THE ROLE OF MONOGLYCERIDE LIPASE IN APOPTOSIS AND INFLAMMATION

By

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Approved

(Sponsor’s signature)

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DISSERTATION ABSTRACT

The Role of Monoglyceride Lipase in Apoptosis and Inflammation

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Monoglyceride lipase (MGL) is lipase that hydrolyzes a variety of monoglycerides, by which it plays an important role in lipid metabolism. Previous studies in our laboratory showed that MGL expression was either reduced or absent in the majority of the cancer tissues that we screened as compared with their normal matching tissues, implying that MGL is a potential tumor suppressor.

To further elucidate the function of MGL in tumorigenesis, we generated MGL-knockout mouse to assess tumor formation in the absence of MGL expression. We found that MGL knockout led to tumor formation in a number of different tissues in mice and lung adenocarcinoma was the predominant phenotype. Our screening of human lung tissues also showed that MGL expression was either reduced or absent in cancer tissues as compared with adjacent normal tissues. The findings altogether strongly suggest that MGL plays a tumor suppressive role in lung cancer. Mechanistically, we determined that MGL deficiency led to enhanced EGFR signaling and up regulation of COX-2 expression in lung cancer cells, both of which were known to promote tumor formation and progression. Moreover, our recent study identified there was direct interaction between MGL and IκB and this interaction was critical for the inhibition of NF-κB activity. These
findings are highly consistent with our observation of inflammatory responses in the MGL-deficient lung tissues.

Previously we found that MGL over-expression induced cell death in multiple cancer cell lines. Recently we determined that MGL was a negative regulator of XIAP. Furthermore, we showed that MGL directly interacted with and destabilized XIAP protein. Our data suggest that this effect of MGL is responsible for the suppression of the anti-apoptotic function of XIAP as well as the induction of apoptosis.

Overall, our previous and recent findings all support the notion that MGL is a tumor-suppressor. In addition, our new studies have demonstrated the important roles of MGL in the modulation of tissue inflammation and apoptosis. These may prove to be significant discoveries that will inspire future studies to elucidate the function of MGL in the context of other diseases other than cancer.
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Chapter 1

GENERAL INTRODUCTION

Monoglyceride lipase

In 1976, a new potential lipase (now known as Monoglyceride lipase, MGL, MAGL, or MGLL) in rodent was first discovered and reported by studies of Tornqvist and Belfrage (1976). From the rat adipose tissue extract, they identified a new protein that processed properties of monoacylglycerol hydrolyzing activity (Tornqvist and Belfrage, 1976). It was estimated this new enzyme protein to have a molecular mass of ~32,900 Dalton (Tornqvist and Belfrage, 1976). Under the assay conditions, the enzyme was found to hydrolyze 1(3)- and 2-monooleoylglycerol at equal rates but did not catalyze the hydrolysis of other lipids such as emulsified trioleoylglycerol, micellar or emulsified dioleoylglycerol, emulsified cholesterol olate or micellar lysophosphatidylcholine. It was therefore considered as an enzyme to have specific monoacylglycerol hydrolase activity (Tornqvist and Belfrage, 1976). Later in 1997, the mouse monoglyceride lipase (MGL) cDNA was first identified and cloned by the Holm’s group from a mouse adipocyte cDNA library (Karlsson et al., 1997). The reported mouse monoglyceride lipase cDNA contained 912 nucleotides and predicted as a protein with 303 amino acids with a molecular mass of 33,218 Dalton. At the time, the cDNA and protein of human monoglyceride lipase had not yet been identified and reported, thus, the authors did not find the mouse monoglyceride lipase cDNA and protein to have extensive homology to any known mammalian cDNAs and proteins (Karlsson et al., 1997). However, the mouse monoglyceride lipase was found to be distantly related to number of microbial proteins, including two bacterial lysophospholipases and a family of haloperoxidases (Karlsson et
The predicted amino acid sequence of mouse monoglyceride lipase harbored a catalytic triad of serine lipase which contained a serine residue (Ser-122) within a GXSXG lipase motif as well as residues of Asp-239 and His-269 (Karlsson et al., 1997). Mouse monoglyceride lipase mRNA was found to be expressed in all mouse organs and when exogenously expressed in COS cells, it exhibited lipase activity for hydrolyzing mono-[3H]olein (MO) and esterase activity for hydrolyzing p-nitrophenyl butyrate (PNPB) (Karlsson et al., 1997). Site-directed mutations at the key residues of the catalytic triad, for example, S122A, D239N and H269A, significantly abolished the activity of mouse MGL as lipase and esterase (Karlsson et al., 1997).

Our laboratory had initiated the studies of human monoglyceride lipase in 1997, while no information was reported about the MGL human homologue. Using a computer-based approach to align redundant ESTs in the dbEST (The Expressed Sequence Tags database) to search for sequences that contain putative novel open reading frames (ORFs), we delineated several EST containing putative ORFs. We then used the SAGE (Serial Analysis of Gene Expression) database to identify those that would potentially exhibit altered expression in cancers. Using such approaches, we had identified a novel human cDNA predicted to encode a protein with molecular mass of 34 kDa (accession number XM_042586). The PROSITE database (a protein database for functional characterization and annotation, prosite.expasy.org) predicted this novel protein to be a lysophospholipase, we therefore named it as LPL34 (Lysophospholipase 34 kD). The amino acid sequence of LPL34 (human MGL) is shown in Fig. 1. A BLAST comparison of the LPL34 sequence with the non-redundant sequence databases revealed
Figure 1. The amino acid sequence of human MGL.

METGPEDPSSMPEESSPRRPQSIPYQDLPHLVNADGQYLFCRYWKPTGT-50
PKALIFVSHGAGEHSGRYEELARMLMLGDLVFAHDHVGHGQSEGERMVV-100
SDFHVFRDVQLQHSVDMQKDYPGPLPVSLLGHSMGGAIALTAERPGHFA-150
GMVLISPLVLANPESATTFKVLAKVLNLVLNLSLGPIDSVLRSNKTE-200
VDIYNSDPLICRAGLKVCFGIQLLNAVSRVERALPKLTVPFLLLQGSADR-250
LCDSKGAYLLMELASQDKTLKIEGAYHVLHKEPEVTNSVFHEINMWV-300
SQRATAGTASPP-313
LPL34 to be highly homologous (84% identity) to the murine monoglyceride lipase (MGL) cDNA reported by Karlsson et al. (Karlsson et al., 1997) (accession number AJ001118). Compared to the reported murine MGL, LPL34 (human MGL) has 10 additional amino acids at its N-terminus (Fig. 1). Amino acid residues (Ser122, Asp-239 and His-249) identified as the lipase catalytic triad for murine MGL are conserved in LPL34 corresponding to residues Ser-132, Asp-249 and His-279. Like murine MGL, LPL34 also harbored the conserved lipase motif ‘GHSMG’ at position 130-134 (Fig. 1). Thus, evidence suggested that LPL34 is the human homologue of murine MGL.

We next investigated whether human MGL cDNA can be expressed and translated into a protein of the expected size. Toward this end, in vitro transcription/translation assay was performed on the MGL cDNA; the in vitro translated human MGL protein product had a molecular mass of ~34 kD. We also subcloned the human MGL cDNA into mammalian expression vectors and expressed it in human cancer cell lines. Exogenously expressed MGL protein also was ~34 kD in human cells which matched the size of endogenous MGL detected by MGL specific antibody generated in our laboratory.

Further studies determined MGL to be a protein encoded by the MGLL gene localized in mouse chromosome 6 or human chromosome 3q21 (Karlsson et al., 2001; Rajasekaran et al., 2016). It was shown that MGL in mice is widely expressed in multiple tissues including those of the spleen, kidney, stomach, lung, liver, brain, skeletal muscle as well as adipose tissue (Karlsson et al., 2001). Similarly, previous studies of human samples from our laboratory detected MGL mRNA or protein expression in multiple tissues too, including those of the lung, kidney, stomach, brain, skeletal muscle, liver, spleen, small intestine, colon, and prostate (Sun et al., 2013). Therefore, MGL is a lipase
that is not limited to adipose tissue but universally present in a large variety of tissues or cells.

**The tumor-suppressive roles of MGL**

Our subsequent studies have identified MGL to have important characteristics of tumor suppressor in human cancer (Sun et al., 2013, Liu et al., 2018, Liu et al., 2020). We showed that the expression of MGL was either absent or reduced in multiple human malignancies, particularly in cancers of lung and colon (Sun et al., 2013, Liu et al., 2018, 2019). Overexpression of MGL suppressed colony formation in tumor cell lines (Sun et al., 2013). Purified MGL was found to selectively interact with several purified phospholipids, including phosphatidic acid and phosphoinositol(3,4,5)P3, phosphoinositol(3,5)P2, phosphoinositol(3,4)P2 and several other phosphoinositides (Sun et al., 2013). MGL knockdown resulted in increased Akt phosphorylation whereas overexpression of MGL suppressed Akt phosphorylation (Sun et al., 2013; Liu et al., 2018). Thus, our studies identified MGL to be an important negative regulator for regulating the PI3-K/Akt signaling.

To investigate the role of MGL in tumorigenesis, MGL knockout mice were generated. Our results demonstrated that MGL-deficient (MGL+/−, MGL−−) mice exhibited a higher incidence of neoplasia in multiple organs, including the lung, spleen, liver and lymphoid tissues (Liu et al., 2018). Importantly, lung neoplasms were found to be the most common neoplastic changes in the MGL-deficient mice (Liu et al., 2018). MGL deficiency was associated with activation of EGFR, ERK and pro-inflammatory
molecules such as COX-2 and TNF-α (Liu et al., 2018) in the MGL-deficient mouse lung tissues and in human lung cancer cells. Mouse embryonic fibroblasts (MEFs) from MGL-deficient animals showed characteristics of cellular transformation including increased cell proliferation, foci formation and anchorage-independent growth (Liu et al. 2018). Our results thus indicate that MGL plays an important role in controlling tumorigenesis in animals.

Our laboratory also investigated whether overexpression of MGL would impair cell survival or even induce cell death. We found that MGL overexpression led to remarkable apoptosis in multiple cancer cell lines such as H1299 lung cancer cells, HeLa cervical cancer cells, and cancer cells extracted from a spontaneously developed tumor from a MGL-knockout mouse (Liu et al., 2020). Our mechanistic studies showed that MGL negatively regulated the X-linked inhibitor of apoptosis protein (XIAP) in multiple cancer cell lines and in mouse tissues including lung and liver, whereas over-expression of XIAP strongly inhibited MGL’s ability to cause cell death (Liu et al., 2020). The detailed study of this function of MGL will be elaborated in Chapter 2 of this dissertation.

Similar to our findings regarding the potential tumor-suppressive role of MGL in mouse liver (Liu et al., 2020), another group showed that MGL was expressed in normal hepatocytes but not in hepatocellular carcinoma (HCC) cells (Rajasekaran et al., 2016). Moreover, MGL expression levels were inversely correlated with the expression levels of a pro-tumorigenic protein, staphylococcal nuclease and tudor domain containing 1 (SND1). SND1 was shown to be an interacting protein of MGL and promotes its ubiquitination and proteosomal degradation (Rajasekaran et al., 2016). Mechanistic studies demonstrated that overexpression of MGL inhibited HCC cell proliferation,
blocked cell cycle progression, and suppressed xenograft tumor growth (Rajasekaran et al., 2016). Interestingly, MGL over-expression reduced AKT phosphorylation in HCC cells and this effect is important for MGL’s role in the inhibition of cell proliferation and cell cycle progression (Rajasekaran et al., 2016). Furthermore, the inhibition of AKT by MGL is independent of MGL lipase activity (Rajasekaran et al., 2016). Taken together, these findings suggested that MGL was a tumor-suppressor in HCC, which is highly consistent with the findings from our mouse model study (Liu et al., 2020).

Another study investigated the relationship between MGL and Yes-associated protein (YAP) of the Hippo pathway, and identified MGL as a transcriptional target of YAP (Tang and Wang, 2015). The study also found that MGL expression was reduced in head and neck squamous cancer cell lines as compared with that of normal human oral keratinocytes, while overexpression of MGL or the lipase-dead MGL S132A similarly suppressed the anchorage-independent growth of the cancer cell lines (SCC23 and SCC9) (Tang and Wang, 2015). Although MGL overexpression inhibited AKT phosphorylation in SCC23 cells, MGL S132A did not clearly affect AKT phosphorylation, indicating that MGL inhibited cell transformation probably independently of its regulation of AKT (Tang and Wang, 2015). By using MCF10A human normal mammary epithelial cell line, the study showed that YAP was able to promote cell transformation and this effect was inhibited by MGL expression induced by YAP (Tang and Wang, 2015).

The lipase activity of MGL may also be important for its tumor-suppressive function. Although 2-AG exhibits anti-tumor effects as mentioned previously, one study showed that 2-AG levels were higher in low-grade and high-grade glioma tissues as compared with that in non-tumor tissues (Wu et al., 2012). Consistently, MGL lipase activities and
mRNA levels are lower in the glioma tissues when compared with those of the non-tumor tissues (Wu et al., 2012). These results also implicate a tumor-suppressive role of MGL in glioma.

Generally, studies to date support the tumor-suppressive role of MGL in tumorigenesis.

The oncogenic characteristics of MGL

So far, several studies have characterized the function of MGL in the context of tumor formation and progression (Xiang et al., 2018; Nomura et al., 2010; Hu et al., 2014; Tang and Wang, 2015). A number of studies implied that MGL also exhibited oncogenic characteristics.

In one study, it was shown that the serine hydrolase activities of MGL were elevated in aggressive cancer cell lines such as a melanoma cell line C8161, an ovarian cancer cell line SKOV3, and a breast cancer cell line 231MFP, when compared with their non-aggressive counterparts (Nomura et al., 2010). Moreover, in contrast to the situation in normal tissues where MGL generally did not regulate the levels of free fatty acids (FFAs), MGL appeared to be important for the production of FFAs in aggressive cancer cells (Nomura et al., 2010). Knockdown of MGL by shRNA in aggressive cancer cells suppressed cell survival and migration and reduced xenograft tumor growth and its FFA contents, suggesting that MGL activity is critical in maintaining the aggressiveness of cancer cells (Nomura et al., 2010). Interestingly, overexpression of MGL but not MGL lipase-dead mutant, MGL-S122A, in non-aggressive cancer cells increased cellular FFA
levels and promoted cell migration and survival, recapitulating the features of aggressive cancer cells (Nomura et al., 2010). Consistently, the aggressiveness of MGL-depleted cancer cells could be rescued by addition of FFAs such as palmitic acid and stearic acid (Nomura et al., 2010). These findings further indicated that MGL lipase activity played an important role in enhancing the pathogenicity of cancer cells by facilitating cancer cells in generating free fatty acids (FFAs) from stored lipids to promote cell invasion, survival and xenograft tumor growth (Nomura et al., 2010). Surprisingly, the FFAs generated by MGL did not seem to enhance cancer cell pathogenicity via β-oxidation to generate energy for cancer cells. The FFAs generated by MGL are composed of abundant pro-tumorigenic signaling lipids or lipid metabolites including lysophosphatidic acid (LPA) and prostaglandin E2 (PGE2), and their levels were increased in aggressive cancer cells as compared with those of the non-aggressive cancer cells (Nomura et al., 2010). Furthermore, LPA and PGE2 appeared to be important in mediating MGL’s role in promoting cancer pathogenicity (Nomura et al., 2010). In addition, 2-AG is a substrate of and hydrolyzed by MGL as mentioned above. Many studies have demonstrated that 2-AG suppressed cancer cell growth in several types of cancers, including pancreatic cancer, prostate cancer, and breast cancer (Carracedo et al., 2006; Nithipatikom et al., 2004; De Petrocellis et al., 1998). Therefore, MGL may promote tumor growth via its capability of hydrolyzing 2-AG. Altogether, these findings suggested that enhanced MGL lipase activity plays an important role in cancer pathogenicity.

