individuals developed pleural plaques with and without pleural effusions, which were sometimes termed malignant. However, the deadliest disease ascribed to asbestos exposure is malignant mesothelioma (MM), which affects only a small subset of exposed individuals (Norbet et al., 2015, Neumann et al., 2013, Broaddus et al., 2011). An underlying thread found in these asbestos-related diseases is their latency period, which ranged in duration from 10-60 years after initial exposure. The level of exposure and time span of exposure also plays a role in the latency period of these diseases (Hesterberg et al., 1998, Craighead, 2011). Asbestos fibers, especially the amphiboles such as crocidolite, are non-degradable and persist in parts of the body where they are eventually deposited (typically the pleural cavity). Macrophages and other phagocytic cells attempt to clear the asbestos fibers, but they only succeed in removing
short fibers (Hesterberg et al., 1998, Davis and Jones, 1988, Donaldson et al., 2013, Murphy et al., 2012). Long asbestos fibers, because of their high aspect ratio, pose a problem for these cells, as they are not able to engulf the fibers fully (Moolgavkar et al., 2001, Sanchez et al., 2009, Davis and Jones, 1988, Murphy et al., 2012). As a result, there are repeated attempts to phagocytose the fibers, which results in frustrated phagocytosis, the accompanying release of superoxide, and the eventual death of the cells involved (Sanchez et al., 2009, Murphy et al., 2013). This process of frustrated phagocytosis and cell death leads to the establishment of a vicious cycle in which inflammatory cells are repeatedly recruited to sites of asbestos fiber deposition accompanied by the secretion of inflammatory factors and chemokines that facilitate an inflammatory environment/niche (Mossman et al., 2013). In this chapter, we cover some of the ways in which asbestos promotes chronic inflammation and how such an environment can engender the development of asbestos-related diseases.

1.3 Asbestos and Oxidant Generation

The surface of the asbestos fiber is a highly reactive one. The stereochemistry of the crystal structure as well as the valency state of iron ions determines the availability of these ions to participate in reactions at the cellular/interstitial fluid-fiber interface (Gazzano et al., 2005). While the chemical structure of chrysotile asbestos lacks iron, studies employing artificially generated iron free chrysotile have shown that chrysotile fibers are capable of drawing iron out of solution onto its surface (Gazzano et al., 2007) in cell free systems as well as under biological conditions. Chrysotile asbestos exposure also cause hemolysis in vivo leading to a buildup of iron on the asbestos fibers (Elferink and Kelters, 1991). Of the
amphibole asbestos fibers, amosite contains the most iron in its chemical structure (28%) followed by crocidolite (27%) by weight (Ghio et al., 2008, Srivastava et al., 2010). However, due to the stereochemistry of the crocidolite fibers and the valency state of iron ions on crocidolite, the iron ions are more motile and crocidolite asbestos generates the most reactive oxygen species (ROS) under both biological and cell free conditions (Srivastava et al., 2010, Pascolo et al., 2013). ROS generated in response to asbestos fibers \textit{in vivo} are not only cytotoxic but genotoxic as well (Srivastava et al., 2010, Aljandali et al., 2001). The mobilization of iron by asbestos in subjects that have been exposed to asbestos leads to a dysregulation of iron homeostasis (Ghio et al., 2008) as the body attempts to detoxify the high iron on the fibers by covering the asbestos fibers with iron storage proteins like ferritin (Pascolo et al., 2013). The increased generation of reactive species also increases the synthesis of intracellular antioxidants like glutathione and glutathione synthetase, extracellular superoxide dismutase (Hillegass et al., 2010b) and thioredoxin (Thompson et al., 2014a) in an attempt to rectify the oxidant/antioxidant balance. Exposure to asbestos also increases lipid peroxidation and generation of reactive nitrogen species (Shukla et al., 2003a, Howden and Faux, 1996, Faux and Howden, 1997, Nymark et al., 2008, Haegens et al., 2005) thus promoting an environment in which signaling cascades that are usually briefly stimulated by various reactive species are upregulated due to a surge in activating stimuli. Among such cascades are the various pro-inflammatory signaling pathways which while useful as the body attempts to clear the foreign bodies quickly become detrimental as they become imbalanced and chronically
active due to the duration of the biopersistent stimulus (Shukla et al., 2003a, Shukla and Mossman, 2004, Shukla and Mossman, 2008, Faux and Howden, 1997).

Asbestos exposure can also lead to the production of peroxynitrites (ONOO⁻) through the reaction of superoxide and nitric oxide (NO), when excess amounts of either are present (Matsuzaki et al., 2012, Nymark et al., 2008). Alveolar macrophages are among the first cells to encounter inhaled asbestos fibers and as they try to clear fibers (Mossman and Churg, 1998, Miserocchi et al., 2008), they undergo a superoxide burst via the activity of NADPH oxidases to produce superoxide to clear pathogens/fibers (Dostert and Petrilli, 2008, Hansen and Mossman, 1987). In the case of asbestos fibers however, the superoxide produced serves to reduce Fe³⁺ ions on the fiber surface and to Fe²⁺ which can also reduce molecular oxygen to superoxide (Wang et al., 2006) leading to the overproduction of superoxide. Infiltrating neutrophils also express inducible nitric oxide synthase and produce large amounts of NO into the extracellular milieu in response to the foreign bodies (i.e. fibers) they encounter (Turci et al., 2012). Under these conditions, the superoxide and NO present can then react to yield peroxynitrites which can attack and damage DNA by forming 8-nitrosoguanine adducts (Zhu et al., 1998, Iwagaki et al., 2003, Murata et al., 2012).

**1.3.1 Effects of Asbestos-Induced Oxidant Generation**

Asbestos fibers have been shown to activate the epidermal growth factor (EGFR) in a ligand-independent manner which was suggested to be more potent than the engagement of the receptor by its endogenous ligands (Zanella et al., 1999, Taylor et al., 2013). Due to the persistence of asbestos fibers and their ability to rapidly bind and dissociate from the
EGFR (Taylor et al., 2013), activation of EGFR by asbestos leads to a sustained activation of the EGFR signaling cascade that is much longer than signaling in response to EGF and other endogenous ligands. Under normal EGFR signaling conditions, hydrogen peroxide (H$_2$O$_2$) is produced in minute quantities as a transient signaling molecule (Hansen et al., 2006, Su et al., 2012). In the presence of asbestos however, sustained signaling by the EGFR would lead to an increase in H$_2$O$_2$ levels in excess of the amount needed for signaling. Whereas H$_2$O$_2$ is normally reduced to two molecules of water by peroxiredoxins or catalase (Goth, 2006, Rhee and Woo, 2011), the production of H$_2$O$_2$ in excess of what the peroxiredoxins or catalase can quickly convert to water, combined with the presence of Fe$^{2+}$ on asbestos fibers will promote Fenton reactions which lead to the generation of the hydroxyl radical (OH$^-$) (Miller et al., 2014). Thus, through the direct or indirect generation of ROS and other reactive species asbestos can promote the destruction of lipid membranes via lipid peroxidation, irreversible oxidation of essential enzymes and DNA damage. One indirect way by which asbestos skews the antioxidant/oxidant balance is through the inhibition of the pentose phosphate pathway. Studies have shown that asbestos-induced oxidants inhibit the rate limiting step of the pentose phosphate pathway by oxidizing glucose-6-phosphate dehydrogenase (G6PD) (Riganti et al., 2002, Riganti et al., 2003). G6PD is required for production of ribulose 5-phosphate for replenishing the pool of reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH provides reducing equivalents for the regeneration of a number of antioxidant proteins including thioredoxin, glutaredoxin and their respective reductases (Berndt et al., 2007). Thus, by inhibiting the PPP and subsequently NADPH, asbestos-induced oxidants further tip the
antioxidant/oxidant scale in favor of oxidants. Asbestos-induced oxidant dependent destruction can lead to the activation of apoptosis via the tumor suppressor, p53 and protein kinase C delta (PKCδ) (Shukla et al., 2003d) or via the activation of tumor necrosis factor alpha (TNFα) (Heintz et al., 2010). Paradoxically, asbestos can cause both cell death and cell proliferation. The key deciding factor is the dose of asbestos delivered. While the dose of asbestos reaching lung epithelial or macrophages and mesothelial cells cannot be determined in real time, it is fair to concede that cells at different locations will receive different doses of asbestos. Long asbestos fibers have long been known to have a higher potential for promoting disease and oxidative stress (Donaldson et al., 2010, Murphy et al., 2011). These fibers cannot pass through the stomata of the pleural mesothelium and thus get lodged in them, potentially increasing the local concentration of asbestos at such loci (Murphy et al., 2011, Murphy et al., 2012, Murphy et al., 2013). Macrophages unable to engulf long asbestos fibers also fuse with other macrophages to engulf the fibers successfully and this sometimes leads to the aggregation of unsuccessful cells on fibers (Donaldson et al., 2013). In such situations cell death may ensue not because of a high dose of asbestos but due to frustrated phagocytosis. So, while some cells may die due to differences in cell death programs initiated in response to asbestos, areas of cells with lower levels of asbestos which are still producing lower levels of oxidants can activate mitogenic pathways that lead to cell survival and proliferation. High asbestos doses on the other hand, promote the release of large amounts of oxidants that promote glutathione depletion, necrosis and apoptosis (Shukla et al., 2003a, Liu et al., 2010). The apoptosis observed in such cases has been attributed to the activation of p53 in response to DNA damage and was
shown to be attenuated with the introduction of iron chelators and antioxidants (Aljandali et al., 2001, Broaddus et al., 1996, Shukla et al., 2003b, Panduri et al., 2006).

Asbestos exposure induces chronic inflammation as macrophages and neutrophils attempt to clear the fibers and die in the process. The death of these cells releases chemokines and other proinflammatory cytokines into the extracellular milieu thereby attracting more inflammatory cells, setting up a vicious cycle of death of inflammatory cells and release of inflammatory molecules and the accompanying release of oxidants that cause DNA damage. The generation of peroxynitrites in response to asbestos exposure leads to the formation of 8-nitroguanine adducts (Chao et al., 1996, Fung et al., 1997, Hiraku et al., 2010, Hiraku et al., 2014). When these adducts are not corrected, DNA replication errors occur causing mutations as the 8-nitrosoguanine is wrongly paired with adenine and eventually a guanine to thymine transversion occurs in whichever gene locus affected is eventually after subsequent rounds of replication. The 8-nitroguanine adduct is actually used as a marker for chronic inflammation (Wang et al., 2016, Ohshima et al., 2006), which is one of the predominant effects of asbestos exposure. Oxidative DNA damage induced by asbestos exposure also affects mitochondrial DNA and this happens at much lower concentrations of asbestos than is required to cause nuclear DNA damage (Shukla et al., 2003b). In addition to direct DNA adduct formation, the DNA replication machinery can also be negatively affected by asbestos-induced oxidant generation and lead to errors in replication (Huang et al., 2011). Evidence suggests that asbestos exposed individuals have higher levels of 8-hydroxydeoxyguanosine (8-OHdG) adducts present in their DNA than non-exposed individuals (Marczynski et al., 2000, Hiraku et al., 2014, Chen et al., 1996).
providing further proof that asbestos exposure causing oxidative DNA damage that can lead to the development of mutations in various genes and potentially contribute to the development of asbestos-induced MM.

Several signaling pathways (apoptosis, inflammatory cytokine secretion and signaling, proliferation, etc.) impinge on mitogen activated protein kinase (MAPK) activation (Shukla and Mossman, 2008). Asbestos activates all of these pathways and more importantly, asbestos activates a number of MAPK proteins (extracellular signal regulated kinases 1, 2 and 5 (ERK 1, 2 & 5) to name a few) (Scapoli et al., 2004, Zanella et al., 1996, Shukla et al., 2013) and by extension their downstream effectors (Shukla and Mossman, 2008, Heintz et al., 2010). Two transcription factors important in asbestos-induced pathogenesis, activator protein 1 (AP-1) and nuclear factor kappa B (NFκB) are both redox sensitive and act downstream of MAPK activation (Heintz et al., 2010). AP-1 and NFκB both promote inflammation, cell proliferation, transformation and survival, are activated by asbestos (Flaherty et al., 2002, Janssen et al., 1997), and have been implicated in playing those roles in the context of asbestos-induced diseases (Heintz et al., 2010).

Several inflammatory processes are activated either directly or indirectly by asbestos related oxidants. Chrysotile-induced mitochondrial oxidants altered the expression of 178 genes associated with inflammatory cascades at both the transcriptional and effector protein levels (Huang et al., 2012). Massive parallel sequencing of primary peritoneal and pleural mesothelial cells exposed to crocidolite asbestos also yielded results indicating that the greatest change in gene expression was in genes found in inflammatory networks, many of which have been implicated in some way in MM development (Dragon et al., 2015).
Several of these genes are regulated by the redox sensitive transcription factors, NFkB and AP-1. While oxidants generated in response to asbestos activate inflammatory networks, the nitrative DNA adducts also provide evidence for the generation of oxidants as a result of chronic inflammation activated in response to the asbestos fibers (Shukla et al., 2003a, Chen et al., 1996, Fung et al., 1997), thereby perpetuating a never ending vicious cycle of oxidant activated inflammation and inflammation related oxidant generation and cellular damage. One other inflammatory cascade that is affected by asbestos-induced oxidant generation and the antioxidant/oxidant imbalance is the inflammasome (Thompson et al., 2014a, Zhou et al., 2010). Oxidation of the cellular antioxidant redox protein, thioredoxin (Trx) leads to the release of its endogenous inhibitor, thioredoxin interacting protein (TXNIP) from Trx (Thompson et al., 2014a, Nishiyama et al., 1999). Association of TXNIP with the inflammasome has been shown to be required for inflammasome activation and important for the pathology of diseases like diabetes (Oslowski et al., 2012) and atherosclerosis (Byon et al., 2015). The inflammatory responses elicited by asbestos play an important role in in the development of almost all the asbestos related diseases (both malignant and non-malignant) and they share the same long latency period albeit with variations. It is currently not known if MM progresses from pleural fibrosis as some patients with pleural fibrosis may develop MM but not all fibrosis/asbestosis patients develop MM.
1.4 Asbestos-Related Diseases

The commonest asbestos-related diseases are pleural in nature and range from benign pleural plaques and benign (or sometimes malignant) pleural effusions to pleural fibrosis, asbestosis, and lung cancer (Manning et al., 2002, Norbet et al., 2015, Prazakova et al., 2014). The less common diseases include the fatal disease MM (primarily pleural and peritoneal, and to lesser extent, pericardial mesothelioma) (Mossman et al., 2013), the rare asbestos-induced IgG-related disease (Onishi et al., 2016), as well as autoimmune diseases that affect the joints (Pfau et al., 2014). The presentation and outcomes of these diseases vary. At times, the disease goes undiagnosed and is only observed post-mortem upon autopsy after death from an unrelated cause.

1.4.1 Pleural Plaques

Pleural plaques, the commonest asbestos-related disease, arises predominantly on the parietal pleura (O'Reilly et al., 2007). These plaques are distinct areas of fibrosis that can be found classically on the dome of the diaphragm, on the back wall of the chest between the seventh and tenth ribs, as well as laterally between the sixth and ninth ribs (Peacock et al., 2000, Becklake et al., 2007, Norbet et al., 2015). Pleural plaques can be diagnosed with a chest x-ray depending on the position of the plaque (Peacock et al., 2000). In addition, examination of such plaques can reveal the presence of asbestos fibers. The plaques are believed to develop as part of an inflammatory response to asbestos fibers (Manning et al., 2002). Although calcification of asbestos-induced pleural plaques is uncommon, there are reports of some calcification in about 10-15% of pleural plaques (Norbet et al., 2015). Pleural plaques are generally presumed to be benign but some
patients experience a small reduction in lung function as well as neuropathic pain (Becklake et al., 2007, Norbet et al., 2015).

1.4.2 Pleural Effusion
The accumulation of fluid in the pleural space of individuals with a history of asbestos exposure may be a benign or malignant manifestation of asbestos-related disease (Prazakova et al., 2014, Manning et al., 2002). As with all asbestos-related diseases, there is a long latency period. Pleural effusions are typically observed within 10-20 years after initial asbestos exposure (Myers, 2012, O'Reilly et al., 2007). However, since other conditions can lead to the development of pleural effusions, diagnosis depends heavily on the exclusion of all other potential causes (O'Reilly et al., 2007). Due to the association of MM with pleural manifestation, a waiting period of 3 years without the manifestation of a neoplasm is required before a pleural effusion incident is deemed benign (Peacock et al., 2000). As such, thorascopic examination of the pleural space and cytological analysis of the effusion is performed to ascertain the presence of malignant cells and asbestos fibers (Peacock et al., 2000). Like many other diseases, the level of morbidity associated with pleural effusions depends on the amount of the effusion and the time it takes to resolve.

1.4.3 Asbestosis
Asbestosis, also known as asbestos-induced pulmonary fibrosis, is an interstitial lung disease that causes fibrosis of the lung parenchyma and honeycombing (Norbet et al., 2015, Manning et al., 2002). In some cases, there are calcifications of the lung interstitia. Asbestosis shares characteristics with idiopathic pulmonary fibrosis and severely compromises lung function, leading to dyspnea and drastic reduction in lung flow volume.
Asbestosis generally occurs within 10 to 30 years after asbestos exposure as a result of unresolved inflammation in response to the presence of long thin asbestos fibers in the small airways and alveoli (Manning et al., 2002). The inflammation causes scarring and thickening of the interstitia and subsequent loss of elasticity of the type II alveoli epithelium, thereby compromising lung function (Norbet et al., 2015).

1.4.4 Lung Cancer
Inhalation of asbestos can also lead to the development of lung cancer. While the majority of lung cancers are related to smoking, asbestos exposure accounts for about 3-8% of all lung cancer cases (Prazakova et al., 2014). Asbestos exposure is believed to increase the risk of developing lung cancer in smokers by as much as 4- to 9-fold (Ngamwong et al., 2015, Swiatkowska et al., 2015). Like other asbestos-associated diseases, asbestos-related lung cancer has a long latency period (20-40 years after asbestos exposure) (Manning et al., 2002, Ngamwong et al., 2015, Swiatkowska et al., 2015). For a diagnosis of lung cancer to be attributed to asbestos exposure, however, the patient has usually been heavily exposed to asbestos with an exposure rate of about 25 fibers/mL per year or more (Prazakova et al., 2014, Roggli and Sporn, 2016). In addition to the presence of asbestos fibers in the lung interstitium, presentation with lung cancer must be found to occur more than 10 years after exposure in order to establish causality (Norbet et al., 2015). In a number of cases of asbestos-related lung cancer, there is concurrent asbestosis. Asbestos-related lung cancer cannot be differentiated phenotypically from lung cancer in non-asbestos exposed individuals, but there appears to be a propensity for this type of lung
cancer to develop in the lower lung compared to non-asbestos related lung cancer (Inamura et al., 2014, Manning et al., 2002). Studies have shown that cumulative asbestos exposure is proportionally related to the development of asbestos-related lung cancer (Inamura et al., 2014).

1.4.5 Malignant Mesothelioma
The deadliest disease attributed to asbestos exposure is MM, which arises in the serosal lining (mesothelium) of the pleura, peritoneum and sometimes the pericardium (Manning et al., 2002, Mossman et al., 2013). Even rarer is the occasional incidence of MM in the tunica vaginalis of the testis (Jankovichova et al., 2015, Trpkov et al., 2011, Chekol and Sun, 2012). As with all asbestos-related diseases, MM has a long latency period (10-40 years) (Mossman et al., 2013). MM is considered an occult cancer that is difficult to detect until it is advanced enough to cause appreciable symptoms. Symptoms usually include chest pain, coughing, dyspnea, pleural effusions (in the case of pleural MM), pain and discomfort with distention of the abdomen due to ascites build up, and night sweats/fever (in peritoneal MM) (Moore et al., 2008, Prazakova et al., 2014). Thin and long asbestos fibers are believed to translocate to the mesothelium outside of the lung, by mechanisms that are still poorly understood, ultimately causing MM in the pleura or peritoneum (Mossman et al., 2013, Murphy et al., 2013). In addition to asbestos fibers, erionite—a non-asbestos fiber—has been shown to be more mesotheliomagenic and is responsible for a MM epidemic in the Cappadocia region in Turkey (Heintz et al., 2010, Dikensoy, 2008). Erionite has been found in different areas here in the United States and has the potential to cause cases of MM because of environmental exposure.
1.5 Mesothelial cells

The mesothelial cell is a unique, squamous epithelial-like cell that is derived from a mesodermal origin (Mutsaers, 2002). They possess both epithelial and mesenchymal characteristics; they display apico-basal polarity, express cytokeratins and form cell-cell junctional complexes (Mutsaers, 2004). Mesothelial cells form a single cell layer known as the mesothelium with a loose basement layer which covers the internal surfaces of the body wall (parietal surface) and the external surfaces of all organs in the serosal cavities (visceral surface) (Mutsaers, 2002). The function of the mesothelium includes the production of glycosaminoglycans for lubrication to prevent the adhesion of organs; hyaluronan production for trapping microbes and also preventing adhesion; antigen presentation for the infiltration of immune cells in the case of infections; cytokine and chemokine secretion as part of an inflammatory response and for the recruitment of immune cells (Mutsaers, 2002, Mutsaers, 2004, Mutsaers et al., 2015). The secretion of extracellular matrix and growth factors by mesothelial cells also aids in their maintenance of the homeostasis of the serosal cavity (Mutsaers, 2002). The regulation of coagulation, fibrinolysis and tumor cell adhesion are also an important function of the mesothelium (Mutsaers et al., 2015). Self-renewal of the mesothelium is a very slow process; at any given time only 0.16-0.5% of mesothelial cells are undergoing mitosis (Mutsaers, 2004). Unlike epithelial cells, mesothelial cells tend to overlap with adjacent cells making it difficult to follow the boundaries of individual cells. On average, a mesothelial cell is about 25 µm in length (Mutsaers, 2002) but only measure about a micron in thickness (Figure
1.1). The luminal surface of the mesothelium is covered in microvilli (Figure 1.2) that help trap the glycosaminoglycan and proteins that form the glyocalyx of the mesothelium [reviewed in (Mutsaers, 2002)]. The function of a mesothelial cell, its state of activation as well as its location affect the observed phenotype of this cell type (Mutsaers et al., 2015). For example, activated mesothelial cells can appear raised and elongated (Mutsaers, 2002). When there is injury to the serosal surface resulting from surgery or denudation of the mesothelial layer due to other factors, repair of the breach is not simply through growth at the edge of injury. Mesothelial cells from distant sites can undergo desquamation and these free floating cells can then migrate to and seed the site of injury and proliferate to repair the damage in addition to growth at the edge of the wound (Mutsaers, 2002, Mutsaers et al., 2015, Mutsaers et al., 2016).

Figure 2.1: Scanning electron micrograph of mouse mesothelial cells cultured from mouse peritoneal wall explants. Small pieces of peritoneal wall were transferred to sterile coverslips placed in a 12 well dish. Cells were fixed, dehydrated and prepared for SEM imaging after 2 weeks in culture.
Figure 1.2: Scanning electron micrograph of mouse parietal mesothelium. A section of the peritoneal wall of wild type mice receiving saline over 8 weeks was oriented mesothelial side up and fixed for SEM. Samples were sputter coated prior to imaging.

In response to injury mesothelial cells also express elevated levels of tissue factor (TF) and other procoagulant factors to promote fibrin deposition (Mutsaers et al., 2015). Fibrin deposition helps in resolution of the injury to the mesothelium but its clearance is required for proper restoration of serosal homeostasis in order to prevent adhesions. To this end, mesothelial cells also secrete tissue factor pathway inhibitor (TFPI) to inhibit the formation of fibrin via the extrinsic coagulation pathway while upregulating the secretion of fibrinolytic enzymes and their activators such as tissue plasminogen activator, plasminogen, urokinase (uPA) and its receptor, uPAR. To regulate fibrinolysis, mesothelial cells also produce plasminogen activator inhibitors, tissue inhibitors of metalloproteinases (TIMPs) as well as tissue factor pathway inhibitor 2 (TFPI2) (Mutsaers et al., 2015). TFPI2
indirectly regulates the activity of matrix metalloproteinases by inhibiting the activation of proteinases such as plasmin and trypsin, among others (Chand et al., 2005, Baker et al., 2002). Interestingly TFPI2 also promotes activation of pro-MMP2 through an unknown mechanism and this function of TFPI2 is thought to promote vasculogenic mimicry in non-vascular cells and some tumor cell lines (Ruf et al., 2003). A careful balance between fibrin deposition and fibrinolysis is maintained by the mesothelium (Mutsaers et al., 2015) and disruption of this balance has been shown to be important in mesothelioma, fibrosis and organ adhesion (Williams et al., 2012, Mutsaers et al., 2016). During injury to the serosal surface, mesothelial cells downregulate TFPI expression in response to thrombin by acting through NFκB thereby promoting coagulation through the tissue factor pathway (Jeffers et al., 2015). Conversely, thrombin may upregulate TFPI2 expression to prevent fibrinolysis through the same pathway as has been observed in macrophages treated with thrombin (Pou et al., 2011) to inhibit fibrinolysis. Thus, if for any reason fibrinolysis is not initiated and fibrin deposition continues after resolution of the injury or insult, this imbalance in fibrinolysis and fibrin deposition may promote fibrosis or organ adhesions at a later stage (Mutsaers et al., 2016).

Infections, asbestos fibers and high glucose peritoneal dialysis (PD) fluid also elicit an inflammatory response in the mesothelium (Hillegass et al., 2013, Mutsaers et al., 2015, Yung and Chan, 2012). Mesothelial cells are phagocytic and take up pathogens for antigen presentation (Shaw et al., 2016) and also attempt to clear asbestos fibers by phagocytosing them (Jaurand et al., 1979). The advanced glycation end products formed from the glucose in the dialysate engage the receptor for advanced glycation end products (RAGE) to
promote inflammation and peritonitis in patients undergoing PD (Zhou et al., 2016). Thus mesothelial cells play an active role in maintaining homeostasis in the serosal cavity and serve as a line of defense against foreign and infectious agents. Disruption of the function of the mesothelium is deleterious to serosal health and proper organ function.

1.6 Asbestos, Inflammation and Asbestos-Related Diseases

All types of asbestos elicit an immune response in the cells they come into contact with, either directly or indirectly through the release of danger associated molecular pattern molecules from cells they encounter (Ballan et al., 2014, Donaldson et al., 2010, Haegens et al., 2007). The amphibole asbestos fibers are bio-persistent compared to the serpentine asbestiform, chrysotile, which can be degraded by bodily fluids and cells due to its more compressible fibril nature and the leaching of magnesium from the fibers (chemical composition: \( \text{Mg}_3\text{Si}_2\text{O}_5(\text{OH})_4 \)) (Manning et al., 2002). A recent review by Acencio et al. (Acencio et al., 2015) clearly summarized the role of inflammatory cytokines in chrysotile- and crocidolite-induced mesothelial cell injury leading to diseases. Studies from our group have also demonstrated that asbestos can cause increased gene expression and protein levels of various inflammatory cytokines (IL-13, bFGF, VEGF, GSF) from mesothelial cells (Shukla et al., 2009) and that many of these cytokines are required for MM development in mice (Hillegass et al., 2010a). Chrysotile asbestos has been shown to elicit an acute immune response 3 and 9 days post inhalation of this fiber type (Haegens et al., 2007). In an examination of mice exposed to chrysotile asbestos for 6 hours a day, an increase in the number of immune cells retrieved from the lungs through bronchoalveolar lavage showed an increase in infiltrating cells in the asbestos-exposed mice when
compared to sham-exposed mice (Haegens et al., 2007). Increases in the number of infiltrating eosinophils and neutrophils were observed, and the number of lymphocytes increased after 9 days of exposure (Haegens et al., 2007).

Amphibole asbestos fibers are generally regarded as the main culprits behind MM, although chrysotile asbestos has also been shown to have mutagenic capabilities and is linked to MM development (Chew et al., 2014, Chew and Toyokuni, 2015, Kanarek, 2011). Among the amphiboles, crocidolite asbestos is classified as the most carcinogenic (Dopp et al., 2005, Ballan et al., 2014), and studies into reactive oxygen species (ROS) production, cell signaling and genotoxicity have generally been carried out using crocidolite or amosite (Dopp et al., 2005). Crocidolite asbestos elicits an acute immune response, and because of its biopersistence can cause chronic inflammation by recruiting inflammatory cells which then die in the process of attempting to clear the asbestos fibers (Moolgavkar et al., 2001). The death of such immune cells then elicits a new wave of inflammatory cell influx, thus leading to a never-ending cycle of inflammation in response to the presence of the fibers. Amphiboles such as amosite and crocidolite also contain iron in their crystal structures (\([(\text{Mg, Fe})_7\text{Si}_8\text{O}_{22}(\text{OH})_2]_n\) and \([\text{NaFe}^{2+}\text{Fe}^{3+}_2\text{Si}_8\text{O}_{22}(\text{OH})_2]_n\), respectively), and the valency state of these iron moieties determines their ability to participate in Fenton reactions (Dopp et al., 2005). These Fenton reactions promote the production of reactive species such as the hydroxyl ion (Dopp et al., 2005, Mossman et al., 2011). Regulators of expression of various cytokines (transcription factors, e.g., NFκB and AP-1) are affected by the redox state of the cell (Shukla et al., 2003c). Raising the oxidation state or altering the redox balance of the cell can thereby lead to their aberrant
activation or inhibition, resulting in the production of more inflammatory cytokines in response to asbestos exposure. Exposure to crocidolite/amosite has been shown to increase the production and secretion of a number of inflammatory cytokines, including but not limited to IL-1β, IL-5, IL-6, IL-8, and IL-18 (Haegens et al., 2007, Hillegass et al., 2013).

While tracheal instillation of asbestos into mice has been shown to result in death of epithelial and mesothelial cells as well as alveolar macrophages, some of these cells appear to escape such death and survive longer than cells from control animals or cells unexposed to asbestos (Nishimura et al., 2013). Alveolar macrophages that survive chrysotile asbestos exposure have been reported to live longer and secrete increased amounts of the fibrogenic factor TGFβ, leading to the conclusion that such alterations contribute directly to the fibrosis observed in response to asbestos exposure (Nishimura et al., 2013, Nishimura et al., 2007, Sullivan et al., 2008). These inflammatory responses to asbestos have been observed both in vitro and in vivo. Recent studies of patients with history of asbestos exposure have shown that these patients have elevated levels of IL-8 and other cytokines, as well as their respective receptors (Comar et al., 2016, Comar et al., 2014). Levels of vascular endothelial growth factor (VEGF), platelet derived growth factor b (PDGFb) and basic fibroblast growth factor (bFGF) were also significantly increased upon exposure to asbestos and further increased in some cases with disease (Comar et al., 2016). VEGF and bFGF are both angiogenic factors that stimulate proliferation of mesothelial cells (Comar et al., 2016, Strizzi et al., 2001, Van et al., 2012) and alter the permeability of the mesothelium (VEGF) (Hillegass et al., 2013, Strizzi et al., 2001).
PDGFβ has been shown to promote the growth of fibroblasts and the progression of fibrosis in lung disease (Safi et al., 1992).

Asbestos fibers can cause systemic changes in inflammatory cytokines (Gavett et al., 2016, Fukagawa et al., 2008, Dragon et al., 2015), and these changes can lead to the creation of a pro-tumorigenic environment (Okada, 2014). The initial site of contact for inhaled fibers is the lung, where alveolar macrophages and epithelial cells encounter the inhaled asbestos. In as early as 3 days after asbestos exposure, the inflammatory profile in the lung changes. An increase in the number of infiltrating eosinophils and neutrophils is observed (Haegens et al., 2007). As cells attempt to clear the foreign objects, the high aspect ratio of the asbestos fibers leads to frustrated phagocytic events that promote death of the phagocytic cells. Consequently, greater numbers of inflammatory cells are recruited to the site of injury. An overproduction of collagen by fibroblasts recruited to these sites of injury promotes parenchymal fibrosis, because the injury is not resolved due to the continued presence of the asbestos fibers. As cells attempt to clear asbestos fibers and resolve the injury, more and more extracellular matrix is deposited and stromal cells are recruited. These may be the underlying contributing factors to the development of asbestosis and potentially lung cancer (Heintz et al., 2010).

Asbestos fibers have been found to translocate to the interpleural space by mechanisms that are still incompletely understood. Studies indicate that asbestos or carbon nanotubes 10 µm or greater in length cause acute inflammation and fibrosis on the parietal surface of the pleural mesothelium in a length dependent manner due to an inhibition of clearance through the pleural stomata (Figure 1.3) (Murphy et al., 2011, Schinwald et al.,
It has been proposed that the length of a fraction of the asbestos fibers interferes with their clearance through the lymphatic drainage, as they cannot go through the pleural stomata. As a result, fibers within a particular length range accumulate on the parietal pleura, leading to the formation of pleural plaques and pleural fibrosis by propagating chronic inflammation and injury to the mesothelium (Donaldson et al., 2010). Recent studies have shown that asbestos-like fibers such as carbon nanotubes are capable of eliciting the same kind of inflammatory and fibrotic responses in the pleural mesothelium as does asbestos (Murphy et al., 2011, Murphy et al., 2012, Nagai and Toyokuni, 2010). Asbestos bodies are not always associated with pleural plaques, suggesting that an intermediate inflammatory mediator may be involved. Macrophages, mesothelial cells and other phagocytic cells undergo frustrated phagocytosis while attempting to clear these high aspect ratio fibers and secrete a host of inflammatory mediators. Thus, pleural macrophages secreting a number of inflammatory cytokines may also play a role in facilitating inflammation-induced injury and repair in the pleura that could promote asbestos-related disease formation (Mossman et al., 2011). One study has shown that supernatants from macrophages exposed to long carbon nanotubes serve as potent activators of mesothelial cells and significantly increase their secretion of inflammatory cytokines such as IL-1β and eotaxin (Murphy et al., 2012). This same phenomenon has been demonstrated for asbestos as well. Studies indicate that one of the inflammatory cascades activated by asbestos in macrophages and mesothelial cells is the activation of the inflammasome (Dostert et al., 2008, Hillegass et al., 2013). The inflammasome is a special component of inflammation, where NOD-like receptor containing pyrin domain 3
(NLRP3), a cytoplasmic protein complex, senses fibers. This results in caspase-1 activation and secretion of the pro-inflammatory cytokines IL-1β and IL-18. There are several NOD-like receptors reported in the literature; however, only activation of the NLRP3 inflammasome in macrophages and mesothelial cells by asbestos has been demonstrated to increase secretion of IL-1β, IL-18 and other pro-inflammatory cytokines as a result of IL-1β signaling (Dostert et al., 2008, Hillegass et al., 2013). Additionally, asbestos-induced inflammasome activation can lead to the release of a pro-inflammatory danger-associated molecule, high mobility group box 1 (HMGB1), from mesothelial cells, which is also a potent inducer of inflammation (Hillegass et al., 2013, Yang et al., 2010, Carbone and Yang, 2012).

Inflammasome activation induced by asbestos plays an important role in the development of MM when coupled with genetic predisposition and cumulative exposure to asbestos (Kadariya et al., 2016b, Hillegass et al., 2010a). In contrast, a recent study of MM tumor cells and tissue arrays showed attenuated levels of the NLRP3 inflammasome and caspase-1 activation (Westbom et al., 2015). Treatment of these tumors with cisplatin caused increased NLRP3 priming and activation. To the contrary, treatment of tumors with a combination of cisplatin and an IL-1 receptor antagonist resulted in better tumor reduction as compared to cisplatin alone. These findings again point towards a tumor promoting role of IL-1β, a pro-inflammatory cytokine (Westbom et al., 2015). Although there are various ways through which the inflammasome is activated by asbestos, NADPH oxidase and thioredoxin interacting protein (TXNIP) appear to play prominent roles (Thompson et al., 2014a, Dostert et al., 2008). This attests to the role of ROS in
inflammasome regulation. The inflammatory profile of patients with MM has been recently suggested to offer insights into the interactions between a tumor and its microenvironment, which promote tumorigenesis (Judge et al., 2016). Analysis of peritoneal ascites fluid from patients with peritoneal MM showed localized elevation in a number of pro-inflammatory cytokines (IL-6, IL-8, MCP-1, MIP-1β, MIP1α, IL-10, VEGF and TNFα) when compared to serum levels from the same patients (Judge et al., 2016). In vivo and in vitro studies of the growth of MM from our group have also demonstrated that inflammation is an active component of MM growth in SCID mice (Hillegass et al., 2010a, Westbom et al., 2014). The risk of developing MM has been shown to rise with increased exposure to asbestos (Mossman et al., 2013, Landrigan and Collegium, 2016). In a small fraction of patients, germline mutations of the BRCA1 associated protein 1 gene (BAP1) have been shown to increase the risk of developing MM even at lower levels of asbestos exposure (Napolitano et al., 2016, Ohar et al., 2016, Testa et al., 2011). It is, however, interesting to note that in a heterozygous BAP1-mutant murine model, exposure to low doses of asbestos led to a stronger inflammatory response and higher incidence of MM when compared to their wild type littermates (Napolitano et al., 2016). This suggests that the higher inflammatory response may play a crucial role in the development of MM in patients with BAP1 mutations as well, indicating a putative link between the function of BAP1 and inflammatory responses (Napolitano et al., 2016).

With the postulated hypothesis of how a fraction of longer asbestos fibers are deposited on the parietal pleura over time, the observation that MM originates from the parietal pleura before spreading to the visceral pleura is somewhat explained (Donaldson
et al., 2010, Murphy et al., 2011). Asbestos activates a number of signaling cascades that are involved in the regulation of inflammatory responses, and this may play a large role in the sustained inflammatory response to asbestos fibers that eventually lead to the development of the various asbestos-related diseases (Shukla et al., 2003c, Nishimura et al., 2013, Haegens et al., 2007, Heintz et al., 2010). In fact, our next-generation sequencing studies of primary mesothelial cells indicate that asbestos exposure predominantly upregulates inflammatory networks (Dragon et al., 2015). Such chronic inflammation eventually leads to DNA damage and may be responsible for the many deletions and mutations observed in MM. Inflammation induced by asbestos has been shown to promote the formation of 8-oxoguanine adducts in nuclear and mitochondrial DNA (Fung et al., 1997, Okada, 2007). The formation of a number of nitrosylation products, including 8-nitroguanine, also results from the generation of ROS as a combined result of Fenton reactions facilitated by asbestos fibers and the induction of the nitric oxide synthase enzyme by inflammatory cells (Fung et al., 1997, Okada, 2007, Hiraku et al., 2010). Thus, not only does asbestos cause mechanical injury to the mesothelium that can lead to aberrant repair, but its chemical composition also promotes inflammation via the recruitment of inflammatory cells (Shukla et al., 2003c, Shukla et al., 2007, Okada, 2007). Asbestos exposure also leads to the activation of cellular pathways that are sensitive to the redox state of the cell and involved in the regulation of inflammation (Thompson et al., 2014a, Shukla et al., 2003c).

A recent study of asbestos-exposed workers from an Italian coastal area showed the presence of elevated chemokines and pro-inflammatory cytokines in serum of asbestos-
exposed workers as compared to healthy controls (Comar et al., 2016). Inflammatory parameters have been suggested to have prognostic significance in MM. For example, lymphocyte-to-monocyte (LMR) ratio (Yamagishi et al., 2015) or neutrophil-to-lymphocyte (NLR) ratio along with red cell distribution width (RDW) (Abakay et al., 2014) could be projected as predictive factors for MM prognosis. Furthermore, inhibition of inflammation by natural compounds (Benvenuto et al., 2016, Pietrofesa et al., 2016) has also been shown to inhibit asbestos-induced inflammation and carcinogenesis in animal models, confirming the role of inflammation in asbestos-induced diseases.
Figure 1.3: Inflammation plays a role in asbestos-related diseases. Asbestos fibers first encounter bronchial epithelial cells when inhaled, and long fibers may become lodged at bifurcations in the bronchi and lead to chronic inflammation at those sites that recruits inflammatory cells. Such chronic inflammation can lead to the development of bronchogenic diseases at those sites. In the lung parenchyma, long and thin asbestos fibers can travel deeper and interact with alveolar macrophages, which will attempt to clear these fibers. While fibers that are short enough to be completely engulfed are cleared, longer fibers will cause frustrated attempts of clearing, leading to more inflammation. The production of various signaling and inflammatory molecules in response to this assault over time contributes to the interstitial lung diseases observed in response to asbestos. Longer asbestos fibers have been found to accumulate on the parietal pleura and are also believed to become lodged in the stomata of the mesothelium at those points where they may potentiate chronic inflammation that contribute to pleural asbestos diseases. It also appears that events occurring in the lung may influence or affect disease in the parietal pleura and vice versa. Taken from Thompson et al. (in press) Springer-Verlag Heidelberg- Book Chapter
1.6.1 Inflammasomes
Inflammasomes are intracellular molecular multiprotein molecular scaffolds that assemble in response to various stimuli to promote the autocatalytic cleavage and maturation of the zymogen, caspase-1 (Martinon and Tschopp, 2007, Lamkanfi et al., 2007). Activation of caspase-1 leads to its processing of the pro-inflammatory cytokines, IL-1β and IL-18 (Launay et al., 2005, Martinon et al., 2007). Inflammasomes are made up of a pattern recognition receptor (PRR), an adaptor protein (apoptosis speck-like protein containing a card domain (ASC)) and the inflammatory caspase, caspase-1 (Martinon and Tschopp, 2007). The name of a given inflammasome is often derived from the PRR that serves as the scaffold to which ASC and caspase-1 are recruited. There are four different families of inflammasomes that contain nucleotide oligomerization domain (NOD) – like receptors (NLRs) as the nucleating PPR on which the inflammasomes assemble. The four family members, NLR containing a pyrin domain (NALP/NLRP), interleukin-1 converting enzyme (ICE) protease activating factor (IPAF), NLR apoptosis inhibiting protein (NAIP) and nucleotide binding oligomerization domain containing protein 2 (NOD2) make up the subgroups into which the approximately 22 NLR proteins are classified (Martinon et al., 2007, Benko et al., 2008). The NLRP/NALP family is the largest group with 14 members and contains the most studied inflammasome to date, the NLRP3 inflammasome. NLRP3 is a unique inflammasome in that it responds to a wide variety of stimuli, unlike the other inflammasomes that typically respond to one or two types of ligands (Benko et al., 2008, He et al., 2016). For example, the NLR containing a CARD domain 4 (NLRC4) responds to pathogen associated molecular patterns (PAMPs) like flagellin, whereas NLRP3 is capable of responding to a number of PAMPs like lipopolysaccharide (LPS) and muramyl
peptides as well as endogenous danger associated molecular patterns (DAMPs) like ATP, in addition to crystalline/fibrous stimuli like uric acid crystals and asbestos (Benko et al., 2008, Sayan and Mossman, 2016, Martinon and Tschopp, 2007, Dostert and Petrilli, 2008). The NLRP3 inflammasome requires two signals for activation (Sutterwala et al., 2014, He et al., 2016). The first signal ‘primes’ the inflammasome by upregulating transcription of NLRP3 and IL-1β (He et al., 2016, Sutterwala et al., 2014). The second ‘activating’ signal serves to trigger oligomerization of NLRP3 and assembly of the inflammasome by recruitment of ASC through its pyrin domain (He et al., 2016, Martinon et al., 2007). ASC binds homotypically to the pyrin domain of NLRP3 and recruits caspase-1 through its caspase activating and recruiting domain (CARD) (Martinon et al., 2007). During assembly of the inflammasome, filaments of ASC also oligomerize (Dick et al., 2016) and recruit several units of procaspase-1 which then undergoes auto-proteolytic cleavage to become active. The whole process has been likened to the assembly of the apoptosome and also resembles a wheel when assembled. This multiprotein scaffold serves to amplify the signal and cause large quantities of IL-1β and IL-18 to be processed and secreted from activated immune cells (Dick et al., 2016). The activation of the inflammasome has been studied predominantly in immune cells, but certain non-immune cells also express inflammasome components and are capable of assembling an inflammasome in response to the appropriate stimuli, although they may not yield as much IL-1β or IL-18 as an immune cell.

The NLRP3 inflammasome has been shown to bear mutations that are responsible for inherited inflammatory diseases like Muckle-Wells syndrome, familial cold auto-inflammatory syndrome, and neonatal onset multisystem inflammatory disease (Aganna et
al., 2002, Dode et al., 2003). NLRP3 has also recently been implicated in inflammatory bowel disease (Chen and Nunez, 2011, Elia et al., 2015, Zaki et al., 2011). All of these suggest that aberrant activation of the inflammasome leads to chronic inflammation. Hence the need for such tight regulation of the activation of the NLRP3 inflammasome.

Chronic inflammation promotes fibrosis by triggering repair mechanisms and activating resident fibroblasts (Artlett, 2012). Under acute inflammatory conditions and injury, the activation of fibroblasts and proinflammatory cytokines that promote fibrosis is resolved upon repair of the injury or resolution of the inflammatory stimulus. When the inflammation becomes chronic or dysregulated however, the repair process that should normally allow for normal growth of tissue to replace dead cells or damaged tissue is corrupted, leading to the development of fibrotic lesions (Artlett, 2012). In non-alcoholic fatty liver disease activation of the inflammasome is necessary for disease progression and liver fibrosis (Wree et al., 2014). The inflammasome product, IL-1β, has been implicated in lung fibrosis as well as the induction of epithelial to mesenchymal transition in different cell types under chronic and acute conditions (Zhang et al., 1993, Kolb et al., 2001). When caspase-1 activity was inhibited in a model of systemic sclerosis myofibroblast differentiation was compromised with lower levels of IL-1β and IL-18, implicating the inflammasome and especially caspase-1 in the pathogenesis of systemic sclerosis (Artlett et al., 2011). In kidney tubular epithelial cells, NLRP3 and ASC have been shown to promote epithelial to mesenchymal transition independent of their inflammasome activity (Wang et al., 2013). In cardiac fibroblasts, mitochondrial localized NLRP3 promoted ROS generation and regulated the differentiation of the fibroblasts into myofibroblasts via
modulation of R-Smad (Bracey et al., 2014) as seen in kidney epithelial cells where NLRP3 also modulated TGFβ signaling via Smad2 (Wang et al., 2013). Thus, the inflammasome components individually as well as a part of the inflammasome play a role in driving cell differentiation and transition from one cell type to the other under varying conditions.