The role of MGL in cancer pathogenicity is also described in hepatocellular carcinoma (HCC) (Zhang et al., 2016). Screening of patient samples demonstrated that MGL levels (mRNA and protein) were higher in HCC samples when compared with
normal liver tissues (Zhang et al., 2016). MGL levels were also higher in poorly differentiated HCC samples as compared with well-differentiated ones (Zhang et al., 2016). Tissue microassay and immunohistochemistry results showed that MGL levels were negatively correlated with the degree of HCC differentiation (Zhang et al., 2016). These results implied that MGL might serve as a predictor of the degree of malignancy of HCC (Zhang et al., 2016). Consistently, MGL levels were found to be associated with poor survival of HCC patients (Zhang et al., 2016). In matrigel invasion assays, when HCC cells, SMMC-7721, were treated with MGL inhibitor JZL184 or depleted of MGL with shRNA, cell invasion was significantly reduced, whereas overexpression of MGL increased invasion of SMMC-7721 (Zhang et al., 2016). These results suggest that MGL lipase activity is likely involved in HCC cancer cell invasion. Moreover, JZL184 treatment and shRNA-mediated knockdown of MGL both reduced HCC cell proliferation and increased cell apoptosis, while MGL overexpression promoted the cell proliferation and reduced cell apoptosis (Zhang et al., 2016). The production of the pro-tumorigenic LPA and PGE2 by MGL was also observed in hepatocellular carcinoma cells (Zhang et al., 2016). In xenograft human HCC tumors in mouse, JZL184 treatment and shRNA-mediated knockdown of MGL all decreased tumor growth, whereas MGL overexpression or high fat diet promoted tumor growth (Zhang et al., 2016). Taken together, these findings indicated that the lipase activity of MGL is involved in the formation and progression of HCC and MGL inhibitor may be useful in treating HCC.

In prostate cancer, the lipase activity of MGL is increased in aggressive cancer cells (PC3 and DU145 cells) as compared with less aggressive cancer cells (LNCaP cells), which is similar to the findings from breast, melanoma, and ovarian cancer cells (Nomura
et al., 2011. Chem Biol.). JZL184 treatment or shRNA mediated knockdown of MGL resulted in reduced PC3 cell migration, invasion, and survival (Nomura et al., 2011. Chem Biol.). In the aggressive PC3 cells, MGL seemed to be responsible for the production of lysophosphatidic acid (LPA), phosphatidic acid (PA), and lysophosphatidyl ethanolamines (LPE), which all contribute to cancer aggressiveness by promoting cell migration, invasion and survival (Nomura et al., 2011. Chem Biol.). These results suggest that LPA, PA, and LPE may account for the oncogenic effects of MGL. Furthermore, palmitic acid only partially rescued cell invasion, migration, and survival that were impaired by MGL ablation, potentially through the activities of LPA, PA, and LPE on G-protein coupled receptors (Nomura et al., 2011. Chem Biol.). The cell migration impaired by MGL ablation is restored by treatment with palmitic acid and a cannabinoid receptor type 1 (CB1) antagonist, rimonabant (RIM), while 2-arachidonoylglycerol (2-AG), the substrate of MGL, suppressed PC3 cell migration (Nomura et al., 2011. Chem Biol.). Altogether, these data suggest that MGL promotes prostate cancer pathogenicity at least partially via the production of LPA, PA, and LPE as well as the degradation of 2-AG in prostate cancer (Nomura et al., 2011. Chem Biol.).

The function of MGL in promoting cancer metastases was also demonstrated by another study that identified MGL as an important protein in maintaining the metastatic property of nasopharyngeal carcinoma (NPC) cells (Hu et al., 2014). In NPC cells with high metastatic potential, there were higher levels of MGL mRNA and proteins than those in cells with less metastatic capacity (Hu et al., 2014). Moreover, human NPC tissues also showed higher levels of MGL mRNA than those in non-cancerous tissues (Hu et al., 2014). Exogenous MGL overexpression was able to increase the metastatic
capacity of NPC cells while shRNA mediated MGL depletion reduced NPC cell migration and invasion (Hu et al., 2014). Furthermore, in vivo study in a mouse model of tumor metastasis demonstrated that MGL expression is negatively correlated with NPC tumor cell metastasis (Hu et al., 2014). Mechanistic studies implied that MGL might enhance epithelial-to-mesenchymal transition (EMT) and increase matrixmetalloproteinase (MMP-2) to promote tumor cell metastasis (Hu et al., 2014).

Through microarray analysis of aggressive cancer cells versus non-aggressive cancer cells, one study identified a set of genes that were overexpressed in aggressive cancer cells, including a number of genes involved in EMT and genetic markers of cancer stem cells (Nomura et al., 2011. Chem Biol.). Since MGL expression is elevated in aggressive cancer cells, it was thus postulated that the tumorigenic pathways that cause EMT and maintain cancer stem cells might account for the increased expression of MGL (Nomura et al., 2011. Chem Biol.). Overall, the findings from this study suggested that MGL contributed to tumor metastasis, although it did not clarify whether this effect of MGL is associated with its lipase activity.

A recent study showed that MGL was an indicator of high-risk gastrointestinal stromal tumors (GISTs) and a predictor of poor prognosis (Li et al., 2016). The transcriptomic dataset of 32 GISTs underwent clustering analyses and the results indicated that MGL was significantly upregulated in high-risk GISTs as compared with non-high-risk GISTs (Li et al., 2016). Further analyses of MGL mRNA levels in GISTs samples and normal tissues demonstrated that MGL levels were the highest in high-risk GISTs, higher in non-high-risk GISTs, but lowest in normal tissues, implying that MGL promotes GIST progression (Li et al., 2016). MGL overexpression was associated with
clinical or pathological findings that predict poor prognosis such as non-gastric location, larger size of tumors, and increased tumor cell mitosis (Li et al., 2016). Moreover, MGL overexpression was indeed associated with shorter disease-free survival (DFS) (Li et al., 2016). Overall, these findings suggest that MGL may promote GIST progression and lead to poor prognosis in patients.

Aside from its roles in the development of GISTs of the gastrointestinal system, MGL was also reported to be involved in colon cancer formation in a Prdm5LacZ/LacZ;ApcMin mouse model (Galli et al., 2014). Prdm5 is a member of the Prdm family of transcriptional regulators and loss of Prdm5 in ApcMin mouse model significantly increased the number of polyps in the small intestine. Microarray analysis of wild-type and Prdm5 knockout MEFs showed that MGL was negatively regulated by Prdm5 (Galli et al., 2014). ChIP-seq analysis further demonstrated that MGL was a direct target of Prdm5 (Galli et al., 2014). Lastly, Prdm5 protein levels were lower in a large proportion of colon cancer samples as compared with normal tissues, whereas MGL protein levels were higher in colon cancer tissues as compared with normal tissues (Galli et al., 2014). These findings altogether implied that Prdm5 loss in colon epithelium led to increased expression of MGL, which then contributed to colon cancer formation.

**Lipase Activity of MGL**

Studies of MGL in the brain have shown that MGL is both a membrane-associated enzyme and a cytosolic protein (Blankman et al., 2007; Dinh et al., 2002). The study from our laboratory identified MGL in cancer cells to be a lipid droplet-associated protein localized in the cytoplasm (Sun et al., 2013). It has been shown that
2-monoglycerides are produced via hydrolysis by lipoprotein lipase (LPL) or hormone-sensitive lipase (HSL) from triglycerides, and subsequently monoglyceride is hydrolyzed by MGL, with the final products being free fatty acids and glycerol (Karlsson et al., 2001). In the protein structure of MGL, there is a catalytic triad formed by Ser-122, Asp-239, and His-269 in both mouse and human MGL (isoform of 303 amino acids) that works as the foundation of the lipase activity of MGL (Karlsson et al., 1997; Tyukhtenko et al., 2016). As a lipase, MGL has been shown to play a significant role in energy metabolism. In a mouse model of MGL deficiency (MGL-knockout), it was found that MGL deletion in mice impaired lipolysis, upregulated monoglyceride levels in the adipose tissue and the liver, and attenuated high fat diet-induced insulin resistance (Taschler et al., 2011).

In terms of lipase activity, it seems that the function of MGL is tissue-specific (Dinh et al., 2002). In tissues that commonly metabolize monoacylglycerol (MG) and triacylglycerol (TG) such as those of the small intestine and liver and adipose tissues, MGL is believed to hydrolyze MG derived from TG, releasing fatty acids and glycerol (Dinh et al., 2002; Chon et al., 2007). In the brain, however, 2-arachidonoylglycerol (2-AG) is the major substrate of MGL (Dinh et al., 2002). 2-AG is a special type of monoglyceride and is a member of the endocannabinoid family (Dinh et al., 2002). Of note, many studies of the lipase function of MGL have extensively investigated the role of MGL in the metabolism of endogenous cannabinoids (endocannabinoids) as well as the physiological outcome following the hydrolysis of 2-AG (Dinh et al., 2002). Studies have demonstrated that in the brain tissue, hydrolysis of 2-AG into arachidonic acid (AA) and glycerol is mostly (approximately 85%) catalyzed by MGL while the rest of 2-AG is
hydrolyzed by other enzymes such as ABHD6 and ABHD12 (Dinh et al., 2002; Navia-Paldanius et al., 2012; Blankman et al., 2007). Further studies showed that MGL in the brain played a major role in the generation of AAs from 2-AG and the AAs subsequently served as the precursor substrate for the synthesis of prostaglandins such as Prostaglandin E2 (PGE2) (Nomura et al., 2011. Science.). MGL inhibition by its inhibitor was able to suppress lipopolysaccharide (LPS)-induced neuroinflammatory responses in the mouse brain (Nomura et al., 2011. Science.). Several MGL inhibitors such as JZL184 and URB602 have been developed to inhibit the lipase activity of MGL (Du et al., 2011; Nomura et al., 2010). One study showed that JZL184 inhibition of MGL lipase activity in cancer cells reduced the production of pro-tumorigenic lysophosphatidic acid (LPA) and prostaglandin E2 (PGE2), and suppressed xenograft tumor growth, implicating the anti-tumor effect of JZL184 (Nomura et al., 2010). Another study demonstrated that URB602 and JZL184 both inhibited NF-κB and COX-2 in hippocampal neurons in culture, implicating the efficacy of MGL inhibitors in treating neuroinflammation, as was also shown by Nomura et al. (Du et al., 2011; Nomura et al., 2011. Science.).

However, the lipase activity of MGL was investigated in cancer cell lines not in human samples. The exact role of MGL as a lipase need to be further investigate in different tumor from patients’ samples. The function of MGL inhibitors need to be studied both in vivo and in vitro. The side effects and off-target effects of MGL inhibitors also requires further investigation.
Overall, studies in last few decades have identified, characterized, and elucidated the lipase activity of MGL. Exploring the potential therapeutic values of MGL inhibitors has become a major interest in the field.

**Lung cancer**

According to the statistics from a new study of global cancer burden, lung cancer is the second most commonly diagnosed cancer and it is still the leading cause of cancer mortality (Sung et al., 2020). Two major types of lung cancer have been described and studied, and they are designated as small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) (Travis et al., 2015). NSCLC is the most common type of lung cancer and accounts for more than 85% of lung cancer cases (Chen, 2014; Malhotra et al., 2017). It is further categorized into three subtypes: squamous cell carcinoma (SCC), lung adenocarcinoma (ADC), and large cell carcinoma (Travis et al., 2015). Among all the subtypes of NSCLCs, SCC and ADC are the two predominant types, and approximately 40% of NSCLCs are SCCs and 50% of NSCLCs are classified as ADCs (Chen et al., 2014).

Regarding the causes of lung cancer, the majority (about 90%) of lung cancers are associated with tobacco smoking (Swanton and Govindan, 2016). Tobacco smoke contains various carcinogens and thus cancers related to smoking usually present with high rates of genetic mutations (Gibbons et al., 2017). For example, smoking plays an important role in the formation and development of lung ADCs (Hu and Chen, 2015). Not surprisingly in the case of lung ADCs, a number of common mutations have been reported and they alter the function of certain oncogenes such as KRAS, EGFR, and
PIK3CA as well as tumor suppressor genes such as TP53 and RB1 (Swanton and Govindan, 2016). Although SCC and SCLC cancers exhibit an even stronger association with smoking than ADCs (Khuder 2001), the mutation landscapes in SCC and SCLC cancers are different from that in ADCs (Swanton and Govindan, 2016). For example, PTEN mutation is common in SCCs, accounting for 10.2% of SCC cases whereas it is only detected in 1.7% of ADCs (Heist et al., 2012). However, there are common alterations in genes such as TP53 and RB1 that are detected in SCCs, SCLCs, and ADCs (Swanton and Govindan, 2016). For decades, chemotherapeutic agents such as cisplatin, carboplatin, and pemetrexed are the major therapeutics for the treatment of lung cancer and nowadays they remain critical for the management of lung cancer (Johnson et al, 2014; Lee, 2018). In the era of personalized medicine, molecular-targeted therapy has become a major field of study in medicine and has also been developed for the treatment of lung cancer. The discovery of genetic mutations in lung cancers has led to the development of a variety of targeted therapies. For instance, several generations of EGFR tyrosine kinase inhibitors (TKIs) have been developed to treat patients with non-small cell lung cancer (NSCLC) harboring activating and correspondingly sensitizing mutations of EGFR (Swanton and Govindan, 2016; Le and Gerber, 2019).

Targeted therapies have improved the survival of patients with tumors that express the specific molecular targets, however, they also have their limitations (Lee, 2019). For example, EGFR mutations in NSCLC have been found in 15% to 30% of patients among different populations (Metro and Crinò, 2012). Anaplastic lymphoma kinase (ALK) rearrangement is detected in 3-8% of lung ADCs (Gao et al., 2013; Cerami et al., 2012) and inhibitors of ALK have been developed for the treatment of ADCs harboring
rearrangements of ALK (Awad and Shaw, 2014). Obviously, the frequency of EGFR or ALK alterations are not high and only account for a small portion of ADCs in lung cancer patients, and this significantly limits the use of EGFR or ALK inhibitors. Importantly, previous studies from our laboratory showed that monoglyceride lipase (MGL) expression was either decreased or lost in 65.5% of human lung cancer tissues as compared with matching normal tissues (Sun et al., 2013; Liu et al., 2018). Future studies of the molecular network regulated by MGL may reveal new targets for the treatment of lung cancer and the corresponding targeted therapy may be effective for as much as 65.5% of lung cancer patients.

**Apoptosis and tumorigenesis**

Apoptosis is a type of programmed cell death that is energy-dependent and genetically determined (Elmore, 2007; Pfeffer and Singh, 2018). It is characterized by distinct morphological characteristics that are different from those of necrosis, another type of cell death (Elmore, 2007). For example, during apoptosis, there is cell shrinkage, the cell membrane is intact, the cytoplasm is retained in apoptotic bodies, and there is no inflammation. As for necrosis, there is cell swelling, the cell membrane is disrupted, the cytoplasm is released, and is usually accompanied by inflammation (Elmore, 2007). Apoptosis is involved in a variety of physiological processes such as embryonic development, normal turnover of cells, immune reactions, and organ atrophy (Elmore, 2007). Apoptosis also plays important roles in pathological processes (Elmore, 2007). For example, deregulated apoptosis has been shown to contribute to the pathogenesis of two
major human disorders, neurodegenerative diseases and cancer (Elmore, 2007; Pfeffer and Singh, 2018).

Studies to date have revealed two major pathways that lead to apoptosis: the mitochondria-related intrinsic pathway and the death receptor-dependent extrinsic pathway (Elmore, 2007). Both pathways require caspases for the induction and execution of cell death (Elmore, 2007; Pfeffer and Singh, 2018). Caspases (cysteine-dependent aspartate-directed proteases) are cysteine proteases that are able to hydrolyze and cleave a target protein at aspartic acid residues (Elmore, 2007). Based on their functions, caspases are classified into categories, the initiator caspases and the executioner caspases (Elmore, 2007). For example, caspase 9 of the intrinsic pathway and caspase 8 of the extrinsic pathway are initiator caspases (Elmore, 2007). The activation of initiator caspases leads to the activation of an executioner caspases such as caspase 3, a shared executioner of the intrinsic and extrinsic pathways that finally results in apoptosis (Elmore, 2007; Tait and Green, 2010). An additional pathway is the perforin/granzyme pathway and it has been shown to be critical for T-cell mediated cytotoxicity (Elmore, 2007). In this pathway, granzyme A is able to induce cell death in a caspase-independent manner whereas granzyme B still requires caspases such as the initiator caspase 10 and the executioner caspase 3 to execute apoptosis (Martinvalet et al., 2005; Elmore, 2007).