1.7 Mesothelial to Fibroblastic Transition/Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition, the process by which epithelial cells gain mesenchymal features and transition into mesenchymal cells is important during development and necessary for organogenesis. This process is under the control of several developmental transcriptional factors and genes that are both spatially and temporally regulated to ensure the proper development of the embryo. From the development of the hair follicle to the formation of blood vessels and digits, EMT is tightly regulated to ensure normal development (Botchkarev and Kishimoto, 2003, Reese et al., 2002). A number of growth factors regulate the process of EMT – transforming growth factor beta family members (TGFβ) (Wu and Zhou, 2010, Moustakas and Heldin, 2007), insulin growth factor (IGF) (Kalluri and Neilson, 2003) and epidermal growth factor (EGF) (Kim et al., 2016) as well as fibroblast growth factor 2 (FGF2) (Kalluri and Neilson, 2003) are among the growth factors that have been demonstrated to induce EMT. An epithelial cell undergoing EMT will degrade its junctional complexes and down regulate the expression of these proteins to enable it to become motile and move to the site where it is needed. The actin stress fibers in the transition cells will also need to reorganize and switch to more fibrous actin structures that enable lamellipodia and pseudopodia required for movement to form and retract in the direction of movement (Kalluri and Neilson, 2003). The
morphology and repertoire of proteins expressed by cells undergoing EMT changes. Changes in the expression of some of these have been identified as hallmarks of the EMT process. For a cell to be considered to be undergoing EMT, it is expected that protein levels of the adherens junctional protein, E-cadherin, (needed in a complex for adhesion of cells to each other) will be decreased to none among other junctional proteins. A number of transcription factors negatively regulate the expression of E-cadherin in addition to regulating the expression of a host of proteins related to mesenchymal and epithelial phenotype. Among these transcription factors a handful have been identified as hallmark EMT factors that are expected to have increased expression in EMT (e.g. ZEB1, Snail, Slug and TWIST1) (Dohadwala et al., 2010, Wu and Zhou, 2010). While all these transcription factors exhibit an inverse relationship between their expression and that of E-cadherin during EMT, cell type specific roles determine the important transcription factor that is upregulated (Kalluri and Neilson, 2003). In some cases, the growth factor or stimulus initiating EMT determines which transcription factor will be observed to be important for the process. One of these transcription factors, Snail family of transcription factors, has been shown to control global epithelial gene expression as well as regulate cell polarity and survival (Wu and Zhou, 2010). Stationary epithelial cells exhibit an apico-basal polarity, demonstrate a cuboidal/cobblestone appearance, mesenchymal cells lack polarity, are loosely arranged in the ECM, tend to have an elongated morphology and do not form cell-cell attachments and are less susceptible to cell death. As such transitioning epithelial cells also lose their apico-basal polarity and also start expressing matrix metalloproteinases
so that they can degrade the basement membrane that hold them in place, appear elongated and are loosely arranged with minimal to no cell-cell contacts.

EMT can be reactivated to produce fibroblasts in tissue repair after injury. In response to inflammatory factors that are released from the injured cells and infiltrating immune cells, fibroblasts may migrate towards the gradient of cytokines and factors released and cells at the site of injury may also undergo EMT to yield the needed fibroblasts/stromal cells (Kalluri and Neilson, 2003). Under such conditions however, the EMT program activated is short lived and reversed once repair is complete or the stimulus is removed (Kalluri and Weinberg, 2009). Deposited excess extracellular matrix is broken down to allow the tissue to return to its normal pre-injury state. If for some reason the fibroblast-like cells persist after repair of the injury they can promote fibrosis (Kalluri and Weinberg, 2009).

Mesothelial cells have been demonstrated to undergo a process similar to EMT during embryogenesis (Batra and Antony, 2014) and contribute to the generation of cardiac myocytes and vascular cells as well as parenchymal cells of the developing lung using lineage tracing studies (Herrick and Mutsaers, 2004, Rinkevich et al., 2012). Because mesothelial cells are not epithelial in nature, the process has been referred to as mesothelial to mesenchymal transition (MMT). Mesothelial cells cultured in osteogenic and adipogenic medium have also been shown to transition into osteoblast and adipocyte-like cells respectively (Lansley et al., 2011). As such, mesothelial cells may be considered a multipotent cell type. Another lineage tracing study demonstrated that pleural mesothelial cells migrated into the lung after bleomycin instillation and transitioned into myofibroblasts, thus providing evidence that mesothelial cells may contribute to the
pathology observed in idiopathic pulmonary fibrosis (Karki et al., 2014). In patients undergoing continuous ambulatory peritoneal dialysis, the dialysate has been observed to compromise the permeability of the mesothelium and promote peritonitis over time (Zhou et al., 2016, Loureiro et al., 2011). In severe cases peritoneal adhesion occurs as the mesothelium becomes fibrotic. The high glucose content of the dialysate has been shown to promote inflammation and mesothelial to mesenchymal transition (Choi et al., 2016). Inflammation and signaling by the receptor for advanced glycation products in the mesothelial cells have been shown to promote this transition and compromise the mesothelium (Raby et al., 2016, Yung and Chan, 2009, Schwenger et al., 2006). In peritoneal dialysis induced fibrosis IL-1β (Strippoli et al., 2008) and TGFβ (Loureiro et al., 2011) have been found to play important roles. Injury to the mesothelial cells leads to the secretion of inflammatory cytokines which then promote the infiltration of immune cells into the peritoneum (Strippoli et al., 2016). Unfortunately, the presence of the immune cells and their responses to the injury upregulates inflammation further and modulates the response of the mesothelial cells (Batra and Antony, 2015) leading to the development of conditions that further promote inflammation and fibrosis. The fibrotic response may start as a repair mechanism as mesothelial cells transition into a more fibroblastic phenotype that deposit collagen and extracellular matrix (Zhou et al., 2016). Without resolution this process continues and fibrosis develops.

Peritoneal dialysis induced peritoneal injury is not the only condition under which IL-1β has been demonstrated to promote fibrosis or EMT. In a study where IL-1β was transiently expressed in the lung via adenovirus transduction, IL-1β expression led to acute lung injury
which progressed to the development of fibrosis after chronic repair (Kolb et al., 2001). In lung transplant patients there is progressive loss of lung capacity due to the development of obliterative bronchiolitis in small and mid-sized airways leading to inflammation and fibrosis of the airways (Borthwick et al., 2010). This loss in lung capacity is believed to be due to dysregulated repair of injury to the airways after bronchiolitis develops and has been attributed to the action of pro-inflammatory cytokines released into the airways by infiltrating macrophages (Borthwick et al., 2010). This was confirmed through a study in which primary human bronchial epithelial cells were either treated with a combination of TGFβ and IL-1β or IL-8 or co-cultured with macrophages. This study demonstrated that TGFβ in conjunction with IL-1β or co-culture with macrophages promoted dysregulated wound repair with increased cell proliferation and characteristics of EMT (Borthwick et al., 2010). In corneal endothelial cells, IL-1β induced endothelial to mesenchymal transition via FGF2 signaling (Lee et al., 2012, Lee and Heur, 2013) in response to corneal injury and is believed to be the mechanism that promotes the formation of retro-corneal fibrous membrane that causes loss of vision (Lee et al., 2012). By this token, the effects of IL-1β on EMT can be argued to be dependent on the effectors induced by IL-1β signaling/inflammation. FGF2 is induced through activation of NFκB downstream of IL-1β signaling in corneal endothelial cells and the EMT induced was reversed by blocking the IL-1 receptor (IL-1R) with the IL-R antagonist (Lee and Heur, 2013). Lee et al demonstrated that IL-1β promoted oral squamous cell carcinoma (OSCC) carcinogenesis and invasiveness by upregulating oncogenic networks and increasing the proliferation of dysplastic cells suggesting that inflammasome activation by carcinogens in tobacco were
important for OSCC development and progression (Lee et al., 2015). In an attempt to recapitulate the mechanisms that could promote MMT in pleural mesothelial cells as a possible mechanism for the development of tuberculous pleurisy, Kim et al demonstrated that TGFβ and/or IL-1β were capable of inducing loss of E-cadherin, as well as increasing levels of the transcriptional repressor of E-cadherin, Snail (Kim et al., 2011) suggesting that inflammation in response to Mycobacterium tuberculosis could account for tuberculous pleurisy seen in some patients.

In cancer, EMT is believed to be reactivated enabling cells that transition to escape the original tumor and migrate to metastatic sites to establish disease or even invade the underlying tissue at the original site as the tumor becomes more aggressive (Diepenbruck and Christofori, 2016). Chronic inflammation plays a role in several cancer types and helps shape the tumor microenvironment to help promote progression and invasiveness of these cancers. In head and neck squamous cell carcinomas (HNSCC), the inflammatory microenvironment is believed to enhance angiogenesis, invasiveness and metastasis (Dohadwala et al., 2010). IL-1β is one of the pro-inflammatory mediators found in the microenvironment of HNSCC. Treating HNSCC cells with IL-1β decreased E-cadherin expression and increased levels of the transcription factors ZEB-1 and Snail, negative regulators of E-cadherin levels (Dohadwala et al., 2010, St John, 2015). This result confirmed the role for a pro-inflammatory mediator found in the HNSCC microenvironment in promoting tumor invasion/metastases through EMT. In addition to promoting EMT in HNSCC cells upregulation of Snail in these cells made them resistant to the chemotherapeutic, elortinib (St John, 2015).
A number of studies have demonstrated that asbestos is capable of inducing EMT in epithelial cells (Kamp, 2009, Gulino et al., 2016, Tamminen et al., 2012) which provides a plausible mechanism for how asbestos causes the asbestos related pulmonary fibrosis referred to as asbestosis. The first cell type to come into contact with inhaled fibers are the bronchial epithelial cells followed by lung macrophages and alveolar epithelial cells. Exposure of these cells to asbestos leads to their uptake and attempts by the cells to detoxify the $\text{Fe}^{3+}$ ions on the fibers leading to an increase in Fenton reactions with the conversion of $\text{Fe}^{3+}$ to $\text{Fe}^{2+}$. This increase in ROS causes oxidative stress and activates a number of redox sensitive signaling pathways that all work to promote proliferation and the development of fibrosis. Signaling pathways like NFκB, EGF signaling HGF/cMET all respond to oxidation and have been shown to play a role in EMT (Kalluri and Neilson, 2003, Kalluri and Weinberg, 2009, Kamp, 2009). In a bronchial epithelial cell line (BEAS2B), exposure to chrysotile over a 72 h period resulted in cells attaining a more fibroblastic appearance while increasing the expression of the mesenchymal markers, vimentin, fibronectin, alpha smooth muscle actin ($\alpha$SMA) and the collagenases, matrix metalloproteinase 2 and 9 (MMP 2&9) at both the protein and transcript level (Gulino et al., 2016). These cells also had displayed a concomitant decrease in protein and transcript levels of the epithelial junctional proteins E-cadherin and β-catenin (Gulino et al., 2016). In these cells asbestos exposure increased ROS levels and signaling via TGFβ, GSKβ and AKT were implicated in the EMT observed as inhibitors of AKT and GSKβ decreased the asbestos mediated increase in Snail levels and rescued some E-cadherin expression although not to basal levels (Gulino et al., 2016). Studies using the lung cancer cell line
(A549 cells) which exhibit characteristics of alveolar type II cells have also demonstrated that asbestos can induce EMT in a mitogen activated protein kinase/extracellular regulated kinase (MAP/ERK) dependent manner (Tamminen et al., 2012). These and other studies have demonstrated that asbestos can promote EMT in epithelial cells, however the same is not the case for mesothelial cells from which MM originates. One study has studied asbestos-induced EMT in MET5A cells (a transformed mesothelial cell line) by looking at mRNA levels of EMT markers.

1.8 Conclusions

Asbestos-related diseases have a long latency period and are the end result of aberrant wound healing responses, chronic inflammation, and (in the case of lung cancer and MM) inflammation-related genetic mutation and deviant gene expression or silencing. The inflammasome and its products, IL-1β and IL-18, may play an important role in promoting a myofibroblastic transition in mesothelial cells as well as in activating fibroblasts to create a microenvironment in which damaged cells survive and go on to acquire mutations and activate gene programs that enable them to become malignant and clonally expand. More studies are needed to help uncover the mechanisms involved in the development of these diseases, as this would lead to discovery of potential biomarkers and therapeutic targets to aid in early diagnosis and treatment.

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CHAPTER TWO: ASBESTOS MODULATES THIOREDOXIN-THIOREDOXIN INTERACTING PROTEIN INTERACTION TO REGULATE INFLAMMASOME ACTIVATION

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Abstract

Background: Asbestos exposure is related to various diseases including asbestosis and malignant mesothelioma (MM). Among the pathogenic mechanisms proposed by which asbestos can cause diseases involving epithelial and mesothelial cells, the most widely accepted one is the generation of reactive oxygen species and/or depletion of antioxidants like glutathione. It has also been demonstrated that asbestos can induce inflammation, perhaps due to activation of inflammasomes.

Methods: The oxidation state of thioredoxin was analyzed by redox Western blot analysis and ROS generation was assessed spectrophotometrically as a read-out of solubilized formazan produced by the reduction of nitrotetrazolium blue (NTB) by superoxide. Quantitative real time PCR was used to assess changes in gene transcription.

Results: Here we demonstrate that asbestos fibers oxidize the pool of the antioxidant, Thioredoxin-1 (Trx1), which results in release of Thioredoxin Interacting Protein (TXNIP) and subsequent activation of inflammasomes in human mesothelial cells. Exposure to asbestos resulted in the depletion of reduced Trx1 in human peritoneal mesothelial (LP9/hTERT) cells. Pretreatment with the antioxidant dehydroascorbic acid (a reactive oxygen species (ROS) scavenger) reduced the extent of asbestos-induced Trx1 oxidation as well as the depletion of reduced Trx1. Increasing Trx1 expression levels using a Trx1 over-expression vector reduced the extent of Trx1 oxidation and generation of ROS by asbestos, and increased cell survival. In addition, knockdown of TXNIP expression by siRNA attenuated asbestos-induced activation of the inflammasome.
Conclusion: Our novel findings suggest that extensive Trx1 oxidation and TXNIP
dissociation may be one of the mechanisms by which crocidolite asbestos activates the
inflammasome and helps in development of MM.

Key words: Asbestos, Malignant mesothelioma, Thioredoxin, Thioredoxin interacting
protein, Inflammasomes.

**Background**

Malignant mesothelioma (MM) is a deadly cancer arising from the mesothelium and its
etiology usually involves asbestos exposure (1). MM is a very invasive and aggressive
disease that is chemo-resistant to most of the standard chemotherapeutic agents. Patients
with MM typically have a poor prognosis with a life expectancy of about 8-12 months after
diagnosis (2). Efforts at understanding how asbestos exposure leads to the development of
MM and other malignancies have not definitively determined how exposure leads to the
formation and progression of this unusual neoplasm. Studies have, however, shown that
apoptosis followed by compensatory proliferation and chronic inflammation induced by
asbestos fibers play a major role in disease progression (3-6). Chronic inflammation
induced by asbestos exposure is believed to be involved in the pathogenic process that
leads to asbestos related diseases like MM (7, 8). Recent work from our group has
demonstrated that asbestos-induced inflammation in mesothelial cells and macrophages
could, in part, be mediated by activation of the inflammasome, a protein complex involved
in the processing of cytokines (9). The exact mechanism by which asbestos activates the
inflammasome is not completely understood, but reactive oxygen species (ROS) are
believed to play a role (6). It has also been reported that a redox-regulated protein,
thioredoxin interacting protein (TXNIP) can bind and activate the Nod-like receptor family
pyrin domain containing 3 (NLRP3) inflammasome (10). ROS induced in response to
asbestos exposure have been shown to initially deplete intracellular levels of reduced
glutathione (11, 12), but the effect of asbestos on another major cellular antioxidant,
thioredoxin (Trx1) is unknown.

Thioredoxin is a small ubiquitously expressed redox active protein that is important for
maintaining the reducing milieu of the cell, in part by reducing protein disulfide bonds that
occur in response to oxidative processes. During reduction of disulfide bonds Trx1 itself
becomes oxidized and in turn reduced by thioredoxin reductase (TR) using electrons from
reduced nicotinamide adenine dinucleotide phosphate (NADPH) (13). Trx1 is inhibited by
thioredoxin interacting protein (TXNIP) via a redox-dependent interaction (14, 15). TXNIP
is only capable of binding to and inhibiting Trx1 in its reduced state (10, 15). In response
to oxidative insults, TXNIP has been shown to bind to and activate the NLRP3
inflammasome (10). Based on these observations and the capacity for asbestos fibers to
generate ROS intra- and extracellularly, we hypothesized that asbestos-induced ROS
generation will oxidize Trx1 causing its dissociation from TXNIP. As a result of this
dissociation, TXNIP would be free to bind to, and activate, the NLRP3 inflammasome.
Here, we show for the first time, that asbestos exposure leads to the irreversible oxidation
of Trx1 and depletes reduced Trx1 levels in LP9/hTERT cells. We also show that over-
expression of Trx1 reduces levels of asbestos-induced ROS. Our results indicate that
oxidation of Trx1 by asbestos results in dissociation of TXNIP and subsequent activation
of inflammasomes, as knockdown of TXNIP by siRNA partially reduced asbestos-induced inflammasome activation as indicated by a reduction in caspase-1 activation.

Materials and Methods

Human LP9 mesothelial cells, an hTERT-immortalized cell line that phenotypically and functionally resembles normal human mesothelial cells, were obtained from Dr. James Rheinwald (Brigham and Women’s Hospital, Harvard University, Boston, MA). All cells were incubated at 37°C in 5% CO₂ and grown to 80–90% confluency as described previously (16). The physical and chemical characterization of the National Institute on Environmental Health Sciences (NIEHS) reference sample of crocidolite asbestos has been reported previously (17). The NIEHS chrysotile reference sample was used for asbestos fiber comparisons. After sterilization under UV light overnight, particulates were suspended in Hank’s balanced salt solution (HBSS) at 1 mg/ml, sonicated for 15 min in a water bath sonicator, and triturated five to ten times through a 22-gauge needle. A volume of this suspension was added to cells in medium to achieve the desired final concentration of 75 x 10⁶ µm²/cm² dish surface area, a concentration known to cause apoptosis and compensatory proliferation of surrounding rat pleural mesothelial and murine alveolar type II epithelial cells (3)(18). Glass beads (Polysciences Inc, Warrington, PA) were used as a non-pathogenic particle control. Dehydroascorbic acid (DHA) and 2,4-Dinitro-1-chlorobenzene (DNCB) were purchased from Sigma (St. Louis, MO). Trx1 expression
vector (pCMV-SPORT6) and pcDNA (empty vector control) used for over-expression studies were obtained from Dr. Nicholas Heintz.

**Western blot analyses:**

Cells grown in 60 mm culture dishes were washed 3x with ice-cold phosphate buffered-saline (PBS), collected in lysis buffer (20 mM Tris pH 7.6, 1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 1 mM Na$_3$O$_4$V, 10 mM NaF, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and incubated on ice for 30 min. Lysates were centrifuged at 14,000 rpm for 15 min at 4°C. Supernatants were collected, and protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA). Cell lysates (40 µg per lane) were resolved by one-dimensional SDS-PAGE and transferred to nitrocellulose membranes according to standard procedures. Equal loading of protein was verified by β-actin (Abcam, Cambridge, MA). Membranes were washed in Tris-buffered saline (TBS), and blocked for 2 h with TBS-Tween (TBST) containing 10% nonfat milk, then incubated with rabbit anti-human Trx1 antibody (Abcam, Cambridge, MA) at 1:5000 dilution in TBST containing 5% nonfat milk and 0.01% sodium azide overnight at 4°C. Membranes were washed four times with TBST for 15 min each time prior to incubation with secondary antibody. Western blots shown are representative blots with their accompanying densitometric analysis.

*Determination of the redox state of thioredoxin:*

The redox state of thioredoxin in response to crocidolite exposure was determined using the redox Western blot method as previously described (19). Briefly, cells were lysed in 6 M guanidine HCl buffer (6 M Guanidine HCl; 50 mM Tris/Cl pH 8.3; 3 mM EDTA, 0.5%
TritonX – 100; 10 µg/ml aprotonin and 10 µg/ml leupeptin) containing 50 mM iodoacetic acid (IAA) for alkylation of the thiol groups of thioredoxin. Cells were incubated in lysis buffer at 37°C for 30 min in the dark. Excess IAA was removed by spinning lysates on Amicon centrifugal concentrating columns with a 10,000 nominal molecular weight limit (NMWL) (EMD Millipore, Billerica, MA). In order to exchange the IAA containing buffer, the lysates were washed 3 times with a HEPES buffer at pH 7.4 and the concentrated lysates were collected in fresh collection tubes by inverting the columns in the tubes. After protein determination by the Bradford method (Bio-Rad, Richmond CA), 40 µg of protein was loaded onto a 15% non–reducing native polyacrylamide gel using a 1X Tris Glycine (pH 8.8) running buffer. The electrophoresis was carried out at 75V for approximately 3.5 h. The redox gel was then washed in 50 mM Tris (pH 8.3) for 5 min and then equilibrated in 1X transfer buffer by washing in buffer 3 times for 5 min each. Thereafter, the proteins were transferred onto a nitrocellulose membrane by wet transfer at 100V for 2 h. The nitrocellulose membrane was then blocked with 10% milk in 1X Tris buffered saline with Tween 20 (TBS-T) for 6 h at room temperature (RT) and incubated in anti-Trx1 primary antibody (1:5000 in 5% milk/TBS-T, Abcam, Cambridge, MA) overnight at 4°C. Goat anti–rabbit secondary antibody conjugated to horseradish peroxidase (1:2000 in 1X TBS-T, Jackson ImmunoResearch Laboratories Inc. West Grove, PA) was used and visualization was done by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ) on X-ray film. Blots were quantified using Quantity One software (Bio-Rad, Richmond, CA). Distribution of redox states of Trx1 was determined as band

67
intensity of reduced or oxidized Trx/ (reduced+semi-oxidized+fully oxidized) as described by Watson et al., (2003) (19).

*Real-Time Quantitative PCR (qRT-PCR):*

Total RNA was prepared using an RNeasy plus mini kit according to the manufacturer’s protocol (Qiagen, Valencia, CA) as described previously (16). Total RNA (1 µg) was reverse-transcribed with random primers using the Promega AMV Reverse Transcriptase kit (Promega, Madison, WI) according to the recommendations of the manufacturer. To quantify gene expression, the cDNA was amplified by TaqMan Real Time Q-PCR using the 7900HT SequencePrism Detector (Applied Biosystems, Foster City, CA). Duplicate assays were performed with RNA samples isolated from at least four independent experiments. Fold changes in gene expression were calculated using the delta-delta Ct method. The values obtained from cDNA and hypoxanthine phosphoribosyl transferase (HPRT) controls helped determine relative gene expression levels for the gene locus investigated. The Assay on Demand primers and probes used were purchased from Applied Biosystems.

Since exposure of cells to chrysotile asbestos had no effect on the oxidation state of Trx1, all subsequent experiments were performed using crocidolite asbestos. All references to asbestos relate to crocidolite asbestos unless otherwise specified.