The intrinsic pathway involves mitochondria and mitochondrial proteins and is activated by intracellular stimuli such as DNA damage, growth factor deprivation, cytokine withdrawal, failed suppression of apoptosis by antiapoptotic proteins, oxidants, and certain toxins (Elmore, 2007; Pfeffer and Singh, 2018). These signals can lead to mitochondrial outer membrane permeabilization (MOMP), the defining step of the
intrinsic apoptosis pathway, and subsequently mitochondrial proteins such as cytochrome c and second mitochondria-derived activator of caspase (SMAC) from intermembrane space are released into the cytosol (Elmore, 2007; Pfeffer and Singh, 2018). In the cytosol, cytochrome c, apoptotic protease activating factor 1 (Apaf-1), dATP, and pro-caspase 9 form the apoptosome, which converts pro-caspase 9 into active caspase 9 (Elmore, 2007; Pfeffer and Singh, 2018). Caspase 9 induces activation of executioner caspases, caspases-3 and caspase-7, which finally lead to cell death (Elmore, 2007; Pfeffer and Singh, 2018).

The extrinsic pathway is primed for activation when death ligands bind to transmembrane receptors such as tumor necrosis factor (TNF) family death receptors (Elmore, 2007; Pfeffer and Singh, 2018). TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand (Fas-L), and tumor necrosis factor (TNF) are common death ligands that can trigger apoptosis (Pfeffer and Singh, 2018). For example, following the binding of tumor necrosis factor TNF-α to its receptor TNFR1, cytoplasmic adaptor proteins including TNF receptor-associated death domain (TRADD), Fas-associated death domain protein (FADD), and receptor interacting protein (RIP) are recruited to the transmembrane receptor to form a death-inducing signaling complex (DISC) together with the initiator caspase 8 (Elmore, 2007). Upon formation of DISC, caspase 8 is activated by an auto-catalytic mechanism, which finally leads to the activation of executioner caspases and apoptosis (Elmore, 2007; Kischkel, 1995; Tait and Green, 2010).

Evasion of apoptosis is a hallmark of cancer while normally apoptosis prevents cancer formation (Elmore, 2007; Pfeffer and Singh, 2018). Cancer cells can either
upregulate anti-apoptotic proteins such as B-cell leukemia/lymphoma-2 (BCL-2) or reduce the expression levels of pro-apoptotic proteins such as BAX to suppress apoptosis (Elmore, 2007; Pfeffer and Singh, 2018). For instance, the tumor suppressor p53 is a typical inducer of apoptosis triggered by DNA damage (Elmore, 2007). In human malignancies loss of p53 or p53 function due to mutations is commonly detected (Elmore, 2007), which supports an important role of apoptosis evasion in tumorigenesis.

Given the importance of apoptosis in the prevention of cancer and its minimal damage to tissues as compared with necrosis, one major goal of cancer therapy is to develop cancer therapeutics to induce apoptosis in cancer cells (Elmore, 2007; Baig et al., 2016). In fact, for the last three decades therapeutic induction of apoptosis by proapoptotic agents has become a mainstay of cancer therapy (Carneiro and El-Deiry, 2020).

The function of XIAP in tumorigenesis

Apoptosis in normal physiology is tightly regulated to maintain tissue homeostasis (Elmore, 2007; Singh et al., 2019). Various proteins have been identified to be important regulators of the apoptosis pathways and are classified as either anti-apoptotic proteins or pro-apoptotic proteins (Singh et al., 2019). Common anti-apoptotic proteins include the Bcl-2 family of proteins such as Bcl-2, Bcl-xL and Mcl-1 (Singh et al., 2019), while Bax, Bak, Bid, Bad, and Bim are typical pro-apoptotic proteins. These protein have been extensively studied in the regulation of the mitochondrial apoptosis pathway (Elmore, 2007; Singh et al., 2019). Another family of proteins, the Inhibitor of apoptosis (IAP) proteins, are also well-established suppressors of apoptosis and they regulate both the
intrinsic and extrinsic pathways (Deveraux and Reed, 1999). Two of its prominent members are X-linked inhibitor of apoptosis protein (XIAP) and survivin (Elmore, 2007). IAPs contain one to three baculovirus IAP repeat (BIR) domain(s) (LaCasse et al., 2008). For example, XIAP has three BIR domains known as BIR1, BIR2, and BIR3 (Abbas R and Larisch, 2020). Additionally, XIAP has a ubiquitin-associated (UBA) domain and a carboxy-terminal RING (really interesting new gene) domain that works as an E3 ligase to mediate protein degradation (LaCasse et al., 2008). Through its BIR1 and BIR2 domains, XIAP inhibits the function of caspases 3 and 7 in the apoptosis pathway (Abbas R and Larisch, 2020). Through its BIR3 domain, XIAP interacts with and inhibits the function of caspase 9 (Schimmer et al., 2006; Abbas R and Larisch, 2020). Furthermore, the anti-apoptotic function of XIAP is also modulated by other proteins such as the mitochondrial proteins Omi/HtrA2 and Smac/DIABLO (Schimmer et al, 2006). During apoptosis, Smac is released from mitochondria into the cytosol where Smac forms a dimer to interact with the BIR2 and BIR3 domains of XIAP, by which Smac prevents XIAP from inhibiting caspase 9 and caspase 3 (Flanagan et al., 2010; LaCasse et al., 2008).

As mentioned above, deregulation of apoptosis is involved in tumorigenesis. It is therefore possible that irregular XIAP function contributes to cancer formation. Indeed, XIAP has been shown to be overexpressed in many tumors (Thompson, 1995; Abbas R and Larisch, 2020). Many studies suggested that overexpression of XIAP was responsible for cancer cell resistance to chemotherapy and was associated with poor prognosis in patients with prostate cancer or acute myeloid leukemia (AML) (Flanagan et al., 2010; Schimmer et al., 2006). Therefore, there has been great interest in the development of
therapeutics that target XIAP for cancer therapy (LaCasse et al., 2008). For example, Smac mimetic compounds (SMCs) have been developed to inhibit XIAP and are tested in clinical trials for cancer therapy either as a single agent or in combination with other therapeutics including checkpoint inhibitors and radiation therapy (LaCasse et al., 2008; Carneiro and El-Deiry, 2020).

**Inflammation and tumorigenesis**

Physiological inflammation is a normal host response to pathogens, tissue injuries and heals tissues through the interplay between a variety of cytokines and leukocytes and is critical for the maintenance of tissue homeostasis, while abnormal inflammatory responses lead to diseases such as cancer (Coussens and Werb, 2002; Greten and Grivennikov, 2019; Taniguchi and Karin, 2018). In 1863, based on histological observation, Rudolf Virchow suggested a potential link between chronic inflammation and cancer (Balkwill and Mantovani, 2001).

To date, inflammation has been shown by decades of research to play important roles in the development, progression, and treatment of cancer of multiple tissue origins (Coussens and Werb, 2002; Greten and Grivennikov, 2019; Taniguchi and Karin, 2018).

Different types of inflammation are associated with cancer, including chronic inflammation and autoimmunity that precede tumorigenesis, cancer cell-induced inflammation, and inflammation triggered by cancer therapy (Greten and Grivennikov, 2019). Typical cancers associated with chronic inflammation include colorectal cancer, lung cancer, hepatocellular carcinoma, and esophageal cancer (Coussens and Werb, 2002;
Greten and Grivennikov, 2019). A large variety of cellular or environmental factors contribute to inflammation in cancer, such as inactivation of tumor suppressors, activation of oncogenes, cell death, hypoxia, carcinogenic microbes, and cancer therapeutic agents (Greten and Grivennikov, 2019). Inflammation in cancer can be a double-edged sword in that it may lead to oncogenic mutations, activation of tumor-promoting signaling pathways, tumor-permissive immunosuppression, or induce acquired resistance to the cancer therapeutic agents, but can also trigger anti-tumor immune responses induced by therapy (Sharma and Allison, 2015; Hou, 2017; Srivatsa et al., 2017; Grivennikov et al. 2010; Greten and Grivennikov, 2019).

Multiple mechanisms have been reported regarding tumor-promoting inflammation. It is been shown by a large number of studies that inflammation exerts its tumor-promoting effects at all stages of tumorigenesis (Grivennikov et al. 2010; Greten and Grivennikov, 2019). Inflammation may cause genetic mutations, epigenetic modifications, promote cell proliferation, enhance cell survival, and contribute to immunosuppression during the formation of a tumor (Grivennikov et al. 2010; Greten and Grivennikov, 2019). In addition, inflammation is often accompanied by the elevated production of certain cytokines such as interleukin-1β (IL-1) and tumor necrosis factor-α (TNFα), increased expression of matrix metalloproteinases MMP2 and MMP9, and enhanced activities of Nuclear factor kappa B (NF-κB) and STAT3 (Signal Transducer And Activator Of Transcription 3), by which inflammation promotes angiogenesis, EMT (epithelial to mesenchymal transition), and tumor invasion, finally leading to cancer metastasis (Grivennikov et al. 2010; Greten and Grivennikov, 2019).
Notably, TNF and COX-2 are both target genes of NF-κB and both are involved in tumor-promoting inflammation (Bassères and Baldwin, 2006; Taniguchi and Karin, 2018). Tumor necrosis factor (TNF) is a cytokine that plays important roles in both host immunity and inflammation (Wajant, 2009). In fact, TNF is identified to be a major player in inflammation and inhibitors of TNF have been developed for the treatment of inflammatory diseases such as rheumatoid arthritis (Balkwill, 2009). Interestingly, in the context of cancer, TNF is also a key player in tumor-promoting inflammation (Balkwill, 2009). But the role of TNF in tumor development and progression depends heavily on the cellular and environmental contexts, and TNF shows both pro-tumor and anti-tumor activities in a context-dependent manner (Balkwill, 2009). Early studies of TNF showed that TNF caused tumor necrosis and shrinkage in both patients and mouse models (Balkwill, 2009). Later studies identified TNF as a ligand that can induce apoptosis through TNF receptors and their downstream apoptosis signaling pathways (Balkwill, 2009; Wang and Lin, 2008). These findings altogether support the anti-tumor activities of TNF. On the other hand, TNF signaling induces inflammation and promotes cell survival via transcription factors including AP1 and NF-κB (Balkwill, 2009; Wang and Lin, 2008). Moreover, TNF in the tumor microenvironment was shown to cause DNA damage, thus contributing to tumor initiation (Balkwill, 2009). Other studies also demonstrated that TNF promotes cell proliferation, migration, angiogenesis, immune evasion, and resistance to chemotherapy (Balkwill, 2009; Wang and Lin, 2008). These discoveries, therefore, revealed the pro-tumor activities of TNF.

Cyclooxygenase-2 (COX-2) is an enzyme that catalyzes the metabolism of arachidonic acid (AA) to produce prostaglandins (PGs) (Liu et al., 2015). COX-2 is not
only a common mediator of inflammatory responses, but also plays important roles in tumorigenesis (Liu et al., 2015; Bassères and Baldwin, 2006; Taniguchi and Karin, 2018). Multiple studies have shown that COX-2 levels are high in tumors of different tissue origins including tumors of the colon, breast, pancreas, liver, and lung (Liu et al., 2015). Mechanistically, COX-2 is able to inhibit apoptosis, enhance cell migration, induce angiogenesis, and promote tumor immune evasion so that it is involved in all stages of tumorigenesis (Gupta and DuBois, 2001; Liu et al., 2015). Selective COX-2 inhibitors have been shown to significantly reduce the risks of colon cancer, lung cancer, breast cancer, and prostate cancer, and therefore they are promising candidate agents for cancer prevention (Göbel et al., 2014; Liu et al., 2015).

In line with the tumor-promoting roles of inflammation, anti-inflammatory drugs including COX-2 inhibitors and aspirin have shown effects in cancer prevention (Gupta and Dubois, 2001; Gierach et al., 2008). Interestingly, Canakinumab (ACZ885, Ilaris), a human anti-IL-1β monoclonal antibody that inhibits IL-1β, was shown to significantly reduce the incidence of lung cancer in a clinical trial of 10,061 patients (Ridker et al., 2017).

**IκBα and NF-κB in tumorigenesis**

Given the close association of inflammation with cancer, studies have shown that molecular components of the inflammatory signaling pathways are commonly involved in cancer development and progression (Coussens and Werb, 2002; Greten and Grivennikov, 2019). A major one of them is Nuclear factor kappa B (NF-κB) (Greten and Grivennikov, 2019; Taniguchi and Karin, 2018). Nuclear factor-kappa B (NF-κB) is a
family of transcription factors including c-Rel, RelA (p65), RelB, NF-κB1 (p105) and NF-κB2 (p100), and they or their proteolytically processed products undergo dimerization to form homodimers or heterodimers to function as either transcriptional repressors or activators to regulate transcription of target genes (Hayden and Ghosh, 2012; Hoesel and Schmid, 2013; Savinova et al., 2009).

There are two major pathways leading to NF-κB activation, the canonical pathway (also known as the classical pathway) and the non-canonical pathway (also known as the alternative pathway) (Oeckinghaus et al., 2009; Taniguchi and Karin, 2018). Extracellular stimuli are the most common triggers of NF-κB signaling, and the typical stimuli include cytokines, bacterial or viral infections, and antigen-receptor interactions (Oeckinghaus et al., 2009; Oeckinghaus et al., 2011). In addition, genotoxic stresses can also induce NF-κB activation (Oeckinghaus et al., 2009).

In the canonical pathway, when there is no stimulation, inhibitory IκB (inhibitor of NF-κB) proteins bind with NF-κB dimers and sequester NF-κB in the cytoplasm, thus blocking its nuclear translocation (Oeckinghaus et al., 2011). The pathway is activated by extracellular stimuli such as the inflammatory cytokine, tumor necrosis factor (TNF), or by the bacterial lipopolysaccharide (LPS), when they are bound to their respective receptors (Oeckinghaus et al., 2011; PMID: 22302935). Upon receptor-ligand binding, adaptor proteins are recruited to the cytoplasmic regions of the receptors, where they can activate downstream IκB kinase (IKK) (Napetschnig and Wu, 2013). Activated IKK phosphorylates IκB, leading to its ubiquitination and proteasomal degradation, and thus NF-κB dimers are released from the cytoplasmic IκB-NF-κB complex and translocated to the nucleus, where NF-κB functions as a transcription factor to transactivate target genes.
(Oeckinghaus et al., 2011; Hayden and Ghosh, 2012; Napetschnig and Wu, 2013). IκB is also present in the nucleus, where it can prevent NF-κB dimers from binding to DNA and even facilitate the shuttling of the NF-κB dimers back to the cytoplasm (Birbach et al., 2002; Huang et al., 2000). Interestingly, nuclear export of NF-κB by IκB is dominant in cells under resting conditions, and therefore NF-κB is localized predominantly in the cytoplasm with a basal transcriptional activity in the nucleus while the cytoplasmic NF-κB can be readily activated upon signal stimulation (Birbach et al., 2002; Huang et al., 2000).

The non-canonical pathway is activated by extracellular stimuli such as the TNF family cytokines, CD40L and lymphotoxin-β (LT-β), when they are bound to their corresponding receptors respectively (Oeckinghaus et al., 2011). In a similar way as in the canonical pathway, the ligand-receptor interaction leads to activation of IKK, and the activated IKK subsequently phosphorylates p100 in a dimer with RelB and p100 is then processed into the smaller NF-κB subunit, p52, which finally forms the transcriptionally active p52-RelB NF-κB complexes (Oeckinghaus et al., 2011; Savinova et al., 2009).

Through its target genes, NF-κB plays important roles in inflammatory responses, immune responses, and the regulation of cell proliferation and survival through their target genes (Oeckinghaus and Ghosh, 2009; Taniguchi and Karin, 2018). To date, it is well-established that NF-κB contributes to tumorigenesis via its versatility of functions and it has been shown that they are activated not only in cancer cells but also in the tumor microenvironment (Oeckinghaus and Ghosh, 2009; Taniguchi and Karin, 2018). More specifically, NF-κB activation promotes cell proliferation and survival but inhibits
apoptosis, mediates tumor-promoting inflammation and epithelial/mesenchymal transition, induces immunosuppression, angiogenesis and cancer metastasis, thereby contributing to its significant role in both cancer development and progression (Taniguchi and Karin, 2018; Bassères and Baldwin, 2006). For example, NF-κB induces expression of cyclin D1 and c-myc to promote cell proliferation and expression of the well-established anti-apoptotic proteins such as X-linked inhibitor of apoptosis protein (XIAP), B-cell lymphoma 2 (Bcl-2), and B-cell lymphoma-extra large (Bcl-xL) to inhibit apoptosis (Bassères and Baldwin, 2006; Taniguchi and Karin, 2018). NF-κB mediates tumor-promoting inflammation through its target genes encoding the pro-inflammatory cytokines such as TNF, IL-1, and interleukin-8 (IL-8), as well as the pro-inflammatory enzyme such as cyclooxygenase 2 (COX-2) (Bassères and Baldwin, 2006; Taniguchi and Karin, 2018).