*Dehydroascorbic acid (DHA) pretreatment:*

In order to investigate whether the ROS generated by crocidolite exposure was responsible for the extensive oxidation of thioredoxin, cells where pretreated with the ROS quencher, dehydroascorbic acid (1 mM) for 1 h before exposure to crocidolite for 8 h.
**Antioxidant pretreatment of cells by N-acetylcysteine (NAC):**

To investigate the role of the asbestos-induced ROS on inflammasome activation, cells were pretreated with 2 mM NAC for 20 h as previously described (Shukla et al., 2004). Briefly, cells were grown to 90% confluency in 60 mm dishes and serum starved by replacing complete medium with 0.5% fetal bovine serum (FBS) supplemented medium for 6 h prior to addition of NAC diluted in HBSS at pH 7.4 (cells were maintained in reducing medium for the entire duration of NAC pretreatment). After pretreatment with NAC, cells were exposed to crocidolite asbestos for 48 h. Thereafter, inflammasome priming was assessed by qRT-PCR of NLRP3 transcript levels while activation was analyzed by Western blot analysis of caspase-1, p20 fragment.

**Treatment with 2,4-Dinitro-1-chlorobenzene (DNCB):**

To obtain a final concentration of 10 µM in culture, 2,4-Dinitro-1-chlorobenzene (DNCB) (an irreversible alkylating inhibitor of TR) was dissolved in DMSO with a final DMSO content of 0.2% which was determined to be non-cytotoxic in previous experiments. For all experiments, cells were pretreated with DNCB for 1 h prior to exposure to crocidolite.

**Lactate dehydrogenase (LDH) activity assay:**

To determine the cytotoxic effects of DNCB and asbestos exposure on LP9 cells, an LDH assay was performed using the LDH kit from Promega (Madison, WI) according to the manufacturer’s direction. Briefly, 50 µl of media were collected from each dish in triplicate into a 96 well plate using cells lysed by the addition of 0.9% Triton X-100 as a positive control. To each of these wells, 50µl of LDH substrate buffer was added and the reaction was incubated on a rotary shaker at room temperature for 30 min in the dark.
the reaction was terminated by the addition of 50 µl stop buffer to each well. Any bubbles present were broken with a hypodermic needle and the plate was read spectrophotometrically at 490 nm in a 96 well plate reader. Cytotoxicity was expressed as a percentage of LDH released relative to the lysis control.

Detection and quantitation of apoptosis:

To determine whether modulation of Trx1 protein levels and oxidation state altered cell death in human mesothelial cells, detection of apoptosis was performed using the ApoStain technique as described previously (Shukla et al., 2003). In brief, cells were grown on glass cover slips and exposed to asbestos with or without DNCB (10 µM) for 8 h. The cover slips were then processed to determine the numbers of apoptotic cells and total cell numbers per field. Five random fields were evaluated at a magnification of 400X on each cover slip.

Assessment of pyroptosis by asbestos

Since asbestos causes inflammasome activation, as measured by caspase-1 activation (4), we were interested in learning if asbestos-induced cell death may be due in part to pyroptosis (caspase-1 dependent cell death) or not. For this purpose we pretreated LP9 cells with a specific caspase-1 inhibitor (40 µM Caspase-1 inhibitor VI (zYVADfmk), EMD Biosciences, Billerica, CA) for 1 h and subsequently with asbestos for 24 h. The number of viable cells was determined after trypsinization and counting of cells on a hemocytometer at the end of the experiment. Media supernatants were also analyzed for the levels of the p20 subunit of active caspase 1 by Western blot analysis.

Transfection procedures:
**Trx1 Over-expression**

Cells at 90% confluence were transfected with pcDNA (empty vector control, 4 µg DNA per 60 mm dish) and human Trx1 over expression vector (pCMV-SPORT6, 4 µg DNA per 60 mm dish) using Lipofectamine 2000 (10 µl) (Life Technologies, Grand Island, NY), following the manufacturer’s protocol. The efficiency of Trx1 protein over expression was determined by qRT-PCR after 48 and 72 h.

**Knockdown of TXNIP**

LP9 cells that were 90% confluent were transfected with either ON-TARGET plus smart pool human TXNIP siRNA (siTXNIP) or ON-TARGET plus non-targeting siRNA (siControl) from Dharmacon (Fisher Scientific, Pittsburgh, PA) using Lipofectamine 2000 (Life Technologies, Grand Island, NY) diluted in a final volume of 500 µl Optimem medium (Life Technologies, Grand Island, NY), as previously described (16). All siRNA were reconstituted to 20 µM before transfection and stored at -20 °C until use. The magnitude of TXNIP knockdown was assessed by qRT-PCR.

To confirm observations of the role of TXNIP in inflammasome activation, a human mesothelioma cell line (HMESO) in which the extracellular signal regulated kinase 2 had been stably knocked down (shERK2) was used. These cells have been previously reported (GSE21750) to have down-regulated expression of TXNIP (several fold). To activate the inflammasome, shERK2 HMESO cells and corresponding control cells stably transfected with non-targeting shRNA (shCon) were treated with 5 µM doxorubicin (Dox) as previously described (20). After 48 h of treatment the shERK2 and shCon HMESO cells, treated with or without Dox, medium supernatants were harvested by centrifuging medium
at 300 x g for 7 min in the cold (4° C) to remove cellular debris. The resulting supernatants, stored in 1 ml aliquots, were concentrated using Amicon centrifugal concentrating columns with a 10,000 nominal molecular weight limit (NMWL) (EMD Millipore, Billerica, MA). 4X sample buffer was added to the concentrated supernatant to a final concentration of 1X before being electrophoresed at 100V for 2 h on a 15% SDS PAGE gel. Immunobloting was performed as described above with capase-1 p20 antibody (Cell Signaling, Danvers MA) to detect caspase-1 activation.

Statistical Analysis

All data were analyzed by one-way ANOVA compared with the respective control group and a Neuman-Keuls posttest for multiple comparisons or the student t-test. Results are presented as the mean ± SEM. All experiments were repeated at least twice. Comparisons with a p value less than 0.05 were considered statistically significant. Statistical analyses were conducted using Graph Pad Prism v6 software.

Results

The effects of crocidolite exposure on thioredoxin 1 (Trx1) expression in human mesothelial cells: To determine the effect of crocidolite exposure on the expression levels of Trx1, LP9/h-TERT cells were exposed to 75 x 10^6 µm^2/cm^2 crocidolite for 8 and 24 h and RNA was extracted. Analysis of fold changes in mRNA levels for Trx1 by qRT-PCR revealed a 1.6 fold increase in Trx1 mRNA levels after 24 h of crocidolite exposure as compared to controls (*p<0.05) (Figure 1a). An assessment of the total Trx1 protein levels after crocidolite exposure showed a decrease in protein levels after 24 h (Figure 1b & c).
In contrast, glass beads (which were used as a negative control) and crocidolite at surface area coverage of $15 \times 10^6 \mu m^2/cm^2$ did not cause a significant increase in mRNA levels at 24 h as compared to untreated controls.

*The oxidation state of thioredoxin 1 (Trx1) after crocidolite exposure:* LP9 cells exposed to $75 \times 10^6 \mu m^2/cm^2$ crocidolite for 8 and 24 h showed a decrease in the proportion of reduced Trx1 as assessed by redox Western analysis as compared to untreated controls (Figure 2a). This decrease in the level of reduced thioredoxin was not observed in cells treated with sodium arsenite, which has been shown previously to oxidize Trx1 in a different cell type (21). In addition, the total amounts of Trx1 observed in crocidolite exposed cells appeared to be lower than the levels in the other treatment groups. A third band that represented fully oxidized thioredoxin was present in cells exposed to either crocidolite or arsenite, but was not observed in lysates from the untreated controls. To determine whether the oxidation of thioredoxin by crocidolite was specific to crocidolite alone, LP9 cells were also exposed to chrysotile asbestos and glass beads. As assessed by redox Westerns, oxidation of thioredoxin was specific to the higher concentration ($75 \times 10^6 \mu m^2/cm^2$) of crocidolite alone (Figure 2c). Levels of reduced and semi-oxidized Trx1 remained the same at 8 and 24 h in response to chrysotile and glass bead exposure.

*Inhibition of thioredoxin reductase (TR) by dinitrochlorobenzene (DNCB) and pretreatment with dehydroascobic acid:* To determine whether inhibition of TR by DNCB would increase asbestos-induced oxidation of Trx1, LP9 cells were pretreated with 10 μM DNCB and exposed to $75 \times 10^6 \mu m^2/cm^2$ crocidolite for 8 and 24 h. A lactate dehydrogenase (LDH) assay performed on the medium from exposed cells showed that
LDH levels were reduced in cells pretreated with DNCB when compared with cells exposed to asbestos alone (Figure 3a). In addition, redox Western blot analysis of cell lysates indicated that the oxidation of Trx1 by asbestos was ameliorated when cells were pretreated with DNCB before exposure to crocidolite asbestos (Fig. 3b). On the contrary, cells exposed to chrysotile asbestos with and without DNCB showed no changes in the redox states of Trx1 when compared to controls (Figure 3c). An Apostain assay to detect chromatin condensation and single strand breaks in nuclear DNA as a measure of early apoptotic events was also conducted to determine the effects of pretreatment with DNCB on asbestos-induced apoptosis. Apostain revealed that cells pretreated with DNCB were protected from apoptosis after 8 h of exposure to crocidolite asbestos (Figure 3d). On the other hand, pretreatment of LP9 cells with DHA before exposure to asbestos slightly increased the amount of reduced thioredoxin in cells compared to asbestos exposure alone (Figure 3e). However, there was no significant increase when compared to control or asbestos exposure alone.

*ROS generation in response to asbestos is modulated by Trx1 in LP9 cells:* To assess ROS generation after exposure to asbestos, 90% confluent LP9 cells were exposed to 75 x 10^6 µm^2/cm^2 asbestos for 24 h and incubated with NBT as described in the methods. Spectrophotometric assessment of the solubilized formazan revealed a significant increase in ROS levels when compared to controls (Figure 4a). Quantitative RT-PCR performed on cDNA from Trx1 over-expressing cells and their respective controls indicated a 4 fold increase in Trx1 levels after 48 h of transfection and this was reduced by approximately 50% 72 h post-transfection (Figure 4b). The effect of Trx1 over-expression on asbestos-
induced ROS generation and cell survival was assessed by exposing 90% confluent LP9 cells transfected with a Trx1 over-expression vector (pCMV-SPORT6) and empty vector transfected controls (pcDNA) to 75 x 10^6 μm²/cm² crocidolite asbestos for 2 h. The levels of ROS generated in response to asbestos exposure were then measured as described previously. Cells over-expressing Trx1 were found to exhibit a trend of reduced ROS levels compared to the null controls exposed to asbestos (Figure 4c). It is to be noted here that the amount of ROS generated by asbestos after 2 h of exposure is significantly lower in magnitude than 24 h after asbestos exposure (Figure 4a). An assessment of the redox state of Trx1 in Trx1 over-expressing cells after asbestos exposure indicated that levels of reduced Trx1 were rescued in over-expressing cells as compared to control cells transfected with empty vector (EV) alone (Figure 4d). Additionally, cells over-expressing Trx1 also had increased cell survival after exposure to crocidolite (Figure 4e).

Effect of N-acetyl-cysteine (NAC) on asbestos-induced inflammasome activation:

Asbestos exposure results in the generation of ROS in LP9 cells, and ROS has been reported to be one of the activators of the NLRP3 inflammasome (6). To determine whether asbestos-induced ROS plays a role in inflammasome activation, LP9 cells were pretreated with NAC and analyzed for inflammasome priming and activation by qRT-PCR and Western blot analysis. A decrease in steady-state NLRP3 transcript levels was observed in cells pretreated with NAC prior to asbestos exposure when compared to cells exposed to asbestos alone (Figure 5a). Concurrently, levels of active caspase 1 secreted into the medium after exposure of LP9 cells to asbestos were also significantly reduced after pretreatment with NAC (Figure 5b).
TXNIP down-regulation attenuated asbestos-induced inflammasome activation:
Validation of the knockdown of TXNIP expression by siTXNIP was determined by qRT-PCR and showed that an approximately 50% reduction in TXNIP mRNA levels was achieved after 48 h of transfection (Figure 6a). Western blot analysis of the cell medium after exposure to asbestos for 48 h indicated that active caspase 1 levels (caspase1-p20) were reduced after knockdown of TXNIP when compared to siControl transfected cells (Figure 6b).

In mesothelioma cell lines with a stable knockdown of extracellular signal regulated kinase 2 (shERK 2), the expression of TXNIP was found to be down-regulated 4-fold (Figure 6c). Activation of the NLRP3 inflammasome by doxorubicin treatment of shERK2 HMESO cells indicated that caspase 1 activation was drastically reduced as compared to control cells stably transfected with non-targeting shRNA (shCon) (Figure 6d) and there was no priming of NLRP3 as measured by mRNA levels of NLRP3 using qRT-PCR (Figure 6c).

Caspase-1 dependent cell death (pyroptosis), which may occur in response to inflammasome activation, may contribute to asbestos-induced cell death in addition to asbestos-induced apoptosis and lytic cell death. Therefore, we sought to determine whether pyroptotic cell death occurred in response to asbestos-induced inflammasome activation. To do so, LP9 cells were pretreated with 40 µM Caspase-1 inhibitor VI (zYVAD-FMK) before exposure to asbestos. When compared to cells exposed to asbestos alone, there was an 18% increase in cell viability, suggesting this fraction of cells may have undergone pyroptotic cell death upon exposure to asbestos (Figure 6e). Immunoblotting for Cas-1 p20
in concentrated medium supernatants also confirmed that the inhibitor attenuated activation of the caspase-1 as expected (Figure 6f).

**Discussion**

Reactive oxygen species generated in response to asbestos exposure have been shown to have deleterious effects in different cell types. Asbestos-induced ROS generation has been shown to cause oxidative damage to mitochondrial and genomic DNA (22, 23) and may modulate the activity/function of various signaling molecules, transcription factors and enzymes that are redox sensitive (11), (22, 24). The high iron content as well as the valency state of iron on crocidolite asbestos fibers has been shown to facilitate Fenton reactions both intracellularly and extracellularly (25, 26). Mitochondrial generated superoxide can react with Fe3+ ions on asbestos fibers to reduce it to Fe2+ (26). Unfortunately, this enables the Fe2+ to react with any H2O2 present in the cell to produce hydroxyl radicals. This can lead to a cycle of oxidation and reduction of Fe3+ to produce more ROS. Additionally, the high aspect ratio of the crocidolite fibers leads to frustrated phagocytosis as mesothelial cells try to unsuccessfully phagocytose the fibers (6). Frustrated phagocytosis promotes a sustained production of superoxide through the activity of membrane bound NADPH oxidases that are activated during the repeated phagocytosis attempts (27). Asbestos exposure has been shown to cause a depletion of reduced glutathione levels *in vitro* (11, 12), but its effects on Trx, another major antioxidant of the cell, is unknown. The ability of asbestos fibers to activate the inflammasome in macrophages and mesothelial cells (4, 6) and the involvement of the endogenous inhibitor of Trx, TXNIP, in activation of the NLRP3 inflammasome (10) led us to hypothesize that oxidation of Trx by asbestos-induced
ROS may cause the dissociation of TXNIP from Trx and lead to activation of the inflammasome. Here we show for the first time that asbestos exposure of human mesothelial cells leads to oxidation of Trx and a compensatory increase in Trx1 transcript levels. We also report that TXNIP is involved in asbestos mediated activation of the inflammasome which may be as a result of the oxidation of Trx1 by asbestos-induced ROS generation.

Quantitative real time PCR showed that asbestos exposure increased the steady-state RNA levels of Trx1 by approximately 1.6 fold after 24 h. The promoter of the Trx gene (TXN) contains an antioxidant response element (ARE) (28). It is therefore likely that ROS generated in response to asbestos exposure led to an increase in Trx1 expression as a compensatory mechanism by activating ARE-dependent gene expression. It has been reported by our group that cells exposed to asbestos show increased expression of the mitochondrial manganese superoxide dismutase (MnSOD) (12). Studies also show that increases in expression of cellular antioxidant proteins (MnSOD and Trx1) occur in response to oxidative stress (11, 12). Exogenous thioredoxin as well as endogenously over-expressed Trx has also been shown to increase MnSOD levels in a redox dependent manner (29). As such, increases in ROS generation in LP9 cells exposed to asbestos coupled with the oxidation of Trx1 and increased transcription of Trx1 could be considered an indication of asbestos-induced oxidative stress. To the contrary, the total intracellular protein levels of Trx1 were reduced by about 67% in response to asbestos exposure after 24 h (Figure 1b, c). This suggests that, ROS generation by asbestos exposure may lead to an increased
oxidation and removal of Trx1 protein as a conjugate, and the cells may increase transcription as a compensatory mechanism. However, Trx1 is secreted into the culture medium in response to many stimuli – it may not be degraded (30, 31). Similarly, we have demonstrated previously that asbestos exposure depletes glutathione from cells while increasing the steady-state RNA levels of γ-glutamylcysteine synthetase (a rate limiting enzyme for glutathione synthesis) at the same time (11).

To determine if asbestos exposure causes changes in the antioxidant capacity of Trx1, the oxidation state of Trx1 was assessed by redox Western blot analysis after exposure of LP9 cells to asbestos. The analysis of the oxidation states of Trx1 after asbestos exposure showed that Trx1 became irreversibly oxidized and levels of reduced Trx1 were drastically decreased compared to control cells. The diminution in the levels of reduced Trx1 may be due to the oxidation of the structural cysteine residues (Cys 62, 69 and 73). Oxidation of these structural cysteines can lead to disulfide bond formation between Cys 62 and 69 which has been shown to reduce the use of Trx1 as a substrate for TR (19). As a result, Trx1 cannot be reduced to its active form. The formation of disulfide bonds between Cys 73 of two adjacent Trx1 molecules leading to their dimerization has also been shown to occur under extremely oxidizing conditions (32). This dimerization may lead to conformational changes in the active site of Trx1 which will make the active site Cys residues inaccessible to TR for reduction. Other studies have shown that strong oxidants can cause the oxidation of Trx1 producing a mixture of Trx1 monomers, dimers and oligomers with no free sulfhydryl groups (33). These findings support the possibility that
asbestos-induced ROS may be involved in directly or indirectly altering the oxidation state of Trx1.

Maintenance of the reducing milieu of the cell is also important for cell survival; thus, imbalances in the ratio of cellular antioxidants and oxidants could lead to deleterious or lethal effects on the cell. To investigate what role perturbations in the redox state of the thioredoxin system plays in asbestos-induced cell death, LP9 cells were pretreated with dinitrochlorobenzene (DNCB), an irreversible alkylating inhibitor of TR (34) and assessed for cell death in response to asbestos exposure. An LDH assay as well as the Apostain assay showed that pretreatment with DNCB protected cells from asbestos induced cell death contrary to all reports in the literature in which treatment of cells with DNCB resulted in cell death (35-37). Recent studies have conversely shown that inhibition of TR leads to increases in GSH levels and a reduction of Trx1 and Trx2 by glutaredoxin in the absence of a functional TR enzyme (38, 39). It is therefore likely that the protection of human mesothelial cells from asbestos-induced cell death after pretreatment with DNCB may be due to increases in GSH and levels of reduced Trx1.

Levels of intracellular GSH are sensitive to ROS levels (40) and are modulated upon exposure of cells to asbestos (11, 12). Reactive oxygen species have also been shown to play a role in the activation of the NLRP3 inflammasome after asbestos exposure (6) and may be linked to the effects of GSH levels on Trx1 oxidation state and the availability of TXNIP to bind to and activate the inflammasome. To test the effect of increased GSH or
reduced ROS levels on inflammasome activation, LP9 cells were incubated with 2 mM NAC for 20 h prior to asbestos exposure and this led to a significant decrease in inflammasome activation, as well as a reduction in priming. This suggests that high GSH levels may buffer reduced Trx1 levels (39) and prevent the dissociation of TXNIP from Trx1. As such, TXNIP is unable to bind to and activate the inflammasome leading to reduced levels of Caspase-1 p20 peptides in the medium supernatant. The reduction in priming of the NLRP3 may also be due to the decrease in ROS levels as GSH levels increased to buffer cells from the ROS generated in response to asbestos exposure since transcription of NLRP3 is under the control of NFκB (a redox sensitive transcription factor) (41-43). Exposure of LP9 cells to NAC also attenuated the increase in Trx1 mRNA induced by asbestos as there was no significant increase when compared to control (data not shown). This implies that the oxidative stress induced by asbestos exposure is ameliorated by NAC.

We have demonstrated previously that crocidolite asbestos causes cell death and compensatory proliferation (3), which may be a required step for crocidolite asbestos-induced cell transformation and MM development. Our results here, indicate that a fraction of total cell death by asbestos is caused by pyroptosis (caspase-1 dependent cell death, Figure 6), a process known to be regulated in part by TXNIP. However, this observation needs to be confirmed using siRNA mediated knockdown of caspase-1 in future studies. We suspect that pyroptosis is prevented by over-expression of Trx1 (Figure 4) which
renders TXNIP unavailable to subsequently induce inflammasome assembly and thus, caspase-1 activation.

Assessment of the effects of Trx1 over-expression on asbestos-induced ROS generation revealed that LP9 cells over-expressing Trx1 had lower levels of ROS after asbestos exposure when compared to vector transfected cells. Although asbestos induced a significant increase in ROS generation in LP9 cells after 24 h, the trend of reduction in ROS levels with Trx1 over-expression at an earlier time point (2 h) were not statistically significant, but reproducible. The reduction in asbestos-induced ROS generation in LP9s over-expressing Trx1 also corresponded to a moderate increase in cell survival which also exhibited a trend. Cells undergoing oxidative stress up-regulate the expression of antioxidant proteins like thioredoxin and MnSOD as well as the antioxidant peptide glutathione to counter the increase in oxidant levels (11, 12, 44). As such, the reduction in asbestos-induced ROS levels upon over-expression of Trx suggests that the increase in Trx1 levels after asbestos exposure may be a compensatory mechanism to restore the antioxidant-oxidant balance that is disrupted by asbestos (22).

Our study also showed that the redox-dependent Trx-TXNIP interaction is involved in asbestos-induced inflammasome activation. When TXNIP, the negative regulator of Trx1 reductase activity, was knocked down in LP9 cells, inflammasome activation was reduced. Cells transfected with siTXNIP had decreased amounts of active Caspase-1 subunit p20 in the medium after exposure to asbestos when compared to control. Additionally, activation
of the inflammasome by the chemotherapeutic doxorubicin in shERK2 HMESO cells, which have a four-fold lower expression of TXNIP, was attenuated, confirming that TXNIP is required for inflammasome activation. In support of our data Zhou et al. (10) demonstrated that knockdown of TXNIP by siRNA in beta islet cells, reduced activation of the NLRP3 inflammasome. Thus our findings corroborate the role of TXNIP in inflammasome activation by asbestos, and relate inflammasome activation via TXNIP to ROS levels in the cell (Figure 7).