NF-κB activation is involved in a large variety of cancers studied to date and the typical examples include gastrointestinal cancers, liver cancer, lung cancer, pancreatic cancer, prostate cancer, Hodgkin’s lymphoma, B-cell lymphoma, and multiple myeloma (Bassères and Baldwin, 2006; Taniguchi and Karin, 2018). Of particular interest to our study of lung cancer, a previous study has shown that NF-κB signaling is required for tumor formation in a mouse model of lung adenocarcinoma (Meylan et al., 2009). NF-κB thus has become an important target for cancer therapy and and also for the study of cancer drug resistance (Taniguchi and Karin, 2018; Hoesel and Schmid, 2013). Although NF-κB inhibitors are not considered as ideal individual agents for cancer treatment due to their effects on cancer immunity, NF-κB inhibitors in combination with
chemotherapeutics have shown promising results in multiple types of cancers (Hoesel and Schmid, 2013).

In our recent studies, we found that MGL interacts with and inhibits XIAP, implying that MGL is an endogenous inhibitor of XIAP. We further showed that such function of MGL is important for its induction of cancer cell apoptosis and may therefore contribute to MGL’s tumor suppressive role, at least in lung tumorigenesis. Moreover, our newest findings suggest that MGL plays an important role in the modulation of tumor-promoting inflammation via its inhibitory regulation of NF-κB signaling pathway. Overall, our studies demonstrated a tumor-suppressive role of MGL in tumorigenesis and multiple mechanisms have been elucidated for such a role of MGL.
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Chapter 2

MONOGLYCERIDE LIPASE MEDIATES TUMOR SUPPRESSIVE EFFECTS BY PROMOTING DEGRADATION OF X-LINKED INHIBITOR OF APOPTOSIS PROTEIN

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Author contributions statement:

I designed experiments, collected and analyzed data and wrote the manuscript. Renyan Liu also designed experiments and collected data. Dr. Christopher Curtiss contributed to the pathological diagnosis. Dr. Ying Huang supervised the entire project and wrote the submitted paper. Dr. M. Saeed Sheikh is my co-advisor who provided regular input and advice and also revised the submitted paper.

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Abstract

We have previously reported that Monoglyceride Lipase (MGL) expression is absent or reduced in various human malignancies and MGL-deficient mice develop tumors in multiple organs. Evidence also suggests MGL to be a tumor suppressor, however, the mechanisms underlying its tumor suppressive actions remain to be investigated. Here, we report a novel function of MGL as a negative regulator of XIAP, an important inhibitor of apoptosis. We found that MGL directly interacted with XIAP and enhanced E3-ligase activity and proteasomal degradation of XIAP. MGL overexpression induced cell death that was coupled with caspase activation and reduced XIAP levels. N-terminus of MGL was found to mediate interactions with XIAP and induce cell death. MGL-deficient cells exhibited elevated XIAP levels and exhibited resistance to anticancer drugs. XIAP expression was significantly elevated in tissues of MGL-deficient animals as well as human lung cancers exhibiting reduced MGL expression. Thus, MGL appears to mediate its tumor suppressive actions by inhibiting XIAP to induce cell death.
Introduction

Several lines of evidence from recent studies have demonstrated that Monoglyceride Lipase (MGL) plays an important role in controlling tumorigenesis (1-4). Our previous studies have shown that MGL expression is frequently reduced in multiple human malignancies including tumors of the lung, colon, rectum, breast, stomach and ovary (1,2). Information from Firehose/RSEM and Oncomine public databases also indicate that MGL (MGLL) mRNA expression is significantly reduced in multiple human malignancies (5,6). We previously showed a significant portion of MGL-deficient mice exhibited lung adenocarcinoma and high-grade dysplasia (2). MGL-deficient animals also exhibited spleen histiocytic sarcoma, ameloblastic adenoma, soft-tissue sarcoma, hepatoma and lymphoma at lower frequencies (2). Depletion of MGL via RNAi or gene targeting significantly enhanced Akt and ERK phosphorylation in cancer cells and MGL-deficient lung tissues (1,2) and overexpression of MGL in cancer cells lacking endogenous MGL suppressed cancer cell growth (1). However, the exact mechanisms via which MGL suppresses cancer cell growth and tumorigenesis remain to be further elucidated.

Induction of cell death is an important cellular function for eliminating the damaged cells with defective DNA and suppressing cancer formation (7-9). X-linked inhibitor of apoptosis protein (XIAP) is a potent negative regulator of apoptosis (10). It is an E3 ligase with three baculovirus inhibitory repeat (BIR) domains and a RING domain (11). XIAP interacts with dimeric and activated caspases-3 and -7 through the BIR 2 domain and prevents caspase-3 from accessing its substrates (12-15). Studies have also shown that XIAP degrades caspases-3 via the proteasome-mediated mechanism (16). Shiozaki et
al (17) showed that XIAP interacts with caspase-9 via its BIR-3 domain; interaction of XIAP and caspase sequesters caspase-9 in a monomeric state that blocks the activation and function of caspase-9. Overexpression of XIAP has been found in variety of human cancers and was associated with anticancer drug resistance and poor survival of cancer patients (18). However, the molecular mechanism(s) leading to XIAP overexpression in cancers remain unclear. The current study identifies MGL to be a novel negative regulator of XIAP and also demonstrates that modulation of XIAP is an important mechanism via which MGL mediates cell death and tumor suppression.
Results

MGL induces cancer cell death that involves activation of apoptotic pathways.

To study the mechanism(s) involving how MGL-deficiency led to development of tumors in animals, we found that exogenously expressed MGL in tumor cells lacking MGL caused significant cell death in human lung (H1299) and cervical (HeLa) cancer cells as well as in MGL-deficient mouse abdominal tumor cells (MADT) (Fig. 1, A & B). Significant portion of RFP-MGL expressing H1299 and HeLa cells showed apoptotic nuclei (Fig. 1C&D). MGL overexpression also led to activation of caspases-3, 8 and 9 (Fig. 2A & B) and increased PARP [Poly (ADP-ribose) polymerase] cleavage (apoptotic marker) in various cancer cell lines (Fig. 2A, B and C). Further, pan-caspase inhibitor Z-VAD-FMK was able to effectively block MGL-mediated apoptotic cell death (Fig 2D & E). Together, these results indicate that MGL induces apoptosis in cancer cells via activation of apoptotic signaling pathways.

MGL negatively regulates XIAP and suppression of XIAP is important for MGL-mediated cell death

To elucidate the mechanism(s) involving MGL-mediated apoptosis, we found that the expression level of XIAP was significantly lower in MGL-overexpressing H1299 (with undetectable endogenous MGL) and HeLa cells (with low levels of endogenous MGL expression) (Fig. 3A). Conversely, MGL knockdown via RNAi elevated the levels of XIAP protein (Fig. 3B). These results appeared to suggest that suppression of XIAP may play a role in MGL-mediated apoptosis. Results presented in Fig. 3C further indicate that
Figure 1. Overexpression of MGL induces cancer cell death and activates apoptotic signaling. A & B. Overexpression of MGL induced cell death in three different tumor cell lines. Forty-eight-hours following transfection of empty vector or MGL expression construct, representative photomicrographs were taken (A) and cell death was evaluated by trypan-blue exclusion assay (B). Each bar indicates the mean values of three-independent experiments (±SE, *p<0.05) (B). C & D. Indicated cancer cells were transiently transfected with pDsRedN1-only (RFP, vector) or RFP-MGL (red) for 48 hours followed by DAPI staining (blue) (scale bar 10μm) (C). RFP-positive dead cells with apoptotic nuclei (fragment or condensed nuclei indicated by yellow arrows) were evaluated under a fluorescent microscope (D). Each bar indicates the mean values of three-independent experiments (±SE, *p<0.05).
Figure 2. MGL induces cell death via activation of apoptotic pathways. A to C. MGL overexpression triggered PARP cleavage (A to C) and activation of caspases 8, 9 and 3, demonstrated by reduction of pro-caspases (pro-casp) and detection of cleaved caspases (cl-casp) (A & B) in various cancer cell lines. D & E. Co-treatment with pan-caspase inhibitor (Z-VAD-FMK, 10 µM) in H1299 cells prevented cell death mediated by MGL overexpression (48 hrs). Photos were taken 48-hours post-transfection; percent of cell death was evaluated as described in legend to Fig. 1. Each bar indicates the mean values of three-independent experiments (±SE, *p<0.01)
while expression of MGL-only induced significant cell death in various human and mouse tumor cell lines, co-expression of XIAP blocked MGL-mediated cell death; cells expressing vector-only and XIAP-only did not show obvious changes in cell viability (Fig. 3C). MGL overexpression also resulted in significant amount of PARP protein cleavage and activation of caspases-3 & 9 (Fig. 3D); co-expression of XIAP inhibited MGL-mediated activation (reduction) of pro-caspase-9, but did not have significantly effect on MGL-mediated pro-caspase-3 activation (Fig. 3D). These results were not surprising since previous studies have shown that XIAP binds to monomeric capspase-9 (pro-caspase-9) to prevent caspase-9 activation (17); however, in the case of caspase-3, it only interacts with and inhibits the dimeric caspase-3/activated caspase-3 not the pro-caspase/monomeric caspase-3 (23,24). Together, these results suggest that MGL is a negative regulator of XIAP and suppression of XIAP appears to play an important role in MGL-mediated cell death.

**XIAP expression is elevated in MGL-deficient human and mouse tumor tissues.**

Fig. 4A shows XIAP protein expression status in MEFs developed from mice with different MGL status, and as it is shown, the expression levels of XIAP was higher in MGL-deficient (MGL\textsuperscript{+/-}, MGL\textsuperscript{-/-}) MEFs (lanes 3-6) than in MGL-proficient (MGL\textsuperscript{+/+}) MEFs (lanes 1&2). XIAP expression level was also generally higher in liver tissues of 5/6 MGL-deficient mice (Fig. 4B, lanes 4-9) compared to that of MGL-proficient mice (Fig. 4B, lanes 1-3). We have shown that significant portion MGL-deficient (MGL\textsuperscript{+/-} and MGL\textsuperscript{-/-}) animals developed lung adenocarcinoma and some animals developed liver tumors or lymphoma (2). Fig. 4C shows lung tissue of a MGL-proficient (MGL\textsuperscript{+/-}) mouse.
Figure 3. XIAP suppression is important for MGL-mediated cell death.  
A. Exogenously expressed MGL reduced XIAP (endogenous) protein levels in cancer cells.  
B. XIAP protein levels were significantly elevated in MGL shRNA (MGL RNAi) expressing cells compared to that of scramble RNA (Scr. RNA) expressing cells.  
C. Exogenous XIAP reversed cell death induced by MGL. Indicated four cell lines transiently expressed either two control vectors (pEBB & pSRα-HA-S) (panel 1), or MGL-only (panel 2) or XIAP-only (panel 3) with control vectors, or combined MGL and XIAP (panel 4) vectors; photomicrographs were taken 48 hours post-transfection.  
D. PARP cleavage and caspase activation induced by MGL overexpression were suppressed by elevated levels of XIAP. Cell lysates for western analyses were collected 48-hours post-transfection.
with no tumor (panel a) and lung tissue of a MGL-deficient (MGL⁺/⁻) mouse with lung
tumor (panel b). Fig. 4C, c & d panels also show the microscopic images of a papillary
predominant lung adenocarcinoma developed in a MGL-deficient mouse (#171). Fig. 4D
shows XIAP protein expression in two groups of mouse lung tissues with different MGL
status. As is shown, XIAP expression was significantly higher in lung tissues from MGL-
deficient mice relative to that from the MGL-proficient animals (compared lanes 2-5 and
7-10 with lanes 1 & 6). Protein expression of XIAP in lung tumor tissues of MGL-
deficient mouse #171 (shown in Fig. 4C, panels b, c) was also significantly elevated (Fig.
4D, red arrows). Likewise, lymphoma developed in the liver tissues of MGL-deficient
mouse (#354) (Fig. 4E) also exhibited much higher XIAP expression (Fig. 4F, right panel)
than that in normal liver tissue of MGL-proficient mouse (#24) (Fig. 4F, left panel). Thus,
it appears that elevated XIAP expression is a common feature in MGL-deficient tissues
and may play an important role in tumor development in MGL-deficient animals.

We also investigated the possible inverse correlation between MGL and XIAP expression
in human lung tissues. MGL expression was significantly lower in tumor tissues relative
to their matched normal tissues (Fig. 5A). In 4/5 lung cancer tissues with lower MGL
expression in tumor (red stars), XIAP expression was elevated (Fig. 5B). Fig. 5C shows
that 9/12 patient lung adenocarcinoma tissues showed a negative correlation between
MGL and XIAP. Together, our results suggest a negative correlation between MGL and
XIAP in vivo. Accordingly, XIAP induction could play an important role in
tumorigenesis in MGL-deficient animals.

MGL alters XIAP protein stability via proteasomal pathway
Chapter 2

A

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C

#24 MGL^{-/+}
Normal lung tissue
adenocarcinoma

D

Mouse Lung tissues

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E

#354 MGL^{+/+}, lymphoma in mouse liver

F

Mouse tissue IHC staining with XIAP antibody

#24 MGL^{++}
#354 MGL^{+/+}
Figure 4. Expression of XIAP is elevated in MGL-deficient mice tissues. A & B. XIAP protein expression in MEFs and liver tissues extracted from MGL-proficient (+/+ or deficient (+/− or −/−) mice. β-actin or Ponceau S staining was used as protein loading controls. C. (a) lung tissue of MGL+/+ mouse; (b) non-tumor and tumor (yellow arrow) lung tissues of MGL+/− mouse; (c & d) H&E staining of MGL+/− lung tumor tissues [shown in (b)] imaged under microscope at 4X (c) or 40X (d) magnification. D. XIAP expression is elevated in MGL-deficient lung tissues. Lysates of Lung tissue used in lanes 2 & 3 (red arrows) were from the same animal (#171) shown in Fig. 4C, b panel. E. H&E staining (right) of lymphoma tissues grown in MGL−/− animal (#354) liver tissue (left, yellow arrows). F. XIAP immunostaining of mouse liver tissues. Left: normal liver tissue (MGL+/+). Right: lymphoma grown in mouse liver (#354).
A) MGL Immunohistochemistry staining
Patient sample 3091427A (lung)

Normal tissue

Tumor tissue

Patient sample 1091945A (lung)

Normal tissue

Tumor tissue

B) Sample #

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MGL

XIAP

Vinculin

C) XIAP-MGL expression in matched human lung normal & cancer samples

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* As shown in the left (samples marked by red star).
Figure 5. Expression of XIAP is elevated in MGL-deficient human tissues. A. MGL immunostaining of human lung normal (a) and tumor tissues (b). B. Representative results of western blot analyses showing negative correlation between MGL and XIAP protein expression (indicated by an aster *). C. Summary of expression patterns of XIAP and MGL in matched human lung normal and tumor tissues.
Studies have shown that XIAP is regulated at the protein level by proteasomal pathway (25). We determined whether MGL alters XIAP protein stability (protein half-life) in MEFs with different MGL-status. Fig. 6A & B show that XIAP protein half-life was much longer in MGL-deficient (MGL$^+$ and MGL$^-$) MEFs than that in MGL-proficient MEFs. In addition, XIAP protein reduction mediated by MGL was reversed by the treatment of proteasome inhibitor MG132 in 293T (Fig. 6C). Further, XIAP protein ubiquitination was also significantly enhanced in cells overexpressing MGL with (6D) or without exogenously expressed ubiquitin (6E). These results together suggest that MGL reduces XIAP protein levels by altering its stability via proteasomal pathway.

**MGL directly interacts with XIAP via its N-terminus**

We further determined whether MGL interacts with XIAP. Fig. 7A shows that, the S-tagged MGL, but not the S-tag-alone, pulled down XIAP in H1299 and HeLa cells. In addition, endogenous MGL and XIAP were co-precipitated by XIAP antibodies not by control IgG (Fig. 7B). These results suggest that MGL interacts with XIAP. To determine the nature of MGL-XIAP interaction, *in vitro* protein interaction assays were performed using the purified recombinant XIAP and MGL proteins. As seen in Fig. 7C, recombinant S-MGL, but not recombinant S-protein-only, pulled down purified recombinant XIAP demonstrating that MGL directly interacts with XIAP.

To study the region(s) of MGL involved in XIAP interaction, we generated a set of MGL deletion variants (illustrated in Fig. 8A and described in legend to Fig. 8A). The expression of MGL-full length and various deletion variants are shown in Fig. 8A and B.
Figure 6. MGL alters XIAP protein stability via proteasomal pathway. A. XIAP protein stability is increased in MGL-deficient MEFs. Protein synthesis was first blocked by treatment of cycloheximide (CHX) for indicated times and reduction of XIAP (protein decay) was then measure by western blot analyses. B. Quantitative results of XIAP protein level in MEFs with different MGL status shown in A. Relative band intensity of XIAP (A, upper panels) was adjusted to the band intensity of the loading control β-actin (A, lower panels). The first time points (20 min) in each cell type were set as 1. Each bar indicates the mean values of three-independent experiments (±SE *p<0.05). C. XIAP protein reduction is reversed in MGL-overexpressing cells following treatment with proteasome inhibitor MG132. Forty-eight hours following transfection of indicated vectors, cells were harvested after treatment of MG132 for indicated times. Untreated (DMSO) samples were also harvested at the 5-hour time point. D. Overexpression of MGL increases XIAP ubiquitination levels. Vector-only or MGL vector was co-transfected with ubiquitin vector (HA-Ub) for 48 hours, then the cells were treated with MG132 for 6 hours. Immunoprecipitation (IP) was performed using XIAP antibody. E. MGL promotes XIAP ubiquitination by endogenous ubiquitin. Cells were transfected with vector-only or MGL expression vector for 48 hours, then treated with MG132 for 6 hours prior to harvesting.
Figure 7. MGL directly interacts with XIAP. A. Exogenous S-tagged MGL, but not S-tag-only, pulls down endogenous XIAP. Cells were transfected with indicated vectors for 48 hours prior to harvesting. S-tag pulldown experiments were performed as described in Materials and Methods. B. Endogenous interactions between MGL and XIAP demonstrated by co-immunoprecipitation. Cells were treated with MGL132 for 6 hours prior to harvesting. Immunoprecipitation (IP) was performed using control IgG or XIAP antibody. C. Recombinant XIAP directly interacts with purified recombinant MGL in vitro.
Figure 8. MGL interacts with XIAP via the C-terminus of MGL. A. A schematic illustration of full-length and deletion variants of MGL. The numbers indicate the amino acid positions of the full-length MGL. MGL deletion variants include the N-terminus-only (1-105); N-terminus plus the central region (1-209); central region-only (106-209); central plus C-terminus (106-313, which contains the entire MGL lipase catalytic triad); or the region of C-terminus-only (210-313). The correct nucleotide sequences of all MGL deletion variants were confirmed by DNA sequencing. B & C. Interaction of endogenous XIAP with exogenously expressed MGL-1-105 and full-length MGL. Lysates from cells (HeLa in B, H1299 in C) expressing (48 hours) S-HA-tagged full-length (FL)-MGL or S-HA-tagged MGL-deletion variants or S-HA-tag-only were assayed for western blotting (inputs) or proceeded for S-tag pulldown assays. The S-tag-pulldown products were assayed for protein interactions using XIAP antibody. The correct-sized MGL protein products are marked with aster “*”.
(inputs, bands indicated by asterisk*). Our results indicate that XIAP was abundantly co-precipitated with variant 1-105 and full-length MGL (Fig. 8B, lanes 6, 14, 8, &16) but not with a polypeptide containing MGL residues 106-313 (Fig. 8B, lane 7) in HeLa cells. Such results indicate that the XIAP-interaction region of MGL resides at the N-terminus. Interestingly, although variant 1-209 also harbored the N-terminus of the protein, it showed remarkably weaker interaction with XIAP in both HeLa and H1299 cells (Fig. 8B, lane 15 and Fig. 8C, lane 7). Thus, these results show that (i) MGL center region (a.a. 106-209) does not mediate MGL-XIAP interaction, (ii) the existence of the center region (a.a. 106-209) may impede the N-terminus region to interact with XIAP, acting through a possible intramolecular negative regulatory mechanism (36).

**XIAP interacts with MGL via its C-terminus**

Used several XIAP deletion variants (Fig. 9A), we investigated the region on XIAP for MGL interaction. XIAP contains three N-terminal BIR domains, an ubiquitin-associated domain (UBA) and a C-terminal RING domain (26) (Fig. 9A). Protein expression of the full-length and deletion variants of XIAP was determined [Fig. 9B & C, inputs (lower panels), bands indicated by aster *]. Our results shown in Fig. 9 indicate that S-tagged MGL-1-105 or FL-MGL abundantly co-precipitated with full-length XIAP protein as well as with XIAP variant without all three BIR domains (ΔBIR, a.a. 331-497) in both 293T and HeLa cells (Fig. 9 B&C, top panels). Consistent with this finding, MGL also did not interact with XIAP deletion variant containing only the BIR domain (BIR1-2-3, a.a. 1-350) (Fig. 9 B&C, top panels). These results indicated that BIR (ΔRING, 1-449) had significantly less interaction with MGL, however, the interaction was not completely
Figure 9. XIAP interacts with MGL via its C-terminus. A. A schematic illustration of full-length and deletion variants of XIAP. The numbers indicate the amino acid positions of the full-length XIAP. B & C. S-tagged MGL-1-105 (left panels in B & C) or FL-MGL (right panels in B & C) was used in the S-tag pulldown down experiments. Protein inputs are shown in the bottom panels; ("*";) sign indicates the correct-size of exogenous XIAP variants. Bands for endogenous XIAP protein is indicated with the sign “§”. For S-pulldown experiments, cell lysates expressing S-MGL-1-105 or S-MGL-full-length (FL) were mixed separately with cell lysates expressing full-length-XIAP (FL-XIAP) or deletion variants or control vector. In HeLa cells, the expression of BIR 1-2-3 was relatively low (Fig. 8C, left lower panel, 1X); therefore, cell lysates with BIR 1-2-3 expressions were increased 5-times [BIR 1-2-3 (5X)] as much in the protein pulldown experiments (Fig. 9C, top panels).
abolished. These results together indicate that RING domain of XIAP is important for MGL interaction and the UBA region may be also be partly involved in this interaction.

**N-terminal region of MGL is sufficient to kill cancer cells**

Next, we sought to determine the region of MGL that is involved in cancer cell killing. As shown in Fig. 10A, variant 1-105 and full-length MGL induced cell death in both HeLa and H1299 cancer cells whereas variant 1-209 did not. In Fig. 8, we show that variant 1-105 and FL-MGL interacted with XIAP while MGL 1-209 did not. Thus, the cell killing potential of MGL appears to be associated with its ability to interact with XIAP. Cells expressing increased amount of MGL-1-105 or FL-MGL exhibited lesser survival (Fig. 10B) suggesting that MGL N-terminal region-alone is sufficient to kill cancer cells. Fig. 10B & C further demonstrate that MGL1-105 and FL-MGL, but not 1-209 and other variants, were able to effectively induce cleavage (activation) of caspase-3 (cl-casp3) and PARP (cl-PARP).

Further studies showed that expression of MGL first 60 a.a. (1-60) was sufficient to induce cleavage (activation) of caspase-3 and PARP (Fig. 10D). As shown in Fig. 10E, deletion of MGL gene significantly enhanced cell survival in MGL-deficient MEFs under anticancer drug (doxorubicin, etoposide and taxol) treatments (p<0.05). These results indicate that MGL plays an important role in regulation of cell death and the death inducing region of MGL resides at its N-terminus. In light of these results, we propose that the N-terminal region of MGL could have a potential to be developed as an anticancer therapeutic.
Chapter 2

A

HeLa

H1299

50 μm

50 μm

50 μm

50 μm

1-105 1-209 MGL-FL

Vector

MTT

Relative Cell Viability

MGL vector conc.

B

HeLa

H1299

Vector 1-105 MGL-1-105 MGL-1-209 MGL-FL

cl-casp3

HA

Vinculin

kDa

15

25

40

130

15

25

40

C

H1299

Vector MGL-1-105 MGL-1-209 MGL-FL

cl-PARP

cl-casp3

HA

α-tubulin

kDa

100

40

25

55

D

H1299

Vector MGL-1-60 MGL-FL

cl-PARP

cl-casp3

XIAP

HA-MGL

α-tubulin

kDa

100

15

55

40

E

MTT

Relative cell viability

DMSO DOX Etop Taxol

+/+ +/- +/-

+/+ +/- +/-

+/+ +/- +/-

+/+ +/- +/-

+/+ +/- +/-

+/+ +/- +/-
Figure 10. N-terminus of MGL is critical for MGL-mediated cancer cell killing. A. N-terminal domain of MGL induces apoptosis. Cells, transfected with indicated vectors for 48 hours, were imaged under a microscope (left) and harvested for MTT assays (right). Each bar indicates the mean values of three-independent experiments (±SE, *p<0.05). B. Expression of MGL N-terminus domain (1-105) and FL-MGL induces caspase-3 cleavage. HeLa cells were transfected with indicated MGL variants for 48 hours prior to harvesting. Cl-casp3; cleaved caspase-3. HA antibody detects all HA-tagged MGL variants. C. Overexpression of full-length (FL) MGL and MGL1-105, but not MGL1-209, induces PARP cleavage (cl-PARP) and activation of caspase-3 (cl-casp3) in H1299 lung cancer cells. D. MGL fragment containing first 60 amino acids is sufficient to induce cell death in H1299 cells. Photomicrographs were taken for cells transfected (48-hours) with indicated vectors (left) and cell lysates were harvested for western blot analysis (right). E. MGL-deficient cells are more resistant to treatment by chemotherapeutic drugs. MTT assays were performed in MGL-proficient (+/+ or -/+) or -deficient (+/-, -/-) MEFs following the treatments (48 hrs) with doxorubicin (DOX, 40 ng/ml), etoposide (Etop, 50 µM) and taxol (10 µM). For bar graphs, each bar indicates the mean values of three-independent experiments (±SE, *p<0.05)
Discussion

In this study, we have identified a novel cellular function of MGL that works as a negative regulator of XIAP. XIAP is one of the major inhibitors of apoptosis and known to inhibit caspases-3, 7 and 9 (27). Overexpression of MGL activated caspases, caused PARP cleavage, and induced apoptosis (Figs. 1-3). We show that lipase dead-mutant of MGL was able to also induce cell death and activates caspases in a manner similar to that induced by the wildtype counterpart (Fig. 11 A-C) and treatment of two different MGL inhibitors URB602 and JZL184 were not able to alter MGL-mediated growth inhibition (Fig. 12). We also found that MGL inhibitor JZL184 was not able to inhibit cell growth at 10 nM (per provider, JZL184 IC50 = 8nM, ref. 45) or at higher concentration of 1μM but inhibited cell growth at 10 μM concentration in several different cell lines (Fig. 13). Together, it appears that, although MGL is a lipase, MGL-mediated apoptosis appears to be independent of its lipase activity.

Our results show that MGL can regulate cellular functions through protein-protein interactions. We show that overexpression of MGL caused significant reduction in XIAP protein levels; conversely, depletion of MGL by RNAi significantly enhanced XIAP protein levels (Fig. 3). Further mechanistic studies demonstrated that MGL altered XIAP protein stability; XIAP protein half-life was significantly longer in MGL-deficient cells (Fig. 6A&B). Furthermore, elevated expression of MGL enhanced XIAP protein ubiquitination (Fig. 6D&E) and treatment of proteasome inhibitor MG132 prevented MGL-mediated XIAP protein reduction (Fig. 6C) and treatment of XIAP inhibitor Embelin (44) did not further enhance MGL-mediated growth suppression (Fig. 14).
Figure 11. The lipase activity is not necessary for MGL to induce apoptosis. Tumor cells were killed by overexpression (48 hrs) of wildtype-MGL (MGL-WT) or lipase-death-MGL (MGL-S132A). Forty-eight-hour post-transfection, microscopic photos were taken (A & C); cell viability MTT assays (B) and western blot (WB) analyses (C, right) were also performed. MTT results presented in B were the means of three-independent experiments. MADT: mouse abdominal tumor isolated from a MGL-deficient (MGL−/−) mouse. D. Expression of RFP-MGL and RFP-MGL-S132A in 293T cells. All expression vectors were confirmed by DNA sequencing.
Figure 12. The lipase activity inhibitors do not affect MGL induced apoptosis. A. Treatment of MGL lipase inhibitor URB602 (25μM) (IC$_{50}$=28μM) or JZL184 (10nM) (IC$_{50}$=8nM) in vector-only or MGL overexpressing H1299 and Hela cells. Vehicle (DMSO) or inhibitors were added and maintained in the culture medium all time (48-hours) during transfection and post-transfection. Results of MTT assays presented are the means of three-independent experiments. B. Treatment of MGL lipase inhibitor JZL184 (10nM) (IC$_{50}$=8nM) in MUM2C (melanoma), C8161 (melanoma), OVCAR3 (ovarian), and SKOV3 (ovarian) tumor cells. Vehicle (DMSO) or inhibitors were added and maintained in the culture medium during post-transfection (24-hours). Results of MTT assays presented are the means of three-independent experiments.
Figure 13. The effects of various concentration of MGL inhibitor JZL184 on different cancer cell lines. Cells were treated with vehicle (DMSO) or MGL inhibitor JZL184 (IC50=8nM) at the indicated concentrations. Cell viability was evaluated by MTT assay. Results presented are the means of three-independent experiments. * indicated significant inhibition of cell viability, p<0.05.
Figure 14. The effect of XIAP inhibitor Embelin on MGL-mediated cell death. Cells, expressing empty vector-only or full-length-MGL (MGL) or MGL1-105 expression vectors were treated with XIAP inhibitor Embelin at the indicated concentrations for 24 hours post MGL transfection. The relative cell viabilities were compared to those with vector only transfection in ctl. group (DMSO 10µM treatment) which were set as 1 in each cell line group. Inhibition of cell viabilities were detected with 10µM Embelin treatment in A549, HeLa, and H1299 cancer cell. Cell viability was evaluated by MTT assay. Results presented are the means of three-independent experiments.
**Figure 15.** A proposed model illustrating the possible mechanism via which MGL induces cell death in cancer cells.
Together, these results demonstrate that MGL negatively controls the XIAP levels by enhancing its protein ubiquitination and degradation. A schematic illustration of the proposed model depicting MGL involvement in regulation of cell death is presented in Fig. 15.

Nomura et al. (47) has previously reported that MGL behaved as an oncogenic protein promoting tumor metastasis due to its lipase activity. Our current and previous findings indicate that MGL can modulate cell function by regulation of proteins, i.e., inhibits the expression of EGFR and phosphorylation of EGFR and Akt/ERK (2) and downregulation of COX-2 (2) and XIAP (current studies). MGL expression is reduced in certain types of tumors, i.e., cancers of lung, liver, breast and others (1-5) but elevated in other types of tumor such as kidney cancer (6). In addition, we note that MGL-mediated downregulation of XIAP and growth suppression/apoptosis, although were seen in most of cancer cell lines studied but was not as evident in others, such as OVCAR3 & SKOV3 (Figs. 16 & 17). MGL knockout animals developed neoplasia in certain tissues such as lung & lymphatic tissues (2). Thus, collective results suggest a tissue/cell specific regulation and function of MGL in different tumor types. It is possible that MGL may have tumor suppressor or oncoprotein dual functions similar to other tumor suppressors as described elsewhere (2).