**Conclusion**

This study has demonstrated that activation of the inflammasome by asbestos is mediated in part by TXNIP as a consequence of alterations in the redox state of Trx1 in the cytosol. Further studies are in progress to offer an understanding of how asbestos-induced activation of the inflammasome and subsequent generation of biomolecules (IL-1β, IL-18, HMGB1 etc.) may lead to mesothelial cell transformation events and mesothelioma development.
List of Abbreviations

12-O-tetradecanoylphorbol-13-acetate TPA
2,2-Dinitro-1-cholorobenzene DNCB
2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid HEPES
Antioxidant response element ARE
Carbobenzoxy-tyrosyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketon zYVAD-fmk
Chrysotile asbestos Chry
Crocidolite asbestos Croc
Dehydroascorbic Acid DHA
Dimethyl sulfoxide DMSO
Dithiotreitol DTT
Doxorubicin Dox
Empty vector EV
Ethylenediaminetetraacetic acid EDTA
Extracellular signal regulated kinase 2 ERK2
Glass beads GB
Hank’s balanced salt solution HBSS
High mobility group box 1 HMGB1
hTERT- immortalized human peritoneal cell line LP9/hTERT (LP9)
Hypoxanthine phosphoribosyl transferase HPRT
Iodoacetic acid IAA
Lactate dehydrogenase LDH
Malignant mesothelioma MM
Manganese superoxide dismutase MnSOD
N-acetylcysteine NAC
Nicotinamide adenine dinucleotide phosphate NADPH
NLR family pyrin domain containing 3 NLRP3
Phosphate-buffered saline PBS
Quantitative real time PCR qRT-PCR/qPCR
Reactive oxygen species ROS
Short hairpin RNA against ERK2 shERK2
Short hairpin RNA non-targeting shCon
Sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS PAGE
TBS-Tween TBST
Thioredoxin 1 Trx1
Thioredoxin Interacting Protein TXNIP
Thioredoxin reductase TR
Tris-buffered saline TBS
Authors contributions: JT, performed experiments and wrote the manuscript with the help of AS; CW, performed NAC and inflammasome related studies; MM, performed statistical analysis and made final figures for the publication. MM also provided technical assistance with many experiments; BM, conceived the initial idea and provided intellectual input; NH, provided all the plasmids used in the study and insightful discussion; PS, helped with redox Western blots; AS, conceived the idea, designed experiments, supervised the study, interpreted data, and helped JT in writing the manuscript.

All authors have read and approved the manuscript.

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References

Figure 2.1: Crocidolite asbestos exposure modulates Trx1 levels in mesothelial cells. (A) LP9 cells were exposed to crocidolite asbestos at $75 \times 10^6 \mu \text{m}^2/\text{cm}^2$ for 8 and 24 h. Glass beads (GB) at the same surface area concentration were used as an inert particulate control. RNA was extracted from the samples and used to prepare cDNA, which was quantified by qRT-PCR (*p< 0.05 compared to untreated controls (NC)). (B) Western blot analysis of LP9 cells for total thioredoxin protein after exposure to crocidolite asbestos, β-actin was used as a loading control. (C) Densitometric analysis of (B) using Quantity One software (n =2 per group).
Figure 2.2: Crocidolite asbestos exposure causes oxidation of Trx1 in human mesothelial cells. (A) LP9/h-TERT cells were exposed to crocidolite at $75 \times 10^6 \, \mu m^2/cm^2$ for 8 and 24 h and 40 µg of protein was run on a 15% native gel and a redox Western blot analysis was performed as described in the Methods section on the immobilized proteins. (B) Quantitation of the blot in (A). (C) Cells were exposed to two concentrations of crocidolite asbestos ($15 \times 10^6 \, \mu m^2/cm^2$ and $75 \times 10^6 \, \mu m^2/cm^2$), as well as chrysotile asbestos ($75 \times 10^6 \, \mu m^2/cm^2$) and a redox Western analysis was performed on the lysates [* p< 0.05 compared to null controls (n = 2 per group)].
Figure 2.3: Inhibition of thioredoxin reductase by DNCB and pretreatment of cells with DHA rescues asbestos-induced oxidation of Trx1. (A) LDH assay to assess lytic cell death after pretreatment of LP9 cells with DNCB and exposure to asbestos (data is presented as a percentage of the lytic control). (B) Effect of DNCB on asbestos-induced Trx1 oxidation. Cells were pretreated with 10 µM DNCB for an hour and then exposed to asbestos for 8 h. Cell lysates were then derivatized with IAA and analyzed for oxidation of Trx1 by redox Western blot and densitometry of redox Western analysis of Trx1 oxidation state was performed. (C) Effect of DNCB pretreatment on chrysotile asbestos-induced oxidation of TRX1 (D) Analysis of apoptosis in response to DNCB pretreatment and asbestos exposure for 8 h as measured by AposTain technique. (E) LP9 cells were pretreated with 1mM DHA for an hour and exposed to asbestos for 8h. Thereafter, the oxidation state of Trx1 was assessed by redox Western blot analysis (* p< 0.05 compared to null controls; †p<0.05 compared to crocidolite asbestos exposure alone (Croc 75) n = 2 per group).
Figure 2.4: Over-expression of Trx1 increases cell survival and ameliorates asbestos-induced ROS generation in LP9 cells. (A) LP9 cells were exposed to two doses of crocidolite asbestos (15 x 10^6 µm^2/cm^2 and 75 x 10^6 µm^2/cm^2) for 24 h and incubated with NBT for 45 min at 37°C. The absorbance of the solubilized formazan formed after incubation with NBT was then read at 630 nm to determine ROS levels after asbestos exposure. (B) Over-expression of Trx1 in LP9 cells using the pCMV-SPORT6 plasmid was confirmed by qRT-PCR 48 and 72 h after transfection. (C) Trx1 transfected cells were exposed to crocidolite asbestos for 2 h and incubated with NBT for 45 min to determine ROS levels. Solubilized formazan was measured spectrophotometrically at 630 nm on a plate reader. (D) Analysis of the oxidation state of Trx1 after asbestos exposure of Trx1 over-expressing LP9 cells was determined by redox Western blot analysis and densitometry of the blot was performed (n = 2 per group). (E) LP9 cells transfected with the Trx1 over-expressing plasmid, pCMV-SPORT6 were exposed to crocidolite asbestos for the times indicated. Cells were then trypsinized and counted to estimate cell survival (*p < 0.05 compared to control; †p < 0.05 compared to Trx1 OE). Cell survival and NTB graphs are the average results of 3 experiments.

Figure 2.5: Asbestos-induced inflammasome priming and activation is attenuated by NAC. (A) LP9 cells pretreated with 2 mM NAC were exposed to 5µg/cm^2 asbestos for 48 h and changes in NLRP3 mRNA levels were assessed by qRT-PCR. (B) Inflammasome activation was assessed by Western blot analysis of the media supernatants from cells exposed to asbestos with and without pretreatment with NAC. Immobilized proteins on the nitrocellulose membrane were probed for the presence of active caspase-1 (p20 fragment) (*p< 0.05 compared to null control; † compared to Croc 75 alone; n = 2 per group).

Figure 2.6: Knockdown of TXNIP decreases inflammasome activation. (A) LP9/hTERT cells (90% confluent) were transiently transfected with siTXNIP or siControl siRNA for 48 h and knockdown of TXNIP expression was assessed by qRT-PCR. (*p< 0.05 compared to siControl) (B) siControl and siTXNIP transfected cells were exposed to crocidolite asbestos for 48 h and the medium were collected, concentrated and analyzed for the presence of caspase-1 (p20) by Western blot analysis. (C) The transcript levels of ERK 1/2 as well as TXNIP were verified in shERK2 HMESO cells by qRT-PCR in the presence and absence of 5 µM Dox along with priming of the inflammasome (*p<0.05 compared to shCon alone; †p<0.05 compared to shCon + Dox; n = 2 per group). (D) Inflammasome activation was assessed by Western blot analysis for the p20 fragment of caspase-1 in the media supernatants after treatment with Dox (*p<0.05 compared to shCon alone; †p<0.05 compared to shCon + Dox; n = 2 per group). (E) LP9 cells were pretreated with 40 µM of the caspase-1 inhibitor VI (cas-1 inh) prior to exposure to asbestos for 48 h and cells were counted to determine survival (*p<0.05 compared to control; †p<0.05 compared to Croc 75 alone). (F) Western blot analysis for Cas-1 p20 fragment in medium supernatant from LP9 cells pretreated with the caspase-1 inhibitor prior to exposure to asbestos (n = 2 per group).
Figure 5

A

NLRP3 fold change

NC  NAC  Croc 75  Croc 75 + NAC

B

Caspase-1 (20kDa) intensity

NC  NAC  Croc 75  Croc 75 + NAC

Caspase-1 (20kDa)
Figure 6

A

B

C

D

E

F

97
Figure 2.7: Role of ROS and antioxidants in asbestos-induced activation of the NLRP3 inflammasome. A simplified schema showing how increased ROS or decreased GSH as a result of asbestos exposure can cause oxidation of Trx1 and release of TXNIP. TXNIP thus released binds to NLRP3 and activates it as represented by caspase-1 activation. NAC on the other hand reduces ROS and elevates GSH levels resulting in inhibition of activation of NLRP3.
CHAPTER THREE: ASBESTOS-INDUCED MESOTHELIAL TO FIBROBLASTIC TRANSITION IS MODULATED BY THE INFLAMMASOME

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Abstract

Despite the causal relationship established between malignant mesothelioma (MM) and asbestos exposure, the exact mechanism by which asbestos induces this neoplasm and other asbestos-related diseases is still not well understood. MM is characterized by chronic inflammation which is believed to play an intrinsic role in the etiology of this disease. We have recently shown that asbestos activates the nod-like receptor family member containing a pyrin domain 3 (NLRP3) inflammasome in a protracted manner leading to an up-regulation of IL-1β and IL-18 production in human mesothelial cells. Combined with bio-persistence of asbestos fibers we hypothesize that this creates an environment of chronic IL-1β signaling in human mesothelial cells which may promote mesothelial to fibroblastic transition (MFT) in an NLRP3 dependent manner. Using a series of experiments, we show that asbestos induces a fibroblastic transition of mesothelial cells with a gain of mesenchymal markers (vimentin and N-cadherin) while epithelial markers like E-cadherin are down-regulated. Use of small interfering RNA against NLRP3, recombinant IL-1β and IL-1 receptor antagonist confirmed the role of NLRP3 inflammasome dependent IL-1β in the process. In vivo studies using wild type and various inflammasome component knockout mice also demonstrated the process of asbestos-induced MFT and its amelioration in caspase-1 knockout mice. Taken together, our data are the first to suggest that asbestos induces MFT in an inflammasome dependent manner.
Introduction

Malignant mesothelioma (MM) is a fatal cancer of the pleural or peritoneal mesothelium. MM has been causally related to asbestos exposure for over 50 years \(^1\) yet the mechanisms involved in the development of the disease are still poorly understood. With a latency period of 10-40 years and a propensity towards resistance and recurrence, MM is in dire need of new drug targets for combination therapy as well as biomarkers for early detection. Chronic inflammation has been implicated in the development and progression of MM \(^2\) and prevalently high circulating cytokines which are incidentally associated with fibrosis (interleukin 6 (IL-6), Platelet Derived Growth Factor and IL-8) have been used as prognosticators of survival for MM patients \(^3,4\). A recent massive parallel sequencing study of immortalized and primary mesothelial cells after asbestos exposure has shown that the most highly upregulated genes after asbestos exposure belong to inflammatory networks \(^5\), further implicating inflammation as an important factor in the development of asbestos-related diseases.

We have previously shown that asbestos activates the nod-like receptor containing a pyrin domain 3 (NLRP3) inflammasome, leading to the secretion of mature IL-1\(\beta\) and IL-18 in human mesothelial cells (HMCs)\(^6\). In these cells however, the activation of the inflammasome is protracted leading to the accumulation of IL-1\(\beta\) and IL-18\(^6\). IL-1\(\beta\) signaling has been shown to upregulate the secretion of the cytokines, IL-6 and IL-8 which have been demonstrated to play a major role in lung fibrosis\(^7\). In addition to promoting fibrosis, IL-1\(\beta\) has been shown to induce epithelial to mesenchymal transition (EMT) in corneal endothelial cells as well as inducing stemness in colon cancer cells through the
activation of transcription factors that regulate the mesenchymal phenotype\textsuperscript{8,9}. On the other hand, NLRP3 and apoptosis speck-like protein containing a CARD domain (ASC) (an adaptor protein for the assembly of the inflammasome) have both been shown to play a role in transforming growth factor beta (TGF\(\beta\)) signaling independent of their inflammasome activity in tubular epithelial cells\textsuperscript{10}. We therefore hypothesized that the NLRP3 inflammasome and its products facilitate mesothelial to fibroblastic transition (MFT) of human mesothelial cells in response to asbestos exposure. As such, asbestos-induced MFT may serve as the initial step in MM tumorigenesis. In this study we investigate the process of MFT in response to asbestos exposure at the transcriptional and translational levels as well as the role of the NLRP3 inflammasome components in the process, using \textit{in vitro} and \textit{in vivo} models. We show for the first time that asbestos induced inflammasome activation results in IL-1\(\beta\) secretion, which promotes MFT through the signaling cascades elicited by IL-8 and IL-6 which may involve tissue factor pathway inhibitor 2 (TFPI2), and fibroblast growth factor 2 (FGF2).

\textbf{Materials and Methods}

\textit{Cell Culture}

Immortalized human peritoneal (LP9/hTERT (LP9)), primary human pleural (HPM-3) and peritoneal (HM3) mesothelial cells were purchased from Brigham and Women’s Hospital (Boston, MA) and were cultured as previously described\textsuperscript{6}. All cells were grown to 90-95\% confluency. All experiments were repeated at least two or more times.
Asbestos fiber preparation for exposure

NIEHS reference samples of crocidolite asbestos have been previously characterized\textsuperscript{11}. Fibers were sterilized and suspended as previously described\textsuperscript{6}.

Cell treatments with asbestos/IL-1β/IL-1 receptor antagonist

To assess the response of human mesothelial cells to long term asbestos exposure, cells were exposed to two pulses of $1 \times 10^{-6}$ um$^2$/cm$^2$ (LP9 and HM3) or $5 \times 10^{-6}$ um$^2$/cm$^2$ (HPM3, as these cells were less sensitive to asbestos) μg/cm$^2$ crocidolite asbestos 48 h apart. Medium was refreshed every 48 h (until cells were harvested) to ensure that cells were not nutrient starved; thus, it was imperative to replace any asbestos fibers lost in the process of switching out media. LP9 and HM3 cells displayed a permanently altered cell shape 10-14 days after the initial asbestos exposure. For HPM3 cells, duration to transition was much shorter (1 week post addition of first asbestos pulse). Thus, cells were maintained in fresh medium for the last 72 h with no ill effects prior to termination of the experiment. A schematic representation of asbestos pulses and medium changes is depicted in Figure 1A. In experiments where the effect of asbestos-induced IL-1β signaling on MFT was being assessed, recombinant IL-1β (Peprotech, Rocky Hill, NJ) or its receptor antagonist (IL-1Ra) (Insight Genomics, Sterling VA) were also replaced during the first 48 hour medium change. HPM3 or LP9 cells were pretreated with two pulses of either 1 ng/mL IL-1β or 150 ng/mL IL-1Ra respectively one hour before asbestos exposure. Both agents were stored in 0.1% BSA solutions and as such,
control cells received equal amounts of 0.1% BSA for the same duration as a vehicle control. Cells receiving both IL-1Ra and IL-1β were pretreated with the antagonist one hour prior to treatment with IL-1β. Effect of asbestos exposure on viability of different human mesothelial cells showed that peritoneal cells are more sensitive to asbestos exposure than pleural mesothelial cells\textsuperscript{12,13}. Precisely, we observed 35-40% cell death in peritoneal mesothelial cells (LP9 and HM3) in response to asbestos exposure as compared to 15-20% cell death in pleural mesothelial cells (HPM3 and HPM4)\textsuperscript{12}. Glass beads (GB) were used as negative control at equal particle surface area concentration and showed no significant effect on viability and other parameters \textsuperscript{6,13}.

\textit{Assessment of cell morphology}

Cells were inspected visually for changes in morphology (acquisition of a spindle-like shape and reorientation/alignment of cells into a whorl) and imaged 48 h after each pulse and at time of harvest using an Olympus IX70 inverted light microscope (Olympus America Inc. Center Valley, PA) in phase contrast with a 20X objective. Cells were also followed visually with each media change to ascertain whether transition in cell morphology remained stable and to determine when transition in shape became more uniform.

\textit{Quantitation of cell morphology}

The effects of asbestos exposure on the morphology of cells was assessed using the integrated morphology analysis module in the MetaMorph Image Analysis software.
(Molecular Devices, Sunnyvale, CA). A total of thirty cells per dish were outlined in MetaMorph to obtain a measure of their perimeters and total area which was then used to determine the shape factor for each cell (Shape factor = \(\frac{4\pi A}{P^2}\) A= area; P= perimeter). The shape factor is a value that ranges from 0 to 1 as a measure of how circular an object is (0 represents a flattened non circular object whereas 1 represents a perfect circle). The change in length of the cells was also determined using the region measurements tool to measure the longest axis (longest chord through the cell) of each of the cells as well. Data obtained from a total of sixty cells per group were then pasted into Graph Pad Prism v 6.0 to yield a min to max plot for each condition and for statistical analysis of the resulting distribution.

**Cell transformation assay**

To determine whether LP9 cells that had undergone MFT were capable of anchorage independent growth, cells were trypsinized at the end of the MFT experiment and used for a CytoSelect™ 96-well cell transformation assay (Cell Biolabs Inc. San Diego, CA) per the manufacturer’s protocol. Wells were monitored for colony formation after 1 week in culture and imaged in phase contrast on the Olympus IX70 microscope each week.

**PCR Array**

To determine whether the transcripts of genes involved in the EMT pathway were altered in response to asbestos exposure, LP9 and HPM3 cells were exposed to asbestos (5 \(\mu g/cm^2\)) for 48 h or 1 week (HPM3 only). As a positive control, LP9 cells were treated with 10 ng/ml TGFβ for 48 h to induce MFT. Cells were then lysed for total RNA extraction using
the Qiagen RNeasy Plus Mini kit as per the manufacturer’s directions. After ensuring the integrity of the RNA samples, 500 ng of RNA was used for cDNA synthesis to be utilized in the Human EMT pathway RT² Profiler PCR Array as described previously\textsuperscript{14}. Data obtained were then analyzed using the online SA Biosciences data analysis template.

Transfection experiments

The role of the inflammasome in asbestos-induced MFT was examined in mesothelial cells using siRNA approaches. Ninety percent confluent LP9 cells were transiently transfected with On-Target Smart Pool© siNLRP3 (ThermoScientific (Life Technologies), Grand Island, NY) or non-targeted control (siControl) constructs 32 h prior to asbestos exposure to study the effects of reduced NLRP3 protein levels on MFT as described before\textsuperscript{14}. Additional experiments were performed with 4 single siNLRP3 and non-target controls (ThermoScientific (Life Technologies), Grand Island, NY) as described above.

Western Blot Analysis

Cells were lysed in 4x sample buffer and boiled at 95°C for 15 minutes as previously described\textsuperscript{15}. For analysis of EMT parameters, 40 μg of protein was loaded on 15, 10 or 7.5 % SDS PAGE gels to resolve proteins. Immunoblotting for EMT markers was performed on transferred proteins using E-cadherin, Snail, Slug, N-cadherin, Vimentin and ZO-1 (Cell Signaling Technology, Danvers, MA). Western blot analysis was performed on media supernatants or peritoneal lavage fluid (PLF) after concentration. Equal volumes of media supernatants or PLF were concentrated using StrataClean resin beads (Agilent
Technologies, Santa Clara, CA) as previously reported\textsuperscript{16}. An equal volume of 4X sample buffer was added to beads after media had been aspirated and boiled for 5 min at 95°C. Thereafter 10-15 μL of each sample was resolved on a 15 % SDS PAGE for subsequent immunoblotting for pro-inflammatory cytokines IL-6, IL-8, FGF2, TFPI2 and the danger associated molecule HMGB1 (Abcam, Cambridge, MA).

**ELISA for IL-18 and IL-1β**

Media supernatants from *in vitro* experiments or PLF from *in vivo* experiments were concentrated in Amicon centrifugal filtration units with a molecular weight limit of 10 kDa (Millipore, Billerica MA) as described previously\textsuperscript{6}. The levels of IL-1β and IL-18 secreted in response to asbestos exposure were then measured using the Human QuantiKine IL-1β/IL-1f2 Immunoassay (R&D Systems, Minneapolis, MN) and Human IL-18 ELISA kits (MBL, Woburn, MA) respectively following the manufacturer’s directions. Values are expressed as pg (IL-1β /IL-18)/ mL of total culture supernatant initially collected.

**Mouse models of asbestos exposure**

To study in vivo effect of asbestos on mesothelial cell transition we selected intraperitoneal injection model of asbestos exposure developed by Goodlick *et al*\textsuperscript{17} and recently used by various groups\textsuperscript{18,19}. All these studies demonstrated the development of early inflammation\textsuperscript{18}, peritoneal fibrosis and eventually MM in mice\textsuperscript{17,19} in response to asbestos. Inhalation or aspiration models were not considered for this study as asbestos exposure by either model has not been shown to cause peritoneal fibrosis or MM in mice. Age (8-10
weeks) and sex matched NLRP3−/− and Cas-1−/− mice (C57/BL/6 background, obtained from Dr. Matthew Poynter (UVM) and bred in our facility) as well as their wild type C57BL/6 (Charles River Laboratory) counterparts were housed in isolators with ad libitum access to food and water according to the University of Vermont Institutional Animal Care and Use Committee guidelines (#12-004). Mice (6-8 mice per group) received either 500 μL of 0.9% sterile saline or 500 μL of 200 μg/mL crocidolite asbestos ip once a week over the course of 8 weeks as previously described. As a negative control, mice were injected with glass beads (GB, Polysciences Inc., Warrington, PA) at an equal surface area concentration (500 μL of 1.076 mg/mL). Mice were euthanized with an overdose of sodium pentobarbital after which the peritoneal walls and diaphragms were harvested, fixed in 4% PFA with a specific orientation of mesothelial lining facing outward. Cross sections of paraffin embedded peritoneal walls were made, hematoxylin and eosin (H&E) stained and examined histologically as demonstrated previously by Donaldson et al. Images were obtained using an Olympus BX50 with a Retiga Magnafire 2000R camera (Olympus America Inc., Center Valley, PA) (20X objective). Peritoneal wall sections were also stained for collagen (Col 1α1) (PhophoSolutions, Aurora CO), pan-cytokeratin (Antibodies-online.com, Atlanta, GA), alpha smooth muscle actin (αSMA) (Sigma Aldrich, St Louis, MO) and vimentin (Vim) (Cell Signaling Technologies, Danvers, MA). Immunofluorescently stained sections were imaged on a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss, Peabody, MA). All mice were confirmed for their genotype at the beginning and end of the experiment.
**MetaMorph Analysis for quantitation of thickening of peritoneum**

Thickening of the parietal submesothelium after asbestos exposure was determined as follows: Images of peritoneal wall sections obtained with an Olympus BX50 light microscope and imported into the offline version of the MetaMorph imaging analysis software (Molecular Devices, Sunnyvale, CA) and calibrated for measurements. Using the line tool, the distance from the edge of the mesothelium to the border between the muscle cells underneath was measured and exported into Excel. Five measurements were taken for each of the five images taken per sample and the average ±SEM of the 25 measurements were used to plot a graph of the increase in thickness of the parietal mesothelium in response to asbestos exposure.

**PLF collection and analysis for total cell counts, differentials and cytokines**

At the time of harvest, peritoneal lavage fluid (PLF) was collected from each animal as previously described \(^{23}\). The resultant lavage fluid was drawn out for the measurement of cytokines [by ELISA (IL-1β and IL-18) and Western blot analysis (FGF2, IL-6 and TFPI2)] and identification of inflammatory cell infiltrating the peritoneum after asbestos exposure. Cells collected from PLF were used for cytospin preparation for differential cell counts after determination of total cell numbers as previously described \(^{23}\).

**Immunohistochemistry and Immunocytochemistry**
To assess EMT marker expression in response to asbestos exposure, formalin fixed paraffin embedded sections of peritoneal walls were deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. Thereafter samples were immunofluorescently stained as previously described\textsuperscript{24}. Peritoneal wall sections were stained for pan-cytokeratin (AE1+AE3) (Antibodies-online.com, Atlanta, GA), vimentin, αSMA and Col1α1. Primary antibodies were used at a ratio of 1:100 or the recommended dilution from the manufacturer. The appropriate secondary antibody conjugated to Alexa fluor 647 (goat anti rabbit) or Alexa fluor 588 (goat anti mouse) were employed at a dilution of 1:400.

**Statistical Analysis**

All experiments were performed in duplicate or triplicate and repeated at least twice. A one-way analysis of variance (ANOVA) followed by a Newman-Keuls procedure for adjustment of multiple pairwise comparisons or the Student t-test was applied to all data points to establish the significance of observed differences between the various experimental groups. A p value ≤0.05 was considered to be significant. All statistical analyses were performed using the GraphPad v 6.0 software program.

**Results**

*Asbestos exposure induces MFT in mesothelial cells as depicted by changes in gene profile:*

To determine whether asbestos was capable of inducing a mesothelial to fibroblastic transition, LP9 and HPM3 were exposed to asbestos for 48 h or 1 wk with TGFβ as a positive control and an EMT PCR Array was performed. Expression levels of genes related to an epithelial phenotype like E-cadherin (*CDH1*), keratin 19 (*KRT19*), keratin 7 (*KRT7*),
macrophage stimulating 1 receptor \((MST1R)\) which is usually expressed on ciliated epithelial cells and \(F11R\), a regulator of tight junction assembly were reduced after asbestos exposure in both cell lines. Changes in the transcript levels of genes related to fibroblastic phenotype were different in magnitude between the two cell types in response to asbestos. For instance, while \(SNAI1\) was downregulated at 24 and 48 h in LP9 cells after asbestos exposure there was a two-fold increase seen in HPM3 cells after one week of asbestos exposure (Table 1). \(TIMP1\) was upregulated by as much as 36-fold in the LP9s (after 24 h) but did not make the two-fold cutoff in HPM3 cells after asbestos exposure (supplemental tables 1 and 2). \(TFPI2\) levels however, increased in both cell types (Table 1) but to a greater magnitude in LP9 cells (52-fold in LP9s and 13-fold in HPM3). TGF family member, bone morphogenic protein 2 \((BMP2)\) was upregulated to the same extent in both cell lines in response to asbestos (Table 1).

**Asbestos exposure causes morphological changes in mesothelial cells:**

Exposure of LP9 and HM3 cells to asbestos for 2 weeks with fresh medium every 48 h resulted in a change in morphology of the cells to a fibroblastic cell type (Figure 1B). While exposure to 1 \(\mu g/cm^2\) asbestos had no effect on HPM3 cells (data not shown), exposure to 5 \(\mu g/cm^2\) asbestos resulted in morphological changes over the course of one week (Figure 1B). In a colony forming transformation assay only asbestos-exposed LP9 cells which appeared fibroblastic formed colonies after two to six weeks on soft agar and some colonies were still associated with asbestos fibers (Figure 1C). Morphometric analysis of LP9 and HPM3 cells in figure 1B showed that the length of LP9 cells did not change significantly
with exposure to asbestos (Figure 1D). However, there was a significant decrease in the circularity of these cells upon exposure to asbestos (Figure 1D) (* p<0.05 compared to control). For HPM3 cells, the length of the cells increased with exposure to asbestos in a dose dependent manner and the circularity (shape factor) of the cells decreased with exposure to the higher dose of asbestos alone (Figure 1 E) (* p< 0.05 compared to control).

Asbestos exposure causes changes in epithelial and mesenchymal markers in human mesothelial cells:

Assessment of the expression levels of epithelial and mesenchymal markers after asbestos exposure revealed a marked reduction in the expression levels of E-cadherin in both LP9 and HPM3 cells (Figure 2A and 2C respectively). The transcription factors Snail and Slug, both down-regulators of E-cadherin expression, were upregulated in response to asbestos exposure in LP9 cells (Figure 2A). The mesenchymal markers, vimentin and N-cadherin were upregulated in LP9 cells (Figure 2C) while HPM3 only exhibited an increase in vimentin protein levels (Figures 2A). No significant changes in snail or slug protein levels were observed in HPM3 cells. IL-6, IL-8, FGF2 and TFPI2 levels were also markedly elevated in media after 96 h of asbestos exposure as determined by Western blot analysis of media supernatants pooled from both pulses of asbestos exposure (Figure 2B and D). Increases in IL-8 and IL-6 levels in HPM3 cells were however not significant after 96 h (Figure 2B). Beta-actin (Figure 2A) or α-tubulin (Figure 2D) was used as a loading control for lysate samples. For medium samples, Ponceau stain was used to confirm equal loading due to unavailability of an appropriate loading control (data not shown). Assessment of
mRNA levels for E-cadherin (*CDH1*), N-cadherin (*CDH2*), *IL-8*, *IL-6*, *SNAI1* (snail) and *SNAI2* (slug) by qPCR at 48 h and 5 days show that asbestos exposure decreases the transcript levels of E-cadherin as early as 48 h after exposure (Figure 2E). N-cadherin (which is constitutively expressed by mesothelial cells) levels were surprisingly decreased after 48 h as well, and exhibited a decrease in transcript levels over time in moderate but significant amounts which were not affected by asbestos exposure after 5 days (Figure 2E). Transcript levels of *IL-8* increase initially after 48 h of exposure but decrease over time with or without asbestos exposure (Figure 2E). *IL-6* transcript levels also increase significantly with asbestos exposure but only after the first 48 h (Figure 2E). While *SNAI1* levels decrease with asbestos exposure and with time, asbestos exposure significantly increased *SNAI2* transcript levels at both time points (Figure 2E). Levels of IL-1β and IL-18 were also found to be increased in supernatant of HPM3 cells when measured by ELISA (Figure 2F and 2G). Similar results were obtained from LP9 cells published previously.

*The NLRP3 inflammasome augments asbestos-induced MFT:*

In assessing the role of NLRP3 in the asbestos-induced MFT observed, the levels of FGF2, *IL-8*, *IL-6* and TFPI2 were all found to be decreased in medium in response to asbestos exposure in siNLRP3 transfected cells when compared to control transfected cells (Figure 3A). The loss of E-cadherin in response to asbestos exposure was not reversed significantly by inhibition of NLRP3 expression by siRNA. (Figure 3B). Lack of sample material prevented us from measuring IL-1β and IL-18 in this experiment. These results demonstrate a partial role of NLRP3 in asbestos-induced MFT. No significant effect on
morphology change could be demonstrated as that is a long term experiment whereas transient transfection effects last only for a short duration. The reduction of asbestos-induced NLRP3 activation in LP9 cells after siRNA treatment was confirmed by qRTPCR and showed a significant decrease in NLRP3 transcript levels (priming) in response to asbestos exposure after knockdown by siRNA 72 h post transfection (Figure 3C).

We also used four single siNLRP3 constructs [siNLRP3(05-08)] and siNLRP3(06) showed most remarkable effect on NLRP3 mRNA levels (Figure 3C). Of the 4 constructs, only siNLRP3(06) had an appreciable effect on attenuation of IL-6 and IL-8 levels. TFPI2 and FGF2 levels were only slightly decreased in response to asbestos exposure in cells transfected with siNLRP3(06) (Figure 3D) as compared to controls. The effect with the pool of 4 siNLRP3 was certainly more remarkable than single siNLRP3, suggesting that the use of pool of siRNA may be a more effective approach.

*IL-1β signaling regulates asbestos-induced MFT:*

As asbestos activates the NLRP3 inflammasome with a concomitant release of IL-1β in human mesothelial cells⁶, the next step was to demonstrate the role of this cytokine in asbestos-induced MFT. Pretreatment of HMCs (HPM3 and LP9) with the IL-1 receptor antagonist, IL-1Ra (150 ng/ml), prior to asbestos exposure resulted in a delay in the transition of the morphology of HPM3 cells to a more fibroblastic appearance (Figure 4A, Figure S1A (LP9 cells)). Cells exposed to asbestos were generally more elongated and narrower in width than control cells (mean shape factor of ~ 0.4 and median length of about 60 microns), and blocking IL-1R delayed this process at 48 h, as fewer cells were long
and narrow as determined using the morphometric analysis (Figure 4A). Additionally, blocking of the IL-1 receptor resulted in a significant decrease in the levels of IL-6, IL-8 and TFPI2 while increasing the levels of FGF2 secreted in response to asbestos exposure (Figure 4B). Furthermore, pretreatment of cells with IL-1Ra also reduced levels of IL-18 in response to asbestos exposure (Figure 4C). In LP9 cells, pretreatment with IL-1Ra reduced IL-8 and FGF2 levels noticeably while only slight reductions in IL-6 and TFPI2 were observed (Figure S1B). To further confirm the role of IL-1β signaling in asbestos-induced MFT, HPM3 and LP9 cells were treated with two pulses of recombinant IL-1β (0.5, 1, 5 or 10 ng/mL). The greatest change in morphology was observed in the group exposed to 1 ng/mL IL-1β (Figure 4D, S1C and S1D); cells in this group were the most elongated and least circular (Figure 4D and S1D graphs). The change in morphology observed with 0.5 ng/mL was no different from that observed with 1 ng but was inconsistent (not shown). Western blot analysis of media supernatants from cells treated with 2 doses of IL-1β indicated that treatment with the lower dose of IL-1β (1 ng/mL) led to activation of caspase-1 as measured by levels of the p20 subunit of active Cas-1 in the supernatant. Additionally, IL-6 levels were increased by treatment with the lower concentration of IL-1β, but had a minimal effect on TFPI2 secretion. Unlike asbestos exposure, treatment with either concentration of IL-1β failed to increase levels of the danger associated molecule, HMGB1 (Figure 4E). The increase in IL-6, IL-8 and FGF2 induced by IL-1β was attenuated by pretreatment of HPM3 cells with IL-1Ra (150 ng/mL). However, this concentration of IL-1Ra failed to block the transition of HPM3 cells to a more fibroblastic phenotype in response to IL-1β (data not shown). IL-1β treatment also
caused a decrease in E-cadherin and upon pretreatment of cells with a higher dose of IL-1Ra (1 μg/mL), a small increase in E-cadherin was observed (Figure 4F). FGF2 and IL-8 levels were drastically reduced by the 1 μg/mL as well (Figure 4F). The expected blockage of IL-1β-induced MFT was also observed morphologically in LP9 cells pretreated with IL-1Ra (Figure S1E and S1F), suggesting that the previous dose was too low to effectively block all the IL-1 receptors available. Unlike HPM3 cells however, treatment of LP9 cells with IL-1β caused a slight increase in E-cadherin levels but no change in N-cadherin, whereas pretreatment with IL-1Ra restored E-cadherin levels and decreased N-cadherin levels slightly (Figure S1F, lower panel).

Asbestos exposure causes MFT in mice that is dependent on inflammasome activation: Having made these observations in vitro, in vivo asbestos exposure experiments were conducted to confirm whether asbestos had the same effect in C57BL/6 mice. Asbestos exposure caused a significant increase in the thickness of the peritoneal submesothelium when compared to controls at 8 weeks (Figure 5A). Glass beads (which are non-fibrous silicates) were used as a negative control. For asbestos exposed samples, five random fields spanning the thickened sections of the peritoneal walls were imaged. Five random fields of control samples were also imaged to ensure there was no bias in variation of submesothelium thickness within and between samples. An increase in total number of cells infiltrating the peritoneal cavity was observed at early time points in the PLF with a peak on day 9 (Figure 5B). The neutrophil numbers however, dipped after day 3 and peaked again on day 28 (Figure 5C). IL-1β and IL-18 levels peaked on day 3 and progressively declined over the course of the experiment but rose again after 8 weeks (Figure 5D and
Immunohistochemical analysis of the peritoneal wall cross-sections for the expression levels of vimentin, alpha smooth muscle actin (αSMA), collagen 1α1 (Col1α1) and cytokeratin 18 showed increases in expression levels of these proteins after asbestos exposure (Figure 5F). Chrysotile asbestos also caused a significant increase in the thickness of the peritoneal wall, whereas, glass beads (GB) did not cause thickness increases, but occasional sites of GB surrounded by inflammatory cells were observed (6A).

Asbestos-induced thickening of the peritoneal wall is caspase-1 dependent:

The peritoneal walls of inflammasome component knock out mice (NLRP3−/−, ASC−/− and Cas-1−/− respectively) were assessed for asbestos-induced thickening of the submesothelium after H&E staining as described above. NLRP3−/− mice showed decreased thickening of their peritoneal walls when compared to their wild type counterparts; however, it was not significant. On the other hand, Cas-1−/− mice exposed to asbestos had significantly less thickening in the submesothelium (Figure 6A-6C). Glass beads, which were used as a particulate negative control did not cause thickening of the sub-mesothelium (Figure 6A). No significant difference in thickening was observed with ASC−/− mice (data not shown). No significant changes were observed in total cell counts (Figure 6D), but infiltrating neutrophil levels (Figure 6E) were lower in Cas-1−/− mice when compared to WT mice exposed to asbestos. While IL-1β levels in the PLF of Cas-1−/− mice were not significantly different from that in WT mice (Figure 6F), a significant reduction in IL-18 levels was observed in Cas-1−/− after asbestos exposure (Figure 6G). Additionally, the assessment of FGF2 and TFPI2 by Western blot analysis showed drastic reduction in the levels of these
two proteins in response to asbestos exposure in the Cas-1 \(^{-/-}\) mice (Figure 6H). However, IL-6 levels were higher in exposed mice as compared to their saline controls but were inconclusive (Figure 6H).

**Discussion**

The mechanisms involved in asbestos-induced MM are less understood. Chronic inflammation is believed to play an important role in the etiology of this deadly disease\(^4\). Recently, we have demonstrated that asbestos can prime and activate the NLRP3 inflammasome in mesothelial cells\(^6\) resulting in the release of pro-inflammatory cytokines, IL-1\(\beta\) and IL-18. IL-1\(\beta\) has been shown to promote EMT and cancer cell stemness in a number of cell types\(^8,9\). Cell type specific studies on the importance of the inflammasome for cancers with an inflammatory signature have indicated that the inflammasome is a key player in the etiology of these cancers\(^25-28\). These studies, combined with our own observations suggest that the inflammasome may be a key potentiator of asbestos-induced MFT and MM. We therefore hypothesized that activation of the inflammasome in response to asbestos exposure facilitates MFT which may be the earliest event in the process of MM development. Transcript levels of the EMT related genes from PCR Array revealed changes in expression in mesothelial cells that did not exactly mimic changes produced by TGF\(\beta\), the positive control (Table 1). These results suggested that asbestos-induced MFT occurred through pathways which may not be directly downstream of TGF\(\beta\) (like BMP2) signaling but involved some of the same players. Although asbestos exposure led to a 12-fold decrease in Snai1 mRNA levels over a 48 h period in LP9/hTERT cells, increased
protein levels of Snai1 were observed at later time point in HPM3. These gene changes were accompanied by changes in morphology of mesothelial cells to a more fibroblastic type. The differences observed in the gene expression between the two cell types may be due to the differential susceptibility of the peritoneal and pleural mesothelial cells to asbestos-induced regulation of genes as observed in a recent NGS study by our group 12. For in vivo studies we selected a mouse model of asbestos exposure where inflammation, fibrosis and MM development have been demonstrated. In vivo studies of asbestos exposure also showed that asbestos causes a significant increase in the thickness of the parietal peritoneal sub-mesothelium which is accompanied by increased collagen deposition as well as increased vimentin and αSMA expression in the same region. Additionally, cytokeratin expression is maintained in the mesothelium with and without asbestos exposure which suggests that the cells in the submesothelium are mesothelial in origin. A study of the changes observed in the peritoneal wall of continuous ambulatory peritoneal dialysis (CAPD) patients also revealed similar changes in the submesothelium 29 as were observed in the peritoneal walls of mice exposed to asbestos.

We are the first to report that asbestos exposure upregulates FGF2 and TFPI2 secretion both in vitro and in vivo, and may play an important role in the transition from mesothelial to fibroblastic/myofibroblastic phenotype. Support of our findings is derived from other reports that FGF2 in concert with IL-1β promotes EMT in corneal endothelial cells 9, while FGF2 alone is capable of inducing EMT in HERS cells during cementum formation in tooth development 30. Another report has shown that MCF-7 cells undergoing EMT upregulate TFPI2 gene expression in excess of 300-fold 31, which corroborates our
findings. TFPI2 inhibits fibrinolysis, an important function of the mesothelium that prevents adhesion of organs \textsuperscript{32}. Fibrin has also been shown to promote EMT in a study where mesothelial cells in culture were overlaid with fibrin resulting in a fibroblastic transition of the mesothelial cells \textsuperscript{33}. Consequently, over-expression of this protein in response to asbestos exposure could lead to fibrin deposition thereby facilitating the MFT observed in our study. Further studies are underway to determine the exact role these proteins play and the mechanisms they utilize in asbestos-induced MFT. As asbestos exposure also induces the secretion of IL-1β and IL-18 under both \textit{in vitro} and \textit{in vivo} conditions by activating the NLRP3 inflammasome \textsuperscript{6} or by other mechanisms, it is likely that the phenomenon of MFT observed in response to extended asbestos exposure is due to IL-1β signaling. This was confirmed by using IL-1Ra prior to asbestos exposure, which delayed the onset of MFT compared to cells exposed to asbestos alone. However, there was a greater loss of cells after transitioning into a fibroblastic phenotype in IL-1Ra pretreated cells suggesting that IL-1β acts as a pro-survival signal caused by asbestos exposure and may be considered as an early asbestos-exposure biomarker for asbestos related diseases. Secretion of the pro-inflammatory cytokines, IL-8 and IL-6 (both implicated in fibrogenesis) in response to asbestos was significantly reduced by the blockade of the IL-1 receptor suggesting that they are directly or indirectly regulated by IL-1β signaling. Secretion of TFPI2 was also decreased by blockade of IL-1R and this may be an indirect response caused by the reduction in IL-6 signaling. IL-6 signaling has been shown to regulate the expression of TFPI2 \textsuperscript{34}; thus, a decrease in IL-6 levels would explain the decrease in TFPI2 levels in the presence of IL-1Ra after asbestos exposure. Unlike the
other cytokines, FGF2, which is secreted in a non-classical secretion pathway dependent on caspase-1, was upregulated in response to asbestos after pretreatment with IL-1Ra. Since FGF2 can initiate EMT in CECs as well as HERS cells\(^9\),\(^{30}\), it may explain why the mesothelial cells eventually undergo MFT after the delay imposed by the antagonist. It is a possibility that both FGF2 and IL-1β play pivotal roles in asbestos-induced MFT. In fact, IL-1β has been shown to induce FGF2 secretion in human mesothelial cells as well as corneal endothelial cells\(^{35}\),\(^{36}\). The dose of IL-1Ra used in these experiments can be said to be the limiting factor as the use of an approximately 7-fold higher concentration (1 μg/mL) led to a complete blockage of MFT, with a slight reduction in FGF2 secretion (greatest reduction was observed in the lower molecular weight isoforms). Since cells were only exposed to IL-1Ra at this dose for 72 h with and without asbestos exposure, further studies are needed to ascertain the mechanism by which IL-1β initiates/induces MFT in mesothelial cells. The role of IL-1β in asbestos-induced MFT was further confirmed by recombinant IL-1β. Low concentration of IL-1β (1ng/mL) caused MFT, whereas high concentrations were ineffective. This observation and other published reports\(^{37}\) supports our finding that low concentrations of IL-1β secreted in response to asbestos exposure is enough to cause MFT, potentially, in association with other molecules.

As IL-1β can be secreted in response to non-canonical inflammasome activation as well as by other sources including caspase 8 activation\(^{38}\), there was a need to confirm that the asbestos-induced MFT observed was indeed dependent on regulation of the NLRP3 inflammasome. To demonstrate this, NLRP3 was knocked down in LP9/hTERT cells by siNLRP3. Exposure of these NLRP3 inhibited cells to asbestos resulted in a reduction in
TFPI2, IL-6 and FGF2 secretion in the culture supernatant as well as a partial rescue of the loss of E-cadherin expression as compared to control cells exposed to asbestos. NLRP3 and ASC have been reported to promote EMT independently of their inflammasome functions \(^1\); thus, ASC may play a similar role in our system accounting for the incomplete rescue of E-cadherin expression in response to asbestos after NLRP3 knock down. Since these experiments show a partial role of NLRP3 in asbestos-induced MFT, a stably NLRP3 inhibited cell line or recently discovered inhibitors \(^{39},^{40}\) are required to confirm these effects.

To confirm the role of inflammasomes in MFT in an \textit{in vivo} model, we used inflammasome component KO mice. Although ASC and NLRP3 KO mice did not show any significant difference in asbestos-induced peritoneal wall thickening as compared to their WT counterparts, caspase-1 KO mice had significantly attenuated submesothelial thickening after 8 weeks of asbestos exposure when compared to their saline counterparts and WT controls. This implies that caspase-1 is required for asbestos-induced thickening of the parietal mesothelium. Caspases including caspase-1 have been shown to be involved in injury/remodeling/fibrosis in various models \(^{41-45}\), however, we are first to report the involvement of caspase-1 in asbestos-induced MFT. Caspase-1 deficiency caused diminished levels of asbestos-induced IL-18 in the PLF, however, IL-1\(\beta\) levels were not affected. As IL-1\(\beta\) can be derived from sources other than the inflammasome, it is not a surprising observation. In addition, IL-1\(\beta\) regulates its own function by regulating IL-1Ra levels. Our microarray data show that asbestos inhalation results in 4-fold increased IL-1Ra mRNA levels in lungs at 9 days (unpublished data). The initial increase, followed by
decline and then recovery of IL-1β levels in our *in vivo* model could be explained by the regulation of IL-1β by IL-1Ra. To understand the role of IL-1β in MFT, currently we are using Anakinra and a specific NLRP3 inhibitor and plan to use IL-1R KO mice in the future. Furthermore, assessment of IL-1Ra and IL-1R expression is also underway as both of these can play important roles in IL-1β biology. NLRP3 inflammasome has also been shown to be involved in the process of fibrosis in various models. In support of our observations, a Finnish study has recently identified a polymorphism in NLRP3 with increased risk of asbestos-related interstitial lung fibrosis and a CARD8 polymorphism associated with development or calcification of pleural thickening. These SNPs have been linked to enhanced IL-1β production and severe inflammation. No significant effect seen in our NLRP3 KO model could be attributed to global knockdown of NLRP3 gene. As has been reported earlier, NLRP3/ASC deletion from different cell types could have different consequences. To pinpoint the role of mesothelial cell NLRP3/ASC in asbestos-induced MFT, mesothelial cell specific targeted knockdown is required. Taken together our data suggests that asbestos-induced MFT in mesothelial cells is regulated in part by the inflammasome via IL-1β/IL-18 in conjunction with TFPI2 and FGF2. Further studies are needed to dissect the roles played by the different proteins involved and this will help provide a basis for determining the mechanism and identifying biomarkers by which asbestos promotes cell survival that can lead to the development of malignancies like MM.
Acknowledgements: The authors thank the University of Vermont Cancer Center Advanced Genome Technologies Core and UVM Microscopy Imaging Center, for assistance in processing and analyzing samples.
References

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129
Table 3-1

**EMT related gene expression changes in mesothelial cells in response to asbestos exposure.** LP9 and HPM3 cells were exposed to asbestos (5 μg/cm²) for the indicated times and whole RNA extracted from these cells was analyzed for gene expression levels of EMT pathway proteins in an EMT PCR array. TGFβ (10 ng/ml) was used as a positive control.