Previous studies have shown that XIAP forms homodimer (28) and is self-ubiquitinated (autoubiquitination) in a manner depending on its own RING domain which results in protein degradation (25,29,30). MGL does not contain a RING domain and is not expected to be an E3-ligase. MGL may mediate its negative effect on XIAP via one or more mechanisms. For example, MGL may alter the E3-ligase activity of XIAP via direct
Chapter 2

A

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**Figure 16.** A. Protein expression of endogenous XIAP, MGL and cleaved caspase 3 in several different cell lines, MUM2C and OVCAR3 (tumorigenic non-aggressive) (Ref. 47) or C8161 and SKOV3 (tumorigenic aggressive) (ref. 47). Endogenous XIAP and MGL proteins were analyzed by Western blot as described in Materials and Methods. MUM2C and C8161 are melanoma cell lines. OVCAR3 and SKOV3 are ovarian cancer cells. B. Effect of MGL knockdown on XIAP protein expression and cell growth in C8161 and SKOV3 cells. Cells were introduced with MGL shRNA (MGL RNAi) or scrambled shRNA (Scr.). Expression of indicated proteins was analyzed by indicated antibodies (left panels). Cell viability was determined by MTT assays (right). MTT results presented are the means of three-independent experiments. * indicated significant inhibition of cell viability, p<0.05. C. Phase-contrast microscopic images of C8161 and SKOV3 cells introduced with either scramble RNAi (Scr.) or two different MGL RNAis for 5 days post infection.
Figure 17. Effects of exogenously-introduced MGL on various cancer cell lines shown to have different aggressiveness on tumorigenesis (47). A. Phase-contrast photomicrographs were taken forty-eight-hours after transfection of empty vector or MGL expression vector. B. The effect of MGL overexpression on expression of different proteins in indicated cell lines. Cells were transfected with empty-vector alone or MGL expression vector and harvested later for analysis of protein expression using the indicated antibodies. α-tubulin is used as a loading control.
interaction with XIAP (Fig. 7 A-C) resulting in enhancement of XIAP autoubiquitination and degradation. Previous studies have demonstrated that the E3-ligase activity of BRCA1 is significantly enhanced by direct interaction with a protein called BARD1 (31). BRCA1 directly interacts with BARD1 protein via its RING domain and the direct interaction between these two proteins results in the enhancement of BRCA1 E3-ligase activity and autoubiquitination (31). It is also possible that MGL may work to enhance XIAP ubiquitination by facilitating XIAP to form dimers that subsequently promote intermolecular auto-ubiquitination of two XIAP molecules. Previous studies have shown that human MGL crystallized as a dimer (32) although another study has shown MGL to be a monomer (33). Our unpublished results indicate that MGL is able to form homooligomers in cells. We found that S-tagged MGL was able to pull down (precipitate) the RFP-tagged MGL in protein lysates of cells co-expressing these two differently tagged-MGL-constructs. Thus, it is possible that when MGL forms dimers and interacts with XIAP, it brings two XIAP molecules into close proximity that facilitates XIAP’s homodimerization, auto-ubiquitination and protein degradation.

We have further identified the region of XIAP that is responsible for interacting with MGL. Our studies show that deletion of the RING domain (ΔRING) significantly reduced XIAP interaction with MGL compared to the full-length counterpart, whereas deletion of BIR domains (ΔBIR) did not affect XIAP interaction with MGL (Fig. 9B&C). In addition, a fragment containing only the BIR domains (BIR-1-2-3) did not show interaction with MGL (Fig. 9B&C). Thus, our results indicate that the C-terminus of XIAP including the RING domain, rather than the N-terminal BIR domains, interacts with MGL. Previous studies have shown that XIAP N-terminal BIR-2 or BIR-3 domains...
interact with caspases-3, 7 or 9 (12-15,17). Presumably, MGL-XIAP interaction may interfere with XIAP binding to caspases and thus protect caspases from XIAP-mediated protein degradation and inhibition. Smac/Diablo, an inhibitor of XIAP that is released from mitochondria in response to apoptotic stimuli, promotes apoptosis by its interaction with XIAP and averts XIAP interaction with caspases (34). It will be of great interest in the future to investigate whether MGL possesses Smac/Diablo-like function to counter inhibition of caspases and enhances activities of caspases via its interaction with XIAP.

We also show that N-terminus of MGL is important for MGL-mediated cell death and interaction with XIAP. We demonstrate that MGL N-terminal fragment (a.a. 1-105) induces cleavage (activation) of caspase-3 and PARP, and cell death, which is coincident with its interaction with XIAP (Figs. 9 & 10). Another MGL fragment (1-209), which contains the N-terminal first 105 residues plus 104 amino acids at the central region, did not induce caspase-3 activation, PARP cleavage and cell death (Fig. 10 A&B). MGL1-209 also did not or minimally interact with XIAP (Fig. 8 B&C). We also noted that the N-terminal polypeptide 1-105 killed cells better than the FL-MGL when expressed in cells under similar conditions (Fig. 10 A). MGL 1-105 also had stronger interaction with XIAP than FL-MGL (Fig. 8 B&C). Based on these results, we propose that the central region (a.a. 106-209) may harbor regulatory element(s) that functionally suppresses the action of the N-terminus of MGL on cell killing and interaction with XIAP. When the inhibitory central domain (a.a. 106-209) is removed, the suppression on N-terminus-mediated XIAP interaction and cell killing is alleviated, such that the fragment 1-105 is able to work better than the FL-MGL for its XIAP interaction and cell killing. Samuels et al (36) have previously shown that ASPP2 (Apoptosis Stimulating Protein of p53, also
called p53BP-2) stimulates the apoptotic function of p53 via its interaction with p53; the C-terminus of ASSP2 interacts with p53 while the N-terminus acts as intramolecular negative regulatory element to inhibit the p53-ASSP2 interaction. It remains to be determined whether the central region of MGL in fact has a suppressive role on the N-terminus on protein interactions (i.e. with XIAP) and cell killing.

Overexpression of XIAP has been commonly found in human cancers (37-40) and the mechanism(s) for its elevated expression are not very clear. Our studies indicate that MGL deficiency could be one of the important mechanisms leading to XIAP overexpression. We show that XIAP expression is significantly lower in MGL-overexpressing cells and the opposite is also true in MGL-deficient cells (Fig. 3). Such reciprocal expression patterns of these two proteins were also observed in MGL-knockout animal tissues and MEFs as well as in human lung cancer tissues (Fig. 4 & 5). MGL expression is commonly reduced in many human malignancies (1-3, 5, 6). Studies presented here, for the first time, provide evidence suggesting a possible causative link between MGL deficiency and XIAP overexpression.

Our results also indicate that MGL-deficient cells are more resistant to doxorubicin and etoposide, the anticancer drugs commonly used in the clinic (Fig.10 E). These results suggest that reduced or lack of MGL expression may contribute to anticancer drug resistance. Our previous studies have also demonstrated that lack of MGL expression resulted in elevated Akt phosphorylation (1, 2). Thus, our current and previous studies together demonstrate that MGL appears to regulate cell death and thus its tumor suppressive function via at least two mechanisms: one is to inhibit the PI3K/Akt survival pathway and another is to block the function of XIAP by enhancing its protein
degradation. Currently, several clinical trials are under way using SMAC-mimetic LCL161 in combination with other anticancer agents for treatments of relapsed and/or refractory small cell lung cancer, gynecologic malignancies and multiple myeloma (41). Our study has revealed that a small region (a.a. 1-60) on the N-terminus of MGL (MGL-N) has strong pro-apoptotic potential. It will be of great interest to further determine whether MGL-N could have SMAC-mimetic-like function in regulation of cell death and enhancing the sensitivity of anticancer drugs.
Materials and Methods

Cell culture, antibodies and reagents. The MGL-proficient and deficient mouse embryonic fibroblasts (MEFs) were generated from our previous studies (2) and maintained as previously described (2). Mouse abdominal tumor (MADT) cell line was isolated from a tumor grown on the abdominal region of a MGL-/− mouse and maintained in DMEM with 10% fetal bovine serum (FBS). Human lung cancer cell lines A549 and H1299, and cervical cancer HeLa cell line were from the National Institutes of Health (NIH, NCI) and cultured in DMEM supplemented with 10% FBS. Human ovarian cancer cell lines OVCAR-3 and SKOV3, and melanoma cell lines MUM2C and C8161 were from ATCC and cultured in RPMI supplemented with 10% FBS.

The XIAP and caspase-3 antibodies were from BD Transduction Laboratories (San Jose, CA). The Phospho-AKT (Ser473), AKT, Cleaved PARP, cleaved caspase3, cleaved caspase 8, pro-caspase-8 and 9 antibodies were from Cell Signaling (Danvers, MA). The MGL antibody was generated by our laboratory and described in previous studies (1). The GAPDH and vinculin antibodies were from Santa Cruz Biotechnologies (Dallas, Texas). The β-actin and α-tubulin antibodies were from Sigma (St. Louis, MO). The XIAP antibody for immunohistochemical staining was from Abcam (Cambridge, MA). The MGL inhibitor URB 602 was purchased from Sigma-Aldrich (St. Louis, MO, USA) with IC50 = 28 μM (46). The other MGL inhibitor JZL184 were purchased from Cayman Chemical (Ann Arbor, MI, USA) with IC50 = 8 nM (45). Pan-caspase inhibitor Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN, USA). Cycloheximide was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used 10μg/ml for indicated times. Embelin was purchased from Cayman Chemical (Ann Arbor, MI, USA).
Anticancer drugs doxorubicin, etoposide and taxol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purified XIAP protein was purchased from R&D Systems (Minneapolis, MN, USA). The purified S-HA-tag MGL protein was generated from the previous studies (1). The matching human patient normal and tumor samples were from The Cooperative Human Tissue Network, an organization sponsored by the National Cancer Institute. MycoFluor Mycoplasma Detection kit was form Thermo Fisher (Waltham, MA, USA).

MTT assays

MTT cell proliferation assays were performed as previously described (1). Briefly, cells seeded in a 96-well plate or 12-well plate with indicated transfections or treatments, were incubated with 0.5 mg/mL MTT for one hour. The resulting formazan precipitate was dissolved in isopropanol with 0.04 mol/L HCl. Absorbance was read with a Bio-Rad SmartSpec 3100 at 570 nm with background subtraction read at 690 nm.

Expression plasmids. XIAP full-length and deletion variants vector were provided by Addgene (Watertown, MA, USA). pDsRedN1-MGL-WT vector (MGL-RFP) and pSRα-HA-S-tagged MGL expression vector were previously described (1, 2). The pDsRedN1-MGL-S132A construct was generated using the pDsRedN1-MGL-WT (2) as a template and the site-directed mutagenesis kit per protocol provided by the company (Agilent Technologies, Santa Clara, CA). MGL-deletion variants were generated by subcloning
the MGL-cDNA fragments generated by PCR into a pSRα-HA-S-tag expression vector; primers for PCR-cloning as the followings: for MGL-1-60:

5’-ACAGCTAGCATGGAAACAGGACCTGAA-3’ and

5’-CATCCGCGGTCCATGGGACACAAAGAT-3’. For MGL-1-105:

5’-ACAGCTAGCATGGAAACAGGACCTGAA-3’ and

5’-CATCCGCGGAACGTGGAAGTCAGACAC-3’. For MGL-1-209:

5’-ACAGCTAGCATGGAAACAGGACCTGAA-3’ and

5’-CATCCGCGGAACGTGGAAGTCAGACAC-3’. For MGL-106-209:

5’-ACAGCTAGCATGTTCTGTCAGGGATGTG-3’ and

5’-CATCCGCGGAACGTGGAAGTCAGACAC-3’. For MGL-106-313:

5’ ACAGCTAGCATGTTCGTCAGGGATGTG-3’ and

5’-CATCCGCGGAACGTGGAAGTCAGACAC-3’. For MGL-210-313:

5’-ACAGCTAGCATGATCTGCCGGGCAGGGC-3’ and

5’-CATCCGCGGAACGTGGAAGTCAGACAC-3’. DNA sequencing was performed to confirm the integrity of MGL sequence for all expression vectors.

**Animal studies.**

MGL knockout mice were generated from our previous studies and maintained as described (2). Animal studies were performed according to guidelines of the Institutional Animal Care and Use Committee (IACUC) of SUNY Upstate Medical University.
Tissues used for histological studies were fixed in 4% paraformaldehyde and hematoxylin and eosin (H&E) staining was performed as previously described and inspected by a pathologists (CC).

**Lentivirus-mediated knockdown of MGL.**

Different nucleotide sequences targeting the human MGL are designed as follows: shRNA1 5’-ccaggacaagactctcaagat-3’; shRNA2 5’-caactccgtcttccatgaaat-3’; shRNA3 5’-ccaatcctgaatctgcaacaa-3’. MGL knockdown was achieved by shRNA silencing as described previously (1). The shRNA expression was mediated by lentiviruses that are prepared and used according to protocols from Addgene.

**Western blot analysis.**

For western blotting analyses, cell lysate and tissue extraction were performed as described previously (1).

**Immunoprecipitation and S-tag pulldown assays**

Immunoprecipitation was performed as previously described (42,43). S-tag pulldown assay was performed using S-protein agarose beads (EMD Millipore, Billerica, MA). Approximately, 50 µl of bead slurry was washed with buffer and mixed with cell lysate and the mixture was incubated overnight on a rotator at 4° C. The beads were then washed for three times and the associated proteins were denatured at 95° C in SDS-loading buffer, and subsequently analyzed by western blot analyses. Complete triton lysis
buffer (150mM NaCl, 20mM HEPES, 1mM EDTA, 1% Triton-X 100, 1mM PMSF, 20 mM NaF, 20 mM Na$_3$VO$_4$, 1% protease inhibitor cocktail (Sigma-Aldrich) was used for making cell lysate, incubation and washing.

**Statistical analysis.**

Two-tail Student’s t-test was used for statistical analysis. Tests are two-sided. Variance was similar between groups within each experiment.
Acknowledgements: This work is supported by NIH grants CA121850 (Y.H) and DK62136 (Y.H.) and Michael Connolly Lung Cancer Research Grant, SUNY Upstate Medical University (Y.H.).
References


Chapter 3

MONOGLYCERIDE LIPASE SUPPRESSES TUMOR-PROMOTING INFLAMMATION VIA INHIBITION OF NF-κB SIGNALING IN LUNG CANCER

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Author contributions statement:

I designed the experiments, collected and analyzed data and wrote the manuscript. Renyan Liu helped with the design of experiments and data collection. Dr. Christopher Curtiss contributed to the pathological diagnosis. Dr. Ying Huang supervised the entire project and wrote the submitted paper. Dr. M. Saeed Sheikh is my co-advisor who provided regular input and advice and also revised the submitted paper.

Part of this chapter is submitted for publication.
Abstract

We have previously reported that Monoglyceride lipase (MGL)-deficiency promoted lung neoplasia in animals and MGL expression was reduced or absent in the majority of human lung cancers. Thus, evidence suggests MGL to be a tumor suppressor in lung tumorigenesis, however, the mechanisms via which MGL functions as a tumor suppressor remain to be investigated. Here we report that MGL acted as a novel negative regulator of NF-κB and inhibited chronic inflammation in the animal lung tissues. We found that MGL deficiency significantly increased lung lymphoid infiltration and expression of inflammatory molecules such as TNFα, COX-2 and IL1β in the MGL-deficient lung tissues and MEFs. MGL depletion increased NF-κB nuclear translocation, which was associated with induction of COX-2; treatment of NFκB inhibitors repressed COX-2 induction in MGL-depleted lung cancer cells and MEFs. MGL overexpression suppressed NFκB-mediated transactivation. Importantly, we found that MGL negatively regulated NF-κB via a mechanism involving its modulation of IκB, the Inhibitor of NF-κB. We found that expression levels of MGL and IκBα were positively associated in human lung tissues. MGL directly interacted with IκB and stabilized IκB protein by inhibiting IκB proteasomal degradation, an important event for triggering NF-κB nuclear translocation. MGL disrupted IKKβ and IκB interactions by its own association with IκB, and inhibited IκB phosphorylation at Ser32 mediated by IKKβ. In summary, our study demonstrates MGL to be an important modulator of regulation of inflammatory response in the lung tissue and identifies a novel mechanism via which MGL negatively regulates NF-κB activation.
Introduction

Our recent studies indicate that Monoglyceride Lipase (MGL) plays an important role in suppression of tumorigenesis (1-3). We demonstrated that MGL-deficient mice exhibited significantly higher tumor incidence in multiple organs (2). MGL-deficient mice developed lung adenocarcinoma and high-grade dysplasia with a high frequency especially in the older animals (2). MGL-deficient animals also developed other tumor types such as histiocytic sarcoma, soft-tissue sarcoma, ameloblastic adenoma, hepatoma and lymphoma at lower frequencies (2). In addition, splenomegaly (spleen size ≥ 2-4 cm) was a common pathological change in MGL-deficient (MGL<sup>-/-</sup>) mice; decreased or absent MGL expression in several human malignancies including lung cancer was also noted (1, 2). Data from Firehose/RSEM and Oncomine databases also revealed MGL (MGLL) expression to be significantly lower in multiple malignancies including lung cancer (4, 5). We also discovered MGL to negatively affect X-linked Inhibitor of Apoptosis (XIAP) via direct interactions with XIAP and promoting XIAP degradation via ubiquitination/proteasomal mechanism (3). Thus, MGL-induced XIAP degradation highlighted one of the important mechanisms via which MGL functioned as a tumor suppressor (3). Our recent results have also revealed that MGL regulates several important inflammatory molecules including COX-2 and TNFα (2). These findings suggest that MGL appears to also play an important role in regulation of inflammatory responses, however, the molecular mechanisms remain to be investigated.

Inflammation has been implicated as playing an important role in tumorigenesis (7, 8). Persistent chronic inflammatory conditions predispose susceptible cells to neoplastic transformation (9). Chronic inflammation also generates an immunosuppressive
microenvironment that inhibits anticancer immunity and promotes tumor development (10). Interestingly, COPD (Chronic Obstructive Pulmonary Disease), a chronic inflammatory lung disease has been linked to lung cancer development (11). Chronic inflammation is thought to affect the bronchial epithelium and lung microenvironment, which promote pulmonary neoplastic transformation and progression (12). Cigarette smoking is the most important risk factor for COPD and lung cancer; however, about 1/4 of adults with COPD are never smokers (13), and 10-15% of all lung cancer occurs in the never smokers (14). Currently, the cause for non-smokers developing COPD and lung cancer remains unclear yet its understanding is critical to develop treatments for these diseases.

It is well-recognized that Nuclear Factor-κB (NF-κB) plays a central role linking chronic inflammation and cancer (15). NF-κB as a transcription factor plays a key role in regulation of inflammatory response. Inflammatory cytokines, chemokines, growth factors, antiapoptotic molecules and enzymes are its transcriptional targets (15) that are linked to inflammation, cell proliferation, cell survival and tumorigenesis (15, 16). Its activity and nuclear translocation are negatively regulated by the inhibitor of κB (IκB) (17). IκB binds and sequesters NF-κB in cytoplasm and prevents NF-κB translocation to nucleus thereby inhibiting its transactivation function (18). Stimulating signals activate IκB kinase complex (IKKs) which in turn, phosphorylates IκB and triggers IκB proteasomal degradation (18). NF-κB, released from IκB, translocates to nucleus to activate its target genes that are linked to inflammation and tumorigenesis (17). Thus, IκB plays a key role in regulation of inflammatory responses by preventing NF-κB activation.
In this study, we have identified MGL as a novel negative regulator of the NF-κB pathway and demonstrate the mechanisms via which MGL regulates the transactivation functions of NF-κB. We found that MGL directly interacts with IκB and suppresses IκB phosphorylation by IKKβ. Our results also indicate that MGL-deficient mice exhibit high prevalence of lymphocytic infiltration and chronic inflammation in the lung tissues. Our results thus demonstrate a novel mechanism of NF-κB regulation and function thereby establishing an important link between (i) MGL deficiency (commonly seen in lung cancer) and (ii) chronic lung inflammation and lung cancer.
Results

High instance of lung lymphoid infiltration in MGL-deficient animals.

We have previously reported that MGL-deficient mice exhibited increased incidence of high-grade dysplasia and adenocarcinoma in the lung, especially the older MGL-deficient animals (2). Fig. 1A shows a normal lung tissue sample from an MGL\[^{+/+}\] mouse (panel a) and three representative lung adenocarcinoma samples from either the MGL\[^{+/-}\] mice (panels b, c) or MGL\[^{-/-}\] mouse (panel d) (blue arrows). These results are consistent with our previous findings, which showed that MGL-deficiency led to lung adenocarcinoma development (2). In the current study, we found that significantly higher proportion of MGL-knockout (MGL\[^{-/+}\], MGL\[^{-/-}\]) animals exhibited lymphoid perivascular infiltration in their lung tissues as compared to their wild-type counterparts (MGL\[^{+/+}\]) (Fig. 1B, yellow arrows & Fig. 1C). In these MGL-deficient animals, lymphoid perivascular infiltration was seen in multiple areas of the lungs either with or without identifiable lung tumors (Fig. 1B). The lymphoid perivascular infiltration is a clear indication of inflammatory reaction in the lung tissues. Fig. 1C shows the percentage of MGL-proficient and -deficient animals that developed lymphoid perivascular infiltration in the lungs; as it is shown, 29% of MGL\[^{+/+}\] animals showed lung lymphoid infiltration compared to 54% and 78% for MGL\[^{+/-}\] and MGL\[^{-/-}\] animals respectively (Fig. 1C). In addition, MGL-deficient animals (MGL\[^{+/-}\] and MGL\[^{-/-}\]) had higher rates of lymphoid infiltrations (59% and 82% in MGL\[^{+/-}\] and MGL\[^{-/-}\] respectively) at older age (16-22 months) and lower rates (44% and 75% in MGL\[^{+/-}\] and MGL\[^{-/-}\] respectively) at the younger age (12-15 months) (Fig. 1C). Similar findings were not noted in MGL-proficient younger or older animals (Fig. 1C). These results demonstrate a link between
Figure 1 Lung lymphoid infiltration and adenocarcinoma in MGL-deficient mice.

A. Top panel: photographs of lung tissues (with the heart) extracted from an MGL$^{+/+}$ mouse without tumor (a) and from MGL-deficient (MGL$^{+/-}$, MGL$^{-/-}$) animals with lung adenocarcinoma (b – d, blue arrows). Middle and bottom panels: photomicrographs of hematoxylin and eosin (H&E)-stained lung normal tissue (a’, a’’) and cancer (b’-d’, b’’-d’’) tissues. Image for 4X and 40X of the H&E-stained tissues were taken from the same corresponding issues with different magnifications. B & C. High instances of lymphoid perivascular infiltration in MGL-deficient mice. B. Photomicrographs of H&E stained MGL-proficient (MGL$^{+/+}$) mouse (#111) lung tissue without lymphoid perivascular infiltration or MGL-deficient (MGL$^{+/-}$, MGL$^{-/-}$) mice (#107, #33, #183) with lymphoid infiltration (yellow arrows). C. Quantification of animals of various MGL status with lymphoid infiltration.
the MGL deficiency and lymphatic inflammatory response in the lung tissues of MGL-deficient animals and indicate that lung inflammation is more common in the older animals.

**MGL deficiency is correlated with elevated expression of inflammatory modulators.**

We next investigated the expression status of inflammatory molecules/modulators in MGL-proficient and -deficient lung tissues and cells. Tumor necrosis factor alpha (TNFα) is one of the key inflammatory cytokines (reviewed in 19). Immunohistochemistry (IHC) staining-based analysis revealed that TNFα expression was significantly higher in the lung tissues from MGL-deficient (MGL⁻/⁻ and MGL⁻/⁺) animals when compared to those from the MGL-proficient (MGL⁺/+⁻) animals (Fig. 2A). Results in Fig. 2B show expression of COX-2 and IL-1β, the two other key inflammatory molecules; as is shown, expression levels of both were elevated in MGL-knockout mouse embryonic fibroblasts (MEFs) when compared to those in the MGL⁺/+⁻ MEFs (Fig. 2B, left and middle panels). Re-expression of MGL in MGL-knockout MEFs reduced COX-2 levels in these cells (Fig. 2B, right panel). We also tested A549 human lung cancer cells in which MGL was knocked down and noted that the COX-2, IL-1β and TNFα levels to be also significantly elevated in MGL-knockdown cells (Fig. 2C). These results indicate that MGL suppresses the expression of key inflammatory molecules such as TNFα, COX-2 and IL-1β.
Chapter 3

A

Mouse lung tissues, IHC staining for TNFα

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**COX-2**

**Vinculin**

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Figure 2. Elevated expression of inflammatory molecules in MGL-deficient lung tissues and cells. A. Photomicrographs showing TNFα immunohistochemical staining (IHC) of mouse lung tissues from MGL-proficient (MGL\(^{+/+}\)) mouse (left) and -deficient (MGL\(^{+/-}\), MGL\(^{-/-}\)) mice (middle and right). All tissues were co-stained with H&E; 10X and 20X images were taken from the same corresponding tissues. B. Left & middle panels: expression of COX-2, TNFα and IL-1β proteins was elevated in MGL-deficient MEFs compared to that in MGL-proficient MEFs. Right panel: transient re-expression (48 hrs) of MGL in MGL-knockout (MGL\(^{-/-}\)) MEFs reduced COX-2 expression. Vinculin signals serve as loading controls. C. Expression of COX-2, TNFα and IL-1β proteins was elevated in MGL-KD A549 human lung adenocarcinoma cells. Three different MGL RNAis (1, 2, 3) with different MGL mRNA targeting regions were used (see Materials and Methods). Scr, scramble RNAi.
MGL inhibits NF-κB nuclear translocation and transactivation potential.

COX-2, TNFα, and IL-1β are transcriptional targets of nuclear factor-kappa B (NF-κB) (20, 21). We investigated subcellular distribution of NF-κB since that affects its activity (18). Fig. 3A shows that staining of p65 (NF-κB subunit) was mostly cytoplasmic in MGL+/+ MEFs (a&b) but predominantly in the nuclei in MGL+/− and MGL−/− cells (c,d & e,f). Fig. 3B shows that in MGL−/− MEFs without reintroduction of RFP-(red)-tagged MGL, NF-κB (p65, green) was predominantly in the nucleus (yellow arrow). By comparison, in cells with re-expressed RFP(red)-tagged MGL, the p65 distribution was predominantly in the cytoplasm (blue arrow) (Fig. 3B). We further performed subcellular fractionations to determine the distribution of p65. As seen in Fig. 3C, p65 protein was largely distributed in the cytosol from MGL+/+ MEFs, whereas it was predominantly in the nuclei from MGL+/− and MGL−/− MEFs. Together, these results indicate that MGL affects NF-κB subcellular distribution. We also examined MGL effect on NF-κB transactivation; our results indicated MGL overexpression to strongly suppress NF-κB-mediated transactivation in MGL−/− MEFs and 293T cells (Fig. 3D). COX-2 is a transcriptional target of NF-κB (20). MGL knockdown (KD) in A549 lung cancer cells significantly elevated COX-2 mRNA levels, which was reversed by NF-κB inhibitor DMAPT (Fig. 3E). Fig. 3F shows that COX-2 protein was also elevated in MGL-KD A549 lung cancer cells (Fig. 3F, compared lane 1 to lanes 2 & 3) and that MGL deficiency-mediated COX-2 elevation was suppressed by NF-κB inhibitor SN50 in A549 cells (Fig. 3 F, left panel). SN50 also inhibited COX-2 protein levels in MGL−/− MEFs (Fig. 3F, right panel). Together these results indicate that MGL regulates NF-κB
Figure 3. MGL inhibits the nuclear translocation and transactivation of NF-κB. **A.** Immunostaining of NF-κB (p65) (green) in MGL-proficient (MGL+/+) and -deficient (MGL+/−, MGL−/−) MEFs. Cells were also counterstained with nuclear dye DAPI (blue). **B.** Representative images indicating that re-introduction of RFP-MGL (red) into MGL−/− MEFs increased cytosolic distribution of endogenous NF-κB (stained by p65 antibody, green). MGL−/− MEFs expressed exogenous MGL (blue arrow) or without expressed MGL (orange arrow) are indicated. Cell nuclei were co-stained with DAPI (blue). **C.** NF-κB (p65) subcellular distribution in MGL-proficient or -deficient MEFs is demonstrated by cell fractionation assay. HDAC2: nuclear protein marker; α-tubulin: cytosolic protein marker. **D.** Overexpression of MGL suppresses NF-κB transactivation from the regulatory element of its target gene. Cells were transfected with NF-κB luciferase reporter construct along with either the empty vector (Vec) or MGL expression vector (MGL). The data represent results collected from three-independent experiments performed in triplicates for each sample. (*) indicates p-value <0.05. **E & F.** MGL knockdown (MGL-RNAi) in lung cancer cells significantly increased the levels of COX-2 (NF-κB target gene) mRNA (E) and protein (F, left) which was blunted by treatment with NF-κB inhibitor DMAPT (E) or SN50 (F, left). Treatment with NF-κB inhibitor SN50 also suppressed COX-2 level in MGL-deficient MEFs (F, right).
transcriptional activity as well as expression of NF-κB target genes that play important roles in modulation of inflammation.

**A positive link of MGL and IκBα.**

IκBα is a key regulator of NF-κB subcellular distribution (18). Fig. 4A shows that IκBα levels were much lower in MGL^{+/−} and MGL^{−/−} MEFs than in MGL^{+/+} MEFs. MGL knockout (KO) also led to decreased expression of IκBα in MGL-deficient lung tissues (Fig. 4B) and in MGL-KD A549 (lung) and MDA-MB-231 (breast) human cancer cells (Fig. 4C). Inversely, overexpression of MGL in H1299 human lung cancer cells or in MGL-KO MEFs also significantly increased IκBα levels (Fig. 4D). We investigated the expression of MGL and IκBα in human primary lung tumors (T) and their matching normal (N) tissues. Fig. 4E & F shows that expression of MGL and IκBα exhibited a strong positive association. For example, tumors that expressed lower levels of MGL also had relatively lower levels of IκBα compared to their matched normal tissues (Fig. 4E, marked by red stars). These results indicate that MGL positively affects IκBα expression.

**MGL protects IκBα from proteasome-mediated degradation**

Proteasomal degradation is a key regulatory mechanism for IκBα protein stability and levels (22). Fig. 5A shows that IκBα protein decay was faster in MGL-RNAi (MGL-KD) cells than in scramble RNAi cells. These findings indicate that IκBα protein was less stable in MGL-depleted cells. Fig. 5B shows that treatment with proteasome inhibitor MG132 prevented IκBα degradation due to MGL-depletion (MGL-RNAi) (compare lanes
Chapter 3

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Patent: 3091427A
Figure 4. Positive correlation between MGL and IκBα expression in human primary lung tissues and in human and mouse cell lines. A&B. IκBα expression in MGL-proficient (+/+ ) and -deficient (+/- or -/- ) MEFs (A) and lung tissues (B). β-actin or vinculin serves as loading control. N: non-tumor tissue; T: tumor tissue. C & D. IκBα expression in MGL-depleted human cancer cells (C) or MGL-overexpressing (D) human lung cancer cells and MEFs. Scr., scramble shRNAi; MGL-RNAi, 1, 2 & 3, shRNA targeted three-different regions of MGL mRNA. Vec, vector-only control. E. Positive correlation between the expression patterns of IκBα and MGL in matched human primary lung normal (N) and tumor (T) tissues. Red asterisk* indicates a positive correlation between IκBα and MGL expression. F. IHC staining of IκBα and MGL proteins in matched human lung normal and tumor tissues. All tissues were also counterstained with H&E.
Fig. 5C & D shows that IκBα protein ubiquitination levels were significantly lower in MGL overexpressing cells than in vector-only control cells with (D) or without (C) co-expression of exogenous HA-tagged ubiquitin construct. These results suggest that (i) MGL depletion accelerated IκB protein degradation, and (ii) increased levels of MGL suppressed IκBα ubiquitination and proteasomal degradation and thus, increased IκBα protein stability.

**MGL directly interacts with IκBα**

We examined whether MGL regulates IκBα via protein-protein interactions. Fig. 6A shows that exogenous S-tagged MGL, but not S-tag-only, pulled down IκBα protein in both HeLa cervical and H1299 lung cancer cells. Endogenous protein interactions between MGL and IκBα were also demonstrated by co-immunoprecipitation assay, which indicated that IκBα antibody but not the control IgG pulled down endogenous MGL (Fig. 6B). We further determined whether MGL directly interacted with IκBα. Fig. 6C shows results of *in vitro* protein pulldown assays using purified recombinant proteins; as is shown, only the recombinant S-tagged MGL, but not the S-tag-only, pulled down the purified IκBα protein. These findings demonstrate that MGL directly interacts with IκBα protein.
Fig. 5. MGL enhances IκBα protein stability and suppresses IκBα ubiquitination. A. IκBα protein stability is decreased in MGL knockdown (MGL RNAi) cells compared to that in scramble RNAi (Scr) cells. Left panel: cells, expressing scramble RNAi (Scr.) or MGL-RNAi were treated or untreated (time 0) with protein synthesis inhibitor cycloheximide (CHX), IκBα protein decay was analyzed by WB. Right panel: the relative IκBα levels were determined by using the relative band intensity of IκBα (left, upper) adjusted for the band intensity of GAPDH (left, lower). The value at time point “0” in each set of samples was set as 1. The mean values of three-independent experiments for each time point are plotted (±SE *p<0.05). B. Treatment with proteasome inhibitor MG132 (4 hrs) prevents IκBα reduction caused by MGL knockdown. C & D. Overexpression of MGL reduces IκBα ubiquitination levels. H1299 cells were transfected with control vector or MGL expression construct without (C) or with (D) HA-tagged ubiquitin (HA-Ub). Forty-eight hours following transfection, cells were treated with MG132 for 6 hrs; immunoprecipitation were performed using IκBα antibody followed by WB with ubiquitin antibody.
Figure 6. MGL interacts with IκBα. A. Exogenous S-tagged MGL, but not S-tag-only, pulls down endogenous IκBα in cell lysates of HeLa (left) and H1299 (right) cancer cells. Cells were transfected with indicated vectors for 48 hrs before harvesting. B. Endogenous IκB α immunoprecipitates (IP) with endogenous MGL. Cells were treated with proteasome inhibitor MG132 for 6 hours before harvesting. C. Recombinant S-tagged-MGL, but not S-tag-only, pulls down recombinant IκBα in vitro. S-tag pull down assay was performed using recombinant S-tagged-MGL protein or S-tag-only mixed together with recombinant IκBα protein followed by Western Blotting using IκBα antibody.
MGL regulates IκBα phosphorylation

IκBα phosphorylation at serine 32 (Ser32) is a prerequisite for its proteasomal degradation (22, 23). We next investigated whether MGL modulates IκBα protein stability by regulating its phosphorylation. Fig. 7A shows that the Ser32 phosphorylation level of IκBα was significantly higher in MGL-deficient (MGL^+/− and MGL^-/-) cells compared to that in MGL-proficient (MGL^+/+) cells. By contrast, the IκBα total protein was much lower in MGL-deficient cells compared to MGL-proficient cells (Fig. 7A). Conversely, overexpression of HA-tagged MGL decreased IκBα Ser32 phosphorylation while increased total IκBα protein levels in both H1299 and A549 cells (Fig. 7B and C). Together, these results suggest that MGL antagonizes the phosphorylation and degradation of IκBα.