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Figures

Figure 3.1: Asbestos exposure induces a morphological change in human mesothelial cells. A. Schematic representation of asbestos exposure schedule for induction MFT. B. Exposure of LP9, HM3 (peritoneal) and HPM3 (pleural) cells to asbestos [1 (peritoneal cells) \(15 \times 10^{-6} \ \mu m^2/cm^2\) (Asb 15)] and 5 \(\mu g/cm^2\) \(75 \times 10^{-6} \ \mu m^2/cm^2\) (Asb 75) (pleural cells)] for 2 and 1 week respectively resulted in morphological changes to the cells over the duration of exposure. C. Fibroblastic looking LP9 cells from B were grown on soft agar to determine whether the cells were transformed and capable of forming colonies. All micrographs imaged on Olympus IX70 inverted microscope (scale bar = 50 \(\mu m\)). D and E. Quantitative depiction of changes in morphology of LP9 (D) and HPM3 (E) cells as determined using the MetaMorph image analysis software. The integrated morphology and regional measurement tools were used to determine the shape factor (circularity) and longest axis (length) of cells respectively (* p < 0.05 compared to control).
Figure 3.2: Asbestos alters markers of a mesothelial to fibroblastic transition in HMCs. A. Western blot analysis performed on cell lysates from HPM3 exposed to asbestos one week after the first dose (Asb 75 \(5 \mu\text{g/cm}^2\) asbestos). B. Western blot analysis of pooled culture supernatants from HPM3 cells in A. 96 h after the first asbestos pulse (48 h after second pulse). C. Western blot of LP9 cell lysates two weeks after the first asbestos pulse (Asb 15 \(1 \mu\text{g/cm}^2\)). D. Western blot analysis of pooled culture supernatants from LP9 cells in C. 96 h after the first asbestos pulse. E. Fold change of \(CDH1\) (E-cadherin), \(CDH2\) (N-cadherin), \(IL-8\), \(IL-6\), \(SNAI1\) (Snail) and \(SNAI2\) (Slug) transcripts in HPM3 cells exposed to asbestos \(5 \mu\text{g/cm}^2\) for 48 h or 5 days (5d) as determined by qRT-PCR (*\(p < 0.05\) compared to 48 h control; † \(p < 0.05\) compared to 48 h asbestos exposure; ‡ \(p < 0.05\) compared to 5 d control). F. IL-1\(\beta\) and G. IL-18 cytokine levels in pooled culture supernatants from A 48 h after first asbestos pulse (* \(p < 0.05\) as compared to control). (Control = 0)
Figure 3.3: Asbestos-induced MFT is partially dependent on NLRP3. A. Western blot analysis of culture supernatants from siNLRP3 (and scrambled controls) transfected LP9 cells 48 h after asbestos exposure (5 μg/cm²). B. Western blot analysis of E-cadherin levels 10 days after asbestos exposure. α-Tubulin was used as a loading control. C. NLRP3 transcript level fold changes 72 h after transfection of LP9 cells with scrambled control (siControl) or a pool of siNRLP3 constructs. D&E. NLRP3 transcript level fold change after transfection of LP9 cells with individual siNLRP3 constructs (05-08). Transfected cells were exposed to 5 μg/cm² asbestos (for 48 h) prior to RNA extraction (* p < 0.05 compared to control; †p<0.05 as compared to siControl-Asb). F. Western blot analysis of culture supernatant from LP9 cells transfected with siNLRP3(06) and scrambled control 48 h after asbestos exposure.
Figure 3.4: IL-1β signaling regulates asbestos-induced MFT. A. Phase contrast micrograph of HPM3 cells pretreated with 150 ng/mL IL-1Ra 1 h prior to asbestos exposure. Images were obtained at 20X, 48 h after initial asbestos pulse (scale bar = 50 µm); changes in morphology were also quantified using MetaMorph image analysis software as described in Figure 1 (* p < 0.05 compared to control; † p < 0.05 compared to asbestos alone). B. Western blot analysis of cytokines (FGF2, IL-6 and IL-8) and TFPI2 levels in pooled culture supernatants 96 h after initial asbestos pulse and densitometric analysis of blots (* p< 0.05 compared to control; † p < 0.05 compared to asbestos alone). C. IL-18 levels measured by ELISA 96 h after first asbestos pulse with and without IL-1ra. D. Phase contrast micrographs of dose dependent MFT in HPM3 cells exposed to IL-1β at indicated concentrations (imaged at 20X on Olympus IX70 inverted microscope, scale bar = 50 µm) and morphometric analysis using the MetaMorph image analysis software as described in Figure 1 (* p <0.05 compared to control). E. Western blot analysis of TFPI2, IL-6, Caspase-1 and HMGB1 levels 96 h after HPM3 cells were treated with 1 or 5 ng/mL IL-1β. F. Western blot analysis of FGF2 and IL-8 levels in concentrated media supernatant, and immunobLOTS of E-cadherin and N-cadherin expression in cell lysates from HPM3 cells pretreated with 150 ng/ml or 1µg/ml IL-1Ra with and without IL-1β (1 ng/ml) treatment (β-actin was used as a loading control for cell lysates).
Figure 3.5: Asbestos exposure induces mesothelial to fibroblastic transition in the parietal peritoneal mesothelium in vivo. A. Wild type C57/BL6 mice were exposed to 500 μL of 200 μg/mL asbestos in saline or saline alone for 3, 7, 14, 28 or 56 days (8 weeks). Cross sections of peritoneal walls were stained by hematoxylin and eosin (H&E) for histological analysis of the submesothelial layer. Peritoneal wall sections were imaged on the Olympus BX50 inverted microscope at a magnification of 20X (Scale bar = 50 μm). Single cell layer of parietal mesothelium (arrow), thickened peritoneal wall after asbestos exposure (double headed arrow). B. Total and C. Differential cell counts in PLF showing the relative proportions of immune cells infiltrating the peritoneal cavity in response to asbestos exposure at 3, 9, and 14 days, 4 and 8 wks. D. IL-1β and E. IL-18 levels in the PLF of asbestos exposed mice over the course of time as measured by ELISA. Cytokine levels reported as pg/mL of PLF collected. (* p ≤0.05 compared to saline controls). F. Peritoneal wall sections from animals exposed to asbestos for 8 weeks were immuno-fluorescently stained to ascertain levels of cytokeratin (green), vimentin, collagen (Col1α1) (red) and alpha smooth muscle actin (αSMA) after asbestos exposure [nuclei were stained with DAPI (blue)]. Smaller panels to the right are individual fluorescent channels. Sections were imaged on the Zeiss LSM 510 META confocal laser scanning microscope at a magnification of 100X (scale bar = 50 μm).
Figure 3.6: Caspase-1 is important for asbestos-induced mesothelium thickening in mice. A. Micrographs of peritoneal wall sections from C57/BL6 wild type and NLRP3−/− and Cas-1−/− mice after 8 weeks of asbestos exposure (100 µg weekly) stained with hematoxylin and eosin for histological analysis. Glass beads (GB) at an equal surface area (538 µg weekly) were used as a negative control for particulate exposure. Single cell layer of parietal mesothelium (arrow), thickened peritoneal wall after asbestos exposure (double headed arrow). Images were obtained on Olympus BX50 as indicated above (scale bar = 50 µm). B. and C. Graphical representation of peritoneal wall thickness in asbestos exposed versus saline controls for wild type and knockout (NLRP3−/− and Cas-1−/−) mice. (* p ≤0.05 compared to saline controls, † p ≤0.05 compared to wild type asbestos). D. Total cell counts per mL of PLF retrieved from wild type, NLRP3−/− and Cas-1−/− saline and asbestos exposed mice. E. Differential cell counts of cells in PLF of Cas-1−/− and wild type mice after 8 weeks of asbestos exposure. (* p ≤0.05 compared to saline controls, † p ≤0.05 compared to wildtype asbestos). F. IL-1β and G. IL-18 levels in PLF of wild type and Cas-1−/− mice as measured by ELISA. (* p ≤0.05 compared to saline controls, † p ≤0.05 compared to wild type asbestos). H. Western blot analysis of TFPI2, FGF2 and IL-6 in saline and asbestos exposed Cas-1−/−-mice.
Figure 3.S 1: Regulation of asbestos-induced MFT by IL-1β signaling in LP9 cells. A. Phase contrast micrograph of LP9 cells pretreated with 1 µg/mL IL-1Ra 1 h prior to asbestos exposure (1 µg/cm²). Images were obtained at 20X, 9 days after initial asbestos pulse (scale bar = 50 µm). B. (top) changes in morphology were also quantified using MetaMorph image analysis software as described in Figure 1 (* p < 0.05 compared to control; † p < 0.05 compared to asbestos alone); (bottom) Western blot analysis of TFPI2, IL-6, FGF2 and IL-8 in concentrated culture supernatant 96 h after asbestos exposure (48 h after 2nd pulse). C. Phase contrast micrographs of dose dependent MFT in LP9 cells exposed to IL-1β at indicated concentrations (imaged at 20X on Olympus IX70 inverted microscope, scale bar = 50 µm). D. Morphometric analysis using the MetaMorph image analysis software as described in Figure 1 (* p <0.05 compared to control). E. Phase contrast micrographs of LP9 cells on day 7 pretreated with two pulses of 1 µg/mL IL-1Ra 1 h prior to each treatment with IL-1β (1 ng/ml). F. (top) Morphometric analysis using the MetaMorph image analysis software as described in Figure 1 (* p <0.05 compared to control); (bottom) immunoblots of E-cadherin and N-cadherin expression in cell lysates from LP9 cells pretreated with 1µg/ml IL-1Ra with and without IL-1β (1 ng/ml) treatment (β-actin was used as a loading control for cell lysates).
Supplemental Tables

Table S3 1: Supplementary Table 1: HPM3 Up-Down Regulation (comparing to control group). EMT PCR Array showing changes in epithelial and mesenchymal gene transcript levels in response to asbestos exposure at the indicated time points.

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Table S3 2: Supplementary Table 2: LP9 Up-Down Regulation (comparing to control group). EMT PCR Array showing changes in epithelial and mesenchymal gene transcript levels in response to asbestos exposure at the indicated time points.

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CHAPTER FOUR: INVESTIGATING THE ROLE OF IL-1 RECEPTOR ANTAGONIST AND INFLAMMASOME INHIBITOR (C Ridley) IN ASBESTOS-INDUCED MFT IN VIVO AND POTENTIAL ROLES FOR TFPI2 AND FGF2 IN VITRO

Introduction

The inflammasome has been implicated in the development and progression of epithelial to mesenchymal transition (EMT/MFT) and fibrosis either through signaling by the components or the products of the inflammasome (NLRP3, ASC and mature IL-1β) (Ludwig-Portugall et al., 2016, Perez et al., 2016, Lee et al., 2012) (Thompson et al. Am J Pathol, in press). The NLRP3 protein has been shown to augment TGFβ signaling independent of its inflammasome function in tubular epithelial cells (Wang et al., 2013). In colitis-associated colon cancer, NLRP3 protein levels were observed to be high in cells with a mesenchymal phenotype which was reversed by knockdown of NLRP3 expression (Wang et al., 2016). This knockdown also corresponded to downregulation of the EMT associated transcription factor Snail levels and was independent of inflammasome activation (Wang et al., 2016). In corneal endothelial cells, IL-1β has been shown to induce EMT via FGF2 signaling as result of corneal injury (Lee et al., 2012). Under inflammatory conditions, IL-1β was shown to act synergistically with TGFβ2 to induce endothelial to mesenchymal transition in human umbilical vein endothelial cells (HUVECs) and cardiac endothelial cells (Maleszewska et al., 2013). In both cell types, endothelial markers were lost and an increase in vimentin and other mesenchymal markers was reported over time.
Inflammation plays an important role in the development and progression of several cancers and the inflammasome has been shown to either promote or curtail tumorigenesis depending on the cell type in which it is activated (Zitvogel et al., 2012, Yazdi and Drexler, 2015, Pertilli, 2017). In mesothelioma, chronic inflammation/inflammasome modulation is believed to play a pivotal role in the development and progression as well as chemoresistance of the disease (Westbom et al., 2014, Westbom et al., 2015, Mossman et al., 2013, Hillegass et al., 2010). Asbestos has been shown to activate the inflammasome in a protracted manner, thus chronic activation of the inflammasome by biopersistent asbestos fibers can potentially promote the development of mesothelioma (Hillegass et al., 2013). The mechanism(s) by which asbestos causes MM is currently unknown and the fact that products and/or components of the inflammasome are capable of inducing or augmenting EMT provides a plausible mechanism by which asbestos exposure would provide the right environment for the development of this deadly disease. A recent study has shown that the inflammasome and IL-1β signaling are important for MM initiation and progression (Kadariya et al., 2016). Other studies exploring modulation of inflammasome activation as a target for drug develop and to aid in investigating the regulation of the inflammasome in cancer and inflammatory diseases have developed and/or tested a number of small molecule inhibitors
and naturally occurring metabolites as inhibitors of the inflammasome (Coll et al., 2015, Ludwig-Portugall et al., 2016, Misawa et al., 2015, Qiu et al., 2016).

In Chapter 3 we show that the inflammasome plays a role in asbestos induced mesothelial to fibroblastic transition with a potential role for TFPI2 and FGF2. To investigate the role of the inflammasome in the MFT process and how TFPI2 and FGF2 facilitated this process we turned to our *in vitro* and *in vivo* models of asbestos-induced MFT. We hypothesized that modulation of inflammasome activation or IL-1β signaling *in vivo* would ameliorate asbestos induced thickening of the parietal mesothelium *in vivo*. We also hypothesized that manipulating TFPI2 levels using an siRNA approach or inhibiting FGF2 signaling by blocking all the FGF receptors would attenuate asbestos-induced MFT *in vitro*. To this end we investigated the effect of inhibiting the inflammasome using the small molecule NLRP3 inflammasome inhibitor, MCC950/CRID3 (Primiano et al., 2016) as well as recombinant IL-1Ra to block IL-1β signaling *in vivo* prior to asbestos exposure. *In vitro*, we either pretreated primary mesothelial cells with the pan FGFR small molecule inhibitor BGJ8-98 or we knocked down TFPI2 expression with a pool of siRNA constructs against TFPI2 prior to asbestos exposure to investigate the role of FGF2 and TFPI2 in asbestos-induced MFT. While we did not observe any significant effects on asbestos-induced sub-mesothelium thickening *in vivo*, preliminary data obtained from our *in vitro* studies indicate that FGF2 signaling and TFPI2 play important roles in asbestos-induced MFT and warrant further studies to elucidate their exact role to determine whether targeting these two pathways would be an effective mode of therapy for MM treatment or biomarkers.
for assessing therapy effectiveness or disease progression or even as serum markers for early detection of MM.

**Materials and Methods**

*Experimental Animals*

Age (8 weeks old) and sex matched C57BL/6 mice were purchased from Charles River Laboratory and housed in microisolators with ad libitum access to food and water. Animals were handled in accordance with the institutional animal care and use committee (IACUC) guidelines (#12-004) of the University of Vermont. Animals received either 50 µL, MCC950/CRID3 (TOCRIS Bioscience (Bio-Techne), Minneapolis, MN) (50 mg/kg) (Coll et al., 2015), Anakinra® (IL-1Ra) (Kineret®, SOBI Inc. Waltham, MA) or sterile saline intraperitoneally 24 hr prior to the start of the experiment. Animals were then randomly grouped (6 animals per group) to receive asbestos (500 µL of crocidolite asbestos 200 µg/mL in sterile saline) or sterile saline the following day. All animals in the respective groups received 50 uL CRID3, Anakinra or saline 1x daily, five times a week not including the day of asbestos injections for 8 weeks. Asbestos was administered once a week for 8 weeks. Mice were euthanized with an overdose of Beuthansia drug at the end of the experiment and peritoneal lavage fluid (PLF), diaphragms (peritoneal side up) and peritoneal walls (mesothelial side up) were collected for analysis of inflammatory cell infiltration/cytokine analysis and sub-mesothelium thickening respectively. Peritoneal walls and diaphragms were fixed in 4% paraformaldehyde and embedded in paraffin before sectioning.
Asbestos fibers

Prior to injections, NIEHS crocidolite fibers were sterilized overnight by UV irradiation and suspended in sterile saline to yield a 200 µg/mL suspension. Asbestos fibers were then sonicated for 15 minutes in a water bath sonicator and triturated 10 times through a 22 gauge needle to obtain a homogenized suspension. Fiber suspension was vortexed between injections to maintain homogeneity of suspension. Each animal received either 500 µL of sterile saline or asbestos on day 0 of the experiment and once weekly thereafter for the duration of the study. For in vitro experiments, a 1 mg/mL suspension of UV irradiated crocidolite was prepared as previously described (Hillegass et al., 2013) and cells were exposed to 1 µg/cm² asbestos per surface per area of the culture dish.

Analysis of PLF

PLF was collected from each animal immediately after euthanasia and processed for total number of infiltrating inflammatory cells and cytokines as previously described (Hillegass et al., 2010). Cytokine levels were assessed in concentrated PLF samples by ELISA (IL-1β and IL-18) as previously described (Westbom et al., 2015).

Cell Culture

Primary human pleural mesothelial (HPM3) and immortalized human peritoneal mesothelial cells [LP9/hTERT (LP9)] were cultured at 37°C, 0.5 % CO₂ in a humidified incubator as previously described (Hillegass et al., 2013). Cells were grown to 90% confluency before being subjected to overnight serum starvation (Medium+ 0.5% serum and appropriate supplements) prior to treatment.
**siRNA Transfections**

To investigate the role of TFPI2 in asbestos-induced MFT, 90% confluent LP9 cells were transfected with either a pool of 4 siTFPI2 constructs (siTFPI2), On-Target Smart Pool® siTFPI2, [ThermoScientific (Life Technologies), Grand Island, NY] or scrambled siRNA constructs (siControl) [ThermoScientific (Life Technologies), Grand Island, NY] following the manufacturer’s protocol. Lipofectamine 2000® [ThermoFisher Scientific (Invitrogen), Carlsbad, CA] was used as the transfection agent.

**Quantitative Real-Time PCR**

To assess mRNA transcript level changes in genes related to inflammasome activation as well as TFPI2 transcript levels in response to siRNA mediated gene knockdown and asbestos exposure, cells were lysed for RNA extraction using the RNeasy Plus kit (Qiagen, Valencia, CA) as per the manufacturer’s instructions. One microgram of RNA was used for synthesis of cDNA following the manufacturer’s protocol for the Promega RT-PCR kit (Promega, Madison, WI). Samples were then submitted for quantitative real-time PCR (qPCR) to ascertain fold changes in mRNA transcript levels for *IL-1B, NLRP3, PYCARD, E-cadherin, SNAI1 and TFPI2*.

**Western Blot Analysis**

Western blot analyses were conducted on the culture supernatants to determine the effects of TFPI2 knockdown and FGF2 inhibition on the asbestos-induced upregulation of the secretion of IL-6, IL-8, TFPI2, FGF2 as well as inflammasome activation (indirectly through the secretion of the p20 subunit of caspase-1 into the culture medium) as well as
levels of the DAMP, HMGB1 which is secreted into the culture medium in response to asbestos exposure and inflammasome activation.

Equal volumes of culture supernatant were first concentrated using StrataClean® resin beads (Agilent Technologies, Santa Clara, CA) as previously described (Westbom et al., 2015). Equal volumes (10 µL) of the concentrated supernatant were then loaded onto 15% SDS PAGE gels to resolve constituent proteins after which proteins were transferred onto a nitrocellulose membrane and immunoblotted with the appropriate primary and secondary antibodies [IL-6, IL-8, FGF2, TFPI2 and HMGB1; rabbit polyclonal antibodies from (Abcam, Cambridge, MA) and caspase-1 (Cell Signaling Technology, Danvers, MA) used at 1:500 dilution; secondary antibody – goat anti rabbit conjugated to hydrogen peroxidase (1:5000), (Jackson ImmunoResearch Laboratories Inc. West Grove, PA)].

**Quantitation of Submesothelium Thickening**

Cross-sections of the peritoneal walls of mice exposed to asbestos with and without treatment with CRDI3 or Anakinra and their respective controls were stained with hematoxylin and eosin to enable histological assessment of changes to thickness of the submesothelium. Five images spanning the length of each section were captured with a Retiga Magnafire camera on a BX50 Olympus light microscope (Olympus America Inc., Center Valley, PA) utilizing the 20X objective. Images were then imported into MetaMorph Image Analysis Software to measure the thickness of the submesothelium as previously described (Thompson et al. Am J Pathol, *in press*).
**ELISAs for IL-1β and IL-18**

Culture supernatants and PLF samples were concentrated using the Amicon centrifugal filtration units (10K molecular weight limit) (Millipore, Billerica, MA) as previously described (Westbom et al., 2015). Levels of IL-18 and IL-1β were measured using IL-18 ELISA kits (MBL, Woburn, MA) and Quantikine IL-1β/IL-1f2 Immunoassay (R&D Systems, Minneapolis, MN) respectively as per the manufacturer’s instructions. Human and mouse kits for the two cytokines were used for the culture supernatant and PLF samples respectively. Cytokine levels are reported as pg IL-1β/IL-18 per mL of starting material.

**Results**

Effects of modulating inflammasome activity and IL-1 signaling on asbestos-induced submesothelial thickening: Assessment of the thickness of the submesothelium in response to asbestos exposure with and without the inflammasome inhibitor, CRID3/MCC950 or the IL-1 receptor antagonist, Anakinra did not yield any significant differences in thickness (Figure 1 A and B). Neither drug was successful in reducing the total number of cells infiltrating the peritoneal cavity in response to asbestos exposure (Figure 1C) and no significant changes in the profile of the cells infiltrating the cavity were observed (Figure 1D). As was expected in response to blocking signaling through the IL-1 receptor however, IL-1β levels significantly increased in response to the administration of Anakinra upon exposure of animals to asbestos, but this was not the case in CRID3 treated animals (Figure 1E). CRID3 treatment failed to modulate the effect of asbestos exposure on IL-1β levels altogether (Figure 1E). The same trend was observed for IL-18 as seen for
IL-1β for both drugs (Figure 1F). Although IL-18 signals through its own receptor, (IL-18R), treatment of animals with Anakinra also increased asbestos-induced IL-18 secretion significantly. All IL-1 members require their respective receptors and the IL-1 receptor accessory protein (IL-1RAcp) for signaling upon ligand binding. It is this interaction between IL-1R1 and IL-1RAcp that IL-1Ra/Anakinra disrupts to prevent signaling.

Role of TFPI2 in asbestos-induced MFT: Transfection of LP9 cells with siRNA targeting effectively downregulated TFPI2 transcript (approximately 21 fold decrease) and protein levels (Figure 2A). Although asbestos-induced induction of IL-1β secretion was slightly reduced in cells with inhibited TFPI2, the decrease was not significant (Figure 2B). Interestingly, in the presence of reduced asbestos-induced TFPI2 levels, levels of active caspase-1 were also reduced (Figure 2C). TFPI2 inhibition also caused a slight decrease in IL-8 levels in response to asbestos exposure, while HMGB1, IL-6 and FGF2 levels remained unaffected (Figure 2C). Morphologically, reduced levels of TFPI2 delayed transition of LP9 cells to a more fibroblastic phenotype during the first 48 h of asbestos exposure (Figure 2D). After 96 h of asbestos exposure however, cells had started appearing more fibroblastic (Figure 2D).

Role of FGF2 in asbestos-induced MFT: Inhibition of FGFR1-4 with BGJ-398 caused a dose dependent decrease in IL-1B transcript levels after asbestos exposure in HPM3 cells (Figure 3A). No effect was observed on NLRP3, PYCARD transcript levels in response to FGFR inhibition or asbestos exposure or the combination thereof after (Figure 3A). By itself, BGJ-398 dose dependently reduced E-cadherin (CDH1) transcript levels but decreased SNAI1 levels independent of dose (Figure 3A). Asbestos exposure also
decreased both of these genes, but pretreatment of the cells with the inhibitor did not result in further decreases in expression beyond the effects of the inhibitor for *SNAI1* whereas *CDH1* levels were just slightly higher in asbestos exposed pretreated cells than inhibitor or asbestos alone (Figure 3A). However, none of these changes were significant. Upon analysis of cytokine release in response to asbestos exposure by Western blot analysis, TFPI2 levels were found to be slightly decreased in the asbestos group that was treated with 5 µM BGJ-398 (Figure 3B). Analysis of secreted IL-1β levels by ELISA 48 h after asbestos exposure showed that 5µM BGJ-398 slightly ameliorated asbestos-induced IL-1β (Figure 3C). By itself, BGJ-398 at this concentration (5µM) had no significant effect on secreted IL-1β levels, unlike the 2 and 10 µM doses (Figure 3C).

**Discussion**

Malignant mesothelioma has a long latency period ranging from 10-60 years after asbestos exposure (Bianchi and Bianchi, 2007, Thompson et al., 2014). It is a deadly disease that is refractory to all current therapeutic modalities approved for its treatment. To compound matters, MM is difficult to diagnose and most cases present in the later stages of the disease (Rodriguez Panadero, 2015) therefore limiting the ability to obtain positive results. As per our knowledge no biomarkers exist that allow for early diagnosis in at risk populations. Epithelial to mesenchymal transition (EMT) has been implicated in tumor development and progression (Lee et al., 2006, Diepenbruck and Christofori, 2016) and its prevention can help stop the dissemination of late stage disease to metastatic sites. Although asbestos exposure has been causatively linked to MM, the mechanisms involved in the development of and progression of this disease remain a mystery. It is essential to
determine which pathways and crucial players drive MM development and progression in order to develop multi-target treatment modalities that will counteract the chemoresistant nature of the disease. In most cases of MM, there is recurrence of the disease even after aggressive treatment involving pleural decortication and removal of the affected lung in eligible patients even though these patients receive chemotherapy and sometimes radiation therapy after surgery (Taioli et al., 2015, Beebe-Dimmer et al., 2016). A biomarker that would allow the effectiveness of therapeutic regimens to be determined before resistance/recurrence would help improve survival beyond the current median of 8-12 months.

Chronic inflammation induced by the mechanical damage caused by asbestos as well as the ROS generated in response to asbestos fibers and the myriad signaling networks activated by asbestos exposure all play a role in the development of this deadly disease (Donaldson et al., 2010, Shukla and Mossman, 2005, Shukla et al., 2003). However, studies on MM samples/patients from all stages of MM are not available to enable an in-depth study of the progression of this fatal disease. Our in vitro and in vivo asbestos exposure models allow for the role of some of these inflammatory networks, such as the inflammasome to be investigated. The inflammasome plays both sides of the field with respect to promoting tumorigenesis or enhancing tumor clearance (Zitvogel et al., 2012, Petrilli, 2017). By understanding which role the inflammasome plays in MM, we will be able to determine how that can be exploited for the detriment of disease progression. Recent studies have confirmed the role of the inflammasome in MM initiation and our lab has also demonstrated that the inflammasome plays a role in asbestos-induced MFT which may
serve as a point of initiation for MM development (Kadariya et al., 2016) (Thompson et al. *Am J Pathol*, in press). In the current study, we attempted to define the exact role of IL-1β signaling in MFT but our model failed to recapitulate the delay we saw *in vitro* when we treated animals with the IL-1 receptor antagonist, Anakinra. Anakinra has a half-life of 4-6 h *in vivo* (Calabrese, 2002) and would require several doses to maintain the appropriate ratio of IL-1β to IL-1Ra required to block IL-1β signaling *in vivo*. Our animals were dosed once daily and we observed increases in IL-1β secretion in animals receiving the antagonist and asbestos compared to those exposed to asbestos only. In our *in vitro* studies we needed a thousand fold more IL-1Ra than IL-1β to inhibit asbestos and IL-1β mediated MFT (Thompson et al. *Am J Pathol*, in press). *In vivo*, where there are many different types of cells expressing the IL-1 receptor and a biologic with a short half-life, it is more difficult to determine the exact dose that would help maintain the proposed >100-fold IL-1Ra/IL-1β ratio to ensure inhibition of IL-1β signaling (Arend, 2002, Arend et al., 1990). IL-1α also uses the same receptor as IL1β and its role in asbestos-induced MFT and inflammation has not been explored. These two IL-1 family members sometimes have opposing functions under inflammatory conditions (Voronov et al., 2013), thus, any regulatory functions of IL-1α that would help balance IL-1β action is also lost when the receptor is blocked. Future studies, employing IL-1β and IL-1Ra deficient mice will have to be conducted to pinpoint the role of IL-1β and the exact stage at which the course towards malignancy is set and cannot be corrected.

We also used an NLRP3 inflammasome specific inhibitor to test the role of NLRP3 pharmacologically but the use of MCC950/CRID3 did not yield encouraging
results. The mechanism by which MCC950 inhibits the inflammasome has yet to be
determined although it has been reported to reduce IL-1β levels *in vivo* and *in vitro*
(Primiano et al., 2016, Coll et al., 2015). Utilizing a house dust mite model of airway
inflammation in mice, as well as a skin inflammation model in which imiquimod cream
was applied behind the ear of test mice, Primiano et al., were able to demonstrate that
CRID3 administered orally (200 mg/kg; 2x daily) reduced IL-1β levels in the
bronchoalveolar lavage fluid and ear swelling in the skin inflammation model (Primiano et
al., 2016). *In vitro*, IL-1β were reduced when human monocytes treated with imiquimod
were pretreated with CRID3 (Primiano et al., 2016). We found no effect of CRID3 on
asbestos-induced IL-1β levels *in vitro* (data not shown) or *in vivo* (Figure 1D). This
suggests that MCC950 was ineffective or failed to block asbestos-induced activation of the
inflammasome in our experimental set up. Other small molecule inhibitors (e.g.
sulforaphane) are being developed and tested for their inflammasome modulating activity
(Yang et al., 2016). In addition, a number of naturally occurring compounds/metabolites
(e.g. resveratrol) are also being tested for their ability to modulate inflammasome activity
(Jiang et al., 2016, Miller et al., 2014, Qiu et al., 2016). Thus, future studies using any of
these pharmacological agents are needed to ascertain whether they can be used as
prophylactic treatment for individuals exposed to asbestos or considered at risk of
developing mesothelioma. Such agents may also be used in conjunction with conventional
chemotherapeutics for MM like cisplatin to reduce the detrimental effects of
inflammasome activation by cisplatin. The inflammasome promotes or hinders cancer
progression depending on the cell type in which it is activated (Zitvogel et al., 2012, Kolb
et al., 2014, Yazdi and Drexler, 2015), therefore it is important to understand the cell types important for inflammasome mediated regulation of asbestos-induced MFT and MM progression. By so doing, therapies targeting inflammasome activity in those cells can be developed by immunotherapy and chemotherapy for delivery of inflammasome modulators to specific cells.

Recent studies have shown that FGF signaling is an attractive target. FGF2, FGF18 and FGFR1 have been found to be overexpressed by mesotheliomas (Marek et al., 2014, Schelch et al., 2014) and recent studies using FGF-ligand trap have shown promise in preclinical studies where it reduced tumor growth (Blackwell et al., 2016). In another study, the use of an FGFR neutralizing antibody used in conjunction with radiotherapy improved the effectiveness of the radiotherapy treatment of the MM (Schelch et al., 2014). FGF2 is a mitogen for mesothelial cells and is secreted in response to injury to the mesothelium (Mutsaers, 2002, Fujikawa et al., 2003). Its secretion from mesothelial cells has also been shown to be induced by IL-1β (Cronauer et al., 1999). Our preliminary studies with the pan FGFR inhibitor, BGJ-398 suggests an interplay between asbestos-induced IL-1β upregulation and FGF signaling. This calls for further study to ascertain the exact role of FGF2 in the process of asbestos-induced MFT and the implications of this role for the treatment and monitoring of MM. Since we see an upregulation of FGF2 levels both in vitro and in vivo after asbestos exposure and it is overexpressed in MM it bears promise as a marker of asbestos exposure and MM progression in conjunction with other markers in order to ensure disambiguation. Further studies are required before the candidacy for FGF2 as an MM biomarker can be confirmed but for now, it shows promise.
Fibrin turnover is an important function of the normal mesothelium (Mutsaers et al., 2016, Mutsaers et al., 2015) and imbalances in fibrinolysis and fibrin deposition have been shown to be important for MFT (Fang et al., 2012, Batra and Antony, 2015) and mesothelioma growth (Williams et al., 2012). The overlay of mesothelial cells with fibrin caused them to down regulate the junctional protein, E-cadherin and induce MFT in human mesothelial cells *in vitro* (Fang et al., 2012). Another study has demonstrated that re-expression of TFPI in a mesothelioma cell line inhibited growth of the cells (Williams et al., 2012). In our studies on asbestos induced MFT, we see elevated levels of TFPI2 which are downregulated upon blockage of the IL-1 receptor or knockdown of the NLRP3 protein (Thompson et al., *Am J Pathol*, in press). Our preliminary data indicate that knockdown of TFPI2 in LP9 cells delays asbestos MFT in a manner reminiscent of blocking the IL-1 receptor. TFPI2 has been shown to regulate the activity of matrix metalloproteinase 2 (MMP2) which may play a role in the clearance of fibrin (Ruf et al., 2003). Although TFPI2 does not inhibit plasminogen activators, it inhibits the activity of plasmin (Chand et al., 2005, Puttabyatappa et al., 2016) thus, indirectly affecting fibrin turnover and promoting MFT. TFPI2 inhibits the activity of MMP2 and MMP9 and in so doing is involved in regulating extracellular matrix remodeling (Chand et al., 2005). Our studies also showed a reduction in caspase-1 activation in the absence of TFPI2. At this time the mechanisms and players involved in how TFPI2 influences asbestos-induced MFT remain to be elucidated. Further studies are required to determine where in the signal cascade TFPI2 functions to promote MFT and whether it can be utilized as a reliable biomarker for disease progression and therapy effectiveness. TFPI2 is considered a tumor suppressor gene and
its promoter is often methylated in a number of cancers. However its role as a potential driver of MFT has yet to be explored.

Altogether, data from this study identify many players for asbestos-induced MM pathogenesis, but, also points to the need for more work to be done to elucidate the importance of these players to be used as biomarkers or drug targets.

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Figures

Figure 4.1: Modulating IL-1β signaling does not attenuate asbestos-induced submesothelium thickening. (A) H&E stains of cross sections through mouse peritoneal walls eight weeks after asbestos exposure. Mice received 50 µL of sterile saline, Anakinra (100 mg/kg) or CRID3 (10 mg/kg) 5x weekly with or without asbestos exposure (1x week) (scale bar = 50 µm). (B) Quantitation of submesothelial thickening by MetaMorph software analysis. (C) Total cell counts per mL of PLF collected (* p<0.05 compared to control). (D) Differential cell counts as a percentage of total number of cells counted from PLF). (E&F) IL-1β and IL-18 levels measured in concentrated PLF as determined by ELISA. Values reported as pg IL-1β or IL-18 per mL of PLF (* p<0.05 compared to control; † p<0.05 compared to asbestos alone).

Figure 4.2: Knockdown of TFPI2 attenuates asbestos-induced MFT. (A) Immortalized peritoneal mesothelial cells (LP9/hTERT) were transfected with control non-targeting siRNA (siCon) or siTFPI2 and exposed to 3 µg/cm² asbestos for 48 h, 32 h post transfection. TFPI2 transcript levels were assessed by qPCR. Protein levels of TFPI2 in control and siTFPI2 transfected cells were also determined by Western blot analysis of culture medium 48 h after exposure to 1 µg/cm² asbestos. (B) The levels of IL-1β secreted into the culture medium 48 h after exposure to 3 µg/cm² asbestos was determined by ELISA. (C) Western blot analysis of concentrated culture medium supernatants to determine the effect of TFPI2 loss on caspase-1, IL-6, HMGB1, FGF2 and IL-8 levels 48 h after exposure to indicated amounts of asbestos. (D) Phase contrast micrographs of transfected cells to assess morphological changes in response to asbestos exposure. (Images obtained with 20X objective). ** p< 0.05 compared to siCon.

Figure 4.3: FGF2 signaling regulates IL-1β and TFPI2 levels in response to asbestos exposure. (A) HPM3 cells were pretreated with increasing doses of the pan FGFR inhibitor, BGJ-398 (2, 5 and 10 µM) 1 hour before two pulses of exposure to 5 µg/cm² asbestos 48 h apart. Transcript levels of EMT, and inflammasome components as well as TFPI2 were then analyzed 1 week after the first asbestos pulse when cells possessed a stable fibroblastic morphology. (B) Western blot analysis of FGF2, IL-6 and TFPI2 levels secreted into the culture medium 48 h after exposure to the second asbestos pulse. * p < 0.05 compared to control, † p < 0.0001 compared to asbestos alone, ‡ p < 0.0001 compared BGJ [2]. (C) IL-1β levels in culture supernatant 48 h after asbestos exposure as determined by ELISA. * p<0.05 compared to control; † p< 0.05 compared to the respective concentration of drug alone; ‡ p<0.05 compared to asbestos alone.
CHAPTER FIVE: CONCLUSION AND FUTURE DIRECTIONS

Through the studies described herein, we have attempted to understand one of the previously unknown inflammatory mechanisms activated by asbestos in mesothelial cells both in vitro and within the context of the peritoneum of the whole animal. Asbestos-related diseases and in particular, MM, have a long latency period during which inflammation and mechanical damage from the fibers promote a chronic cycle of injury and repair of cells in contact with the asbestos fibers (Miller and Shukla, 2012, Murphy et al., 2011). While chronic inflammation has long been proposed to play a major role in the development of MM (Hillegass et al., 2010a, Shukla et al., 2003, Linton et al., 2012, Carbone and Yang, 2012), the exact mechanisms employed by asbestos to induce/foster the growth of this lethal neoplasm remain elusive. MM is difficult to diagnose because of its occult site of origin as well as the fact that its symptoms are shared with a number of maladies (Henderson et al., 2013) leading to misdiagnoses in areas where MM is not often encountered by medical practitioners. It requires both a differential diagnosis and imaging techniques as well as a large panel of differential markers being tested on biopsied tissue to reach a diagnosis (Henderson et al., 2013, Chapman et al., 2012, Rodriguez Panadero, 2015). MM patients are typically diagnosed in the late stages of the disease as the disease does not typically present with overt symptoms until the tumor is large enough or causing pleural effusions to compromise breathing (pleural MM), cause pain (pleural and peritoneal) or distention of the abdomen from the tumor mass or the
buildup of ascetic fluid (Mitsui et al., 2015, Mossman et al., 2013, Neumann et al., 2013). No progress has been made in the last twenty years to improve the prognosis of MM (Rodriguez Panadero, 2015, Kao et al., 2010). In addition to being refractory to all current modes of therapy, there are no biomarkers that allow for early detection. A wide array of genes are upregulated or downregulated in response to asbestos exposure and the largest network of genes affected by asbestos exposure belong to inflammatory cascades (Hillegass et al., 2010b, Dragon et al., 2015). One such inflammatory cascade activated by asbestos in mesothelial cells is the inflammasome. Asbestos activates the NLRP3 inflammasome in a prolonged manner, leading to the secretion of IL-1β and IL-18 (Hillegass et al., 2013). Both IL-1β and IL-18 have been implicated to play roles in the development, progression and maintenance of inflammation driven cancers (Kolb et al., 2014, Muzes and Sipos, 2015). Through our studies we are the first to show that activation of the inflammasome by asbestos in mesothelial cells is partially modulated by the redox state of the cellular redox protein, Trx1 via its interaction with TXNIP which has been shown to be involved in inflammasome activation. Reactive oxygen species generation in response to asbestos exposure upsets the redox balance of cells exposed to asbestos and increases expression of thioredoxin as well as the proportion of semi-oxidized Trx1 in mesothelial cells. This increase in the Trx1 oxidized state led to a dissociation of Trx1 from its negative regulator, TXNIP and promoted inflammasome activation. By knocking down the expression levels of TXNIP
using siRNA, we were able to ameliorate the extent of asbestos induced inflammasome activation. The treatment of shERK2 bearing Hmeso cells that exhibited a 4-fold reduction in TXNIP levels with inflammasome inducing doxorubicin confirmed the role of TXNIP in asbestos-induced inflammasome activation. As inflammasome activation was significantly reduced in these cells after treatment with doxorubicin.

With the increasing evidence that the inflammasome or its individual components were involved in epithelial to mesenchymal transition (Wang et al., 2016, Wang et al., 2013, Lee et al., 2015, Lee et al., 2012), and evidence indicating that asbestos as well as chemotherapeutics used for the treatment of MM both activate the inflammasome (Zitvogel et al., 2012, Westbom et al., 2015, Bruchard et al., 2013), it became imperative to investigate the role of asbestos-induced inflammasome activation in the role of MM development. MM cell lines and tumors both exhibit lower inflammasome activity than normal mesothelial cells. While prolonged asbestos exposure at sub-toxic levels promotes an EMT-like transition in mesothelial cells, our studies demonstrated for the first time that this MFT we observed was in part dependent on asbestos-induced activation of the inflammasome both in vitro and in vivo. In fact, inhibition of IL-1β signaling delayed the transition of mesothelial cells into a fibroblastic phenotype. The treatment of mesothelial cells also recapitulated several of the MFT features observed in response to asbestos and could be inhibited by pretreatment of cells with IL-1Ra. The pretreatment of cells with IL-1β prior to asbestos exposure
however, failed to induce EMT earlier in mesothelial cells, suggesting that other signaling cascades or factors regulating MFT were activated by asbestos in addition to the effects of IL-1β signaling.

Our studies narrowed down a few potential candidates for further study in the asbestos-induced mechanisms controlling MFT and may control MM development and progress. TFPI2, a regulator of fibrinolysis and FGF2, an EMT inducing growth factor and pro-inflammatory cytokine were significantly upregulated in response to asbestos exposure and their levels appear to be regulated in part by inflammasome activity. In response to knocking down NLRP3 levels by siRNA, decreases in levels of IL-6, IL-8, FGF2 and TFPI2 were observed. In vivo studies also revealed a potential role for IL-18 and Cas-1 in asbestos-induced thickening of the submesothelium.

TFPI2 is considered a tumor suppressor gene and its promoter has been demonstrated to be hypermethylated in a number of cancers (e.g. metastatic melanoma, gastric and colorectal cancer) (Ribarska et al., 2010, Hibi et al., 2011a, Takada et al., 2010, Sun et al., 2016). Serum levels of methylated TFPI2 have also been detected in advanced gastric cancer (Hibi et al., 2011b) and hepatocellular carcinoma patients (Sun et al., 2013), and is being considered as a biomarker for metastatic melanoma and a potential prognostic marker for hepatocellular cancer advancement (Lo Nigro et al., 2013, Sun et al., 2016). In contrast to the role of TFPI2 as a tumor suppressor, elevated serum levels have been reported in ovarian clear cell cancer (OCCC) patients and is now being
validated as a biomarker for OCCC (Arakawa et al., 2013, Arakawa et al., 2016). In uveal melanoma, Ruf et al. demonstrated that TFPI2 was important for vascular mimicry and invasiveness of the uveal melanoma cell line MUM-2B compared to its less invasive counterpart MUM-2C (Ruf et al., 2003).

TGFβ2 has been shown to upregulate TFPI2 levels in human trabecular meshwork cells as part of an alteration in turnover of the ECM by these cells as they undergo EMT (Fuchshofer et al., 2009). The growth of the non-invasive breast cancer cell line (MCF-7) in a 3-D scaffold consisting of an artificial ECM oriented in different directions verified the role of the ECM in EMT as these cells displayed changes in morphology and protein expression consistent with EMT (Foroni et al., 2013). These cells expressed increased amounts of TGFβ2 and TFPI2 which potentially facilitated the transition of these cells into a more mesenchymal phenotype (Foroni et al., 2013).

A number of studies have demonstrated that FGF2 induces EMT in different cell types (Lee et al., 2012, Chen et al., 2014, Strutz et al., 2002). Additionally, FGF has been shown to promote the proliferation of mesothelial cells and is overexpressed in mesothelioma (Mutsaers et al., 1997, Marek et al., 2014). Recent studies have also revealed FGF2 as an attractive drug target for reducing MM growth (Schelch et al., 2014, Blackwell et al., 2016) which has the potential of improving the prognosis of MM when combined with the current approved therapeutic modalities. Our studies show that asbestos induces FGF2 expression and secretion. The transcription factor NFκB, which is redox sensitive
and activated by asbestos (Janssen et al., 1995), has been shown to regulate FGF2 expression in endothelial cells (Lee and Kay, 2012) and the same may hold true for mesothelial cells. It is therefore possible that FGF2 may be an early marker of asbestos exposure and further investigation of FGF2 serum levels in asbestos exposed individuals and patients with various asbestos related diseases and MM will be needed to confirm whether FGF2 is a good biomarker of MM development.

Further studies are required to determine the exact roles of TFPI2, Cas-1, FGF2 and IL-18 in MM development and MFT. The use of TFPI2, IL-18, IL-1β and IL-1Ra knock out mice will be required to tease out the roles of these molecules and their respective pathways in MM development. Since the inflammasome exerts cell type specific roles in either promoting or inhibiting tumorigenesis (Yazdi and Drexler, 2015, Allen et al., 2010, Kolb et al., 2014), mesothelial cell and immune cells specific knockout mice will need to be developed to aid in these studies as well. New studies suggest that FGF receptor 1 is important for the growth of MM and the use of an FGF-ligand trap has shown promise in restricting MM growth in pre-clinical studies (Marek et al., 2014, Blackwell et al., 2016). This gives further credence to the need to investigate the role of FGF2 in asbestos-induced MFT. By extension, the roles of both FGF2 and TFPI2 in MM resistance as a result of activation of the inflammasome by chemotherapeutics will have to be investigated. If these two factors do indeed function downstream of IL-1β signaling to promote MFT, it will be imperative to
potentially include an FGF2 neutralizing antibody to treatment modalities involving chemotherapeutics and IL-1Ra in order to circumvent any deleterious effects of IL-1β signaling while promoting pyroptosis. Additionally, serum FGF2 and TFPI2 levels could be monitored during MM treatment as a measure of treatment efficacy and as predictors of recurrence after treatment. Both of these will need to be further studied for candidacy as part of a panel of serum or plasma markers for early MM detection in at risk groups.
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