MGL blocks phosphorylation of IκBα by IKK

The IKK complex, containing IKKα or IKKβ, is responsible for IκBα phosphorylation (24, 25). Specifically, in the classical NF-κB signaling pathway, phosphorylation and degradation of IκBα is dependent on IKKβ (25). Our results show that MGL directly interacted with IκBα (Fig. 6) and reduced IκBα phosphorylation (Fig. 7). We reasoned that MGL could inhibit IκBα phosphorylation by interfering with IκBα phosphorylation by IKKβ. We thus performed in vitro IKKβ kinase assay to determine the effect of MGL on IκBα phosphorylation by IKKβ. We used commercially provided reagents (Promega), including the purified recombinant IKKβ kinase, recombinant full-length IκBα or the IKKtide (a peptide of 20 amino acids with IKKβ phosphorylation site on
Figure 7. MGL negatively regulates IkBα phosphorylation. A. IkBα phosphorylation on Ser32 is elevated in MGL-deficient (MGL+/−, MGL−/) MEFs, by contrast, the levels of total IkBα protein are reduced in MGL-deficient cells. B & C. Overexpression of MGL in two different human lung cancer cells reduces IkBα phosphorylation on Ser32, whereas the levels of total IkBα are elevated.
IκBα) as substrates. The recombinant S-tagged MGL, or the negative controls S-tag-only or BSA was also used in the assays. Fig. 8A shows the IκBα phosphorylation or the IKKtide phosphorylation by IKKβ, detected by luminescent kinase assay. As is shown, the presence of recombinant S-tagged MGL at two different doses (0.2 µg, left panel; 2µg, right panel) significantly inhibited the phosphorylation of IκBα or IKKtide by IKKβ in vitro (Fig. 8A). By contrast, S-tag-only or BSA did not have such effect on IκBα phosphorylation. Fig. 8B demonstrates IκBα phosphorylation mediated by IKKβ in vitro by western blotting using the phospho IκBα (Ser32) antibody. As is shown, in both sets of experiments, the recombinant S-tagged MGL, but not the S-tag-only or the BSA, significantly inhibited Ser32 phosphorylation on recombinant IκBα, mediated by purified IKKβ in vitro. We have demonstrated that MGL directly interacts with IκBα (Fig. 6C). Therefore, it is possible that MGL may suppress IκBα phosphorylation by competitive interaction with IκBα and therefore, obstruct interactions between IκBα and IKKβ. To investigate this possibility, we performed IκBα co-immunoprecipitation assay (Fig. 8C) and found that, in the presence of recombinant S-tagged MGL, the amounts of IKKβ protein in the anti-IκBα precipitated complex was significantly less compared to that in the presence of S-tag-only or BSA recombinant protein (Fig. 8C, top panel). Together these results indicate that MGL, via its interactions with IκBα, appears to disrupt IKKβ association with IκBα and thus, suppresses IκBα phosphorylation by IKKβ.
A  \textit{In vitro} IKKβ kinase assay (IκBα or IKKtide peptide as substrate)

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C  \textit{In vitro} proteins interaction assay

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Figure 8. MGL inhibits IκBα-IKKβ interactions and inhibits IκBα phosphorylation by IKK in vitro.  

A. IKKβ-mediated *in vitro* phosphorylation of a full-length recombinant IκBα or IKKtide peptide was significantly blunted in the presence of recombinant S-tagged-MGL, but not by S-tag-only or bovine serum albumin (BSA) (negative controls). IKKβ activity was detected by luminescent kinase assay (Promega). IKKtide is a fragment (peptide) of IκBα that contains a phosphorylation site for IKKβ-mediated phosphorylation. Amounts of ~0.2 µg (left) or ~2 µg (right) of S-tagged-MGL, S-tag-only or BSA were used in the reactions as indicated. Assays were performed per the protocol provided by the company (Promega) and detailed in Materials and Methods.

B. The presence of MGL significantly reduced *in vitro* phosphorylation of IκBα (recombinant) by IKKβ (recombinant) detected by Western Blotting using phospho-IκBα antibody. Following the *in vitro* IKKβ kinase reactions of the indicated proteins or controls, IκBα phosphorylation was detected by Western blotting using antibody specific to phosphorylated IκBα (p-IκBα, S32). C. The presence of MGL, but not S-tag-only or BSA, reduced *in vitro* protein interaction between IKKβ and IκBα. *In vitro* protein interaction assays were performed as described (Materials and Methods) using the purified IKKβ and IκBα in the presence or absence of recombinant S-tagged-MGL, S-tag-only or BSA (0.2 or 2 µg). After *in vitro* protein reactions, immunoprecipitation (IP) was performed using anti-IκBα antibody followed by Western blotting using the indicated antibodies.
Discussion

In this study we demonstrate the important role of MGL in regulation of inflammatory response in relation to lung tumorigenesis. Our results provide important insights into the molecular mechanisms via which MGL regulates the NFκB-mediated inflammatory response. First, in MGL-deficient animals, lung lymphoid infiltration and expression of inflammatory molecules were significantly elevated when compared to MGL-proficient counterparts. Overall, 78% of MGL-/- and 54% of MGL+/+ animals revealed lung lymphoid infiltration versus only 29% of MGL+/+ animals exhibiting similar changes in the lungs (Fig. 1). Furthermore, lung lymphoid infiltration was concurrent with elevated expression of inflammatory molecules such as TNFα and COX-2 in the lungs of MGL-deficient animals (Fig. 2 and ref. 2). MGL-KD also elevated the expression of TNFα, COX-2 and IL-1β in human lung cancer cells (Fig. 2C). These results indicate that prolonged insufficient MGL expression appears to trigger chronic inflammatory changes in the lung tissues of MGL-deficient animals. In this context, our previous studies (1-3) and information collected from the public databases Firehose/RSEM and Oncomine (Fig. 9, refs 4 & 5) demonstrated that MGL expression was significantly lower (or absent) in the majority of human lung cancers compared to normal lung tissues (1-5). Thus, it is possible that insufficient expression of MGL in human lung tissues could also lead to chronic inflammatory changes that are similar to those found in the MGL-deficient animals. Studies are needed to further investigate this issue.

We also found that the proportion of MGL-deficient animals with lung lymphoid infiltration was significantly higher in the older animals (Fig. 1C). For example, 44% of MGL+/+ and 75% of MGL-/- animals at ages 12-15 months had lung lymphoid infiltration
Disease Summary for MGLL

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<td>Lymphoma</td>
<td>11</td>
</tr>
<tr>
<td>Melanoma</td>
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<tr>
<td>Myeloma</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Pancreatic Cancer</td>
<td>3</td>
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<tr>
<td>Prostate Cancer</td>
<td>4</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>4</td>
</tr>
</tbody>
</table>

Significant Unique Analyses: 17
Total Unique Analyses: 458

Cell color is determined by the best gene rank percentile for the analyses within the cell.
NOTE: An analysis may be counted in more than one cancer type.
Figure 9. The levels of MGL mRNA in cancer and normal tissues (Adapted from ONCOMINE Cancer Microarray database). Red: over-expression of genes; blue: under-expression of genes. The intensity of the color correlates with the significance of the expression status. Data accessed on October 1st, 2021.
versus 59% of MGL\textsuperscript{+/+} and 82% of MGL\textsuperscript{−−} animals showing similar changes at 16-22 months (Fig. 1C). In comparison, lung lymphoid infiltration seen in MGL\textsuperscript{+/+} animals was 38% at 12-15 months and 23% at 16-22 months (Fig. 1C), which was not increased in the older animals (Fig. 1C). These results indicate that continued MGL deficiency can further increase the inflammatory changes when animals become older. In this context, our previous studies have demonstrated that the older MGL-deficient animals had significantly higher incidences of lung neoplasia (2). Thus, increase in chronic lung inflammation due to MGL deficiency is associated with higher incidences of lung neoplasia in the older MGL-deficient animals. These findings indicate that MGL appears to play important roles in inhibition of chronic inflammation, which may be critical for its function as a tumor suppressor particularly in context to lung cancer development.

Our current study also demonstrates that MGL plays an important role in regulation of \(\text{I}\kappa\text{B-NF\kappa B}\) pathway. We show that MGL depletion (by knockout or knockdown) promotes NF-\(\kappa\)B nuclear translocation concomitant with increased expression of NF-\(\kappa\)B target genes such as COX-2 (Fig. 3 A-C, E). Increased COX-2 expression in MGL-depleted cells was inhibited by the NF-\(\kappa\)B inhibitors DMAPT or SN50 (Fig. 3E & F), and overexpression of MGL suppressed NF-\(\kappa\)B activity from its regulatory promoter (Fig. 3D). These results demonstrate that MGL modulates the expression of inflammatory target molecules, such as COX-2, via negative regulation of NF-\(\kappa\)B. Importantly, our results have identified a novel mechanism via which MGL regulates NF-\(\kappa\)B. We have found that \(\text{I}\kappa\text{B}\alpha\) levels positively associated with those of MGL in lung tissues from animals and humans (Fig. 4, B,E,F). \(\text{I}\kappa\text{B}\alpha\) expression was also significantly lower in MGL-knockdown lung cancer cells (Fig. 4C) and elevated in MGL-overexpressing cells
(Fig. 4D). IκBα is an inhibitor of NF-κB; it forms a complex with NF-κB in cytoplasm preventing NF-κB nuclear translocation (18). In this context, IKK (Ikappa B kinase)-mediated phosphorylation of IκBα triggers IκBα proteasomal degradation that leads to reduction of IκBα protein followed by NF-κB translocation to the nucleus to activate its target genes (18). Significantly, we have discovered that MGL directly interacted with IκBα (Fig. 6) and reduced IκBα ubiquitination and degradation (Fig. 5). We have further revealed that the MGL levels were inversely associated with the Ser32 phosphorylation levels of IκBα but positively correlated with the total levels of IκBα (Fig. 7). IκBα Ser32 phosphorylation is higher in MGL-deficient cells and lower in MGL-overexpressing cells (Fig. 7). It is well-established that phosphorylation at Ser32 is a key event that leads to IκBα proteasomal degradation (26, 27). Thus, our results suggest that modulation of IκBα phosphorylation appears to be an important mechanism via which MGL prevents IκBα degradation. It is well-recognized that IκBα phosphorylation at Ser32 is predominantly mediated by IKK complex (α/β), and IKK-mediated phosphorylation induces IκBα polyubiquitination and rapid degradation (26-28). Our study further demonstrates that MGL negatively affected IκBα (in vitro) phosphorylation by IKKβ (Fig. 8, A&B); in addition, MGL inhibited the interactions between purified IκBα and IKKβ (Fig. 8C). Thus, our results indicate that, MGL by directly interacting with IκBα (Fig. 6, B&C), suppresses the interactions between IKKβ and IκBα and thereby, prevents IκBα (substrate) direct phosphorylation. This appears to be an important mechanism via which MGL prevents the phosphorylation of IκBα and thus, protects IκBα from proteasomal degradation. The schematic of a proposed model depicting MGL regulation of IκB-NFκB is shown in Fig. 10.
Ample MGL expression in normal lung tissue

MGL

IKKβ

IκBα

IκBα

NFκB sequestered in the cytosol by IκBα

Low MGL expression in abnormal/cancerous lung tissue

IKKβ

IκBα

P

IκBα

Proteasomal degradation

Increased expression of inflammatory molecules such as COX2, TNFα, IL-1 and lymphoid infiltration

Nuclear localization of NFκB increases lung inflammation & promotes tumorigenesis
Figure 10. The proposed mechanism via which MGL regulates NF-κB-mediated inflammatory response through modulation of IκBα in lung tissues. In normal lung tissues, ample MGL levels would favor MGL-IκB interactions that competitively inhibit IKK-IκB interactions thereby preventing IκB phosphorylation and proteasomal degradation. IκB would keep NF-κB sequestered in the cytosol. NF-κB not being able to translocate to nucleus would not transcriptionally activate the inflammatory target genes. In abnormal conditions, the lung tissues chronically express low or absent levels of MGL, such conditions would favor IKK-IκB interactions, and consequently IκB phosphorylation and proteasomal degradation. NF-κB no longer kept sequestered in cytosol by IκB would translocate to nucleus to transcriptionally activate its target genes involved in promoting inflammatory responses and tumorigenesis.
Chronic inflammation appears to be one of the critical causes underlying cancer promotion, as many solid tumors arise from the sites of chronic inflammation. Chronic inflammatory condition can be caused by infectious pathogens or non-infectious agents such as chemicals. For example, infection with Helicobacter pylori-induced chronic inflammation is causally linked to gastric cancers (29). Asbestos-induced chronic inflammation is also causally linked to malignant mesothelioma (MM) (30). Tobacco-smoking-induced lung cancers are commonly associated with COPD, a chronic pulmonary inflammatory condition (31). Tobacco smoking-induced lung inflammatory response is reported to coincide with deregulated production of cytokines and growth factors (31). Overexpression of COX-2 is also found in lung precursor lesions as well as established human lung cancers (31, 32). Tumor microenvironment, composed of cellular component (including inflammatory cells) and secreted component (including cytokines, chemokine, growth factors), can affect cell proliferation, tumor initiation, progression, and metastasis (7). NF-κB is considered to play an important role in initiation, promotion and progression of lung cancer (33). Cigarette smoke induced proinflammatory cytokine induction has been found to be activated by NF-κB (34). Thus, various lines of evidence demonstrate that activation of NF-κB and chronic inflammation are important in the development of lung cancer.

Our current study unveils that continued insufficient expression of MGL can trigger chronic inflammation in the lungs of animals. Our results indicate that, in addition to the known factors such as pathogens, chemicals and cigarette smoking, absent or reduced expression of tumor suppressor MGL can also lead to chronic inflammation in the lungs coupled with increased incidence of lung cancer. These novel findings are clinically
relevant. For example, it is estimated that about a quarter of the adults with chronic pulmonary inflammation such as COPD are never smokers (13); a significant proportion of lung cancers occur in never smokers with no clearly identifiable etiological factors (14). As mentioned above, absent or reduced MGL expression is rather common in human lung cancer, noted in ~60% of human lung cancers (1-5). Based on our current and previous findings, we propose reduced MGL expression to be an important mechanism underlying chronic inflammation and concomitant lung cancer development in some patients.
Materials and Methods

Animal studies. MGL knockout mice were used as in the previous studies (2, 3), which were generated through the Gene Targeting and Transgenic Facility (GTTF) at University of Connecticut Health Center (Farmington, CT). Our animal studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at SUNY Upstate Medical University. Histological studies were performed as described previously (2, 3) as diagnosed by a pathologist (CMC). Animal organs/tissues were collected and processed for protein analyses as previously described (2, 3).

Cell culture, antibodies and reagents.

Mouse embryonic fibroblasts (MEFs) were prepared and used as previously described (2, 3). Human non-small cell lung cancer cell lines A549 and H1299, and HeLa cervical cancer cell line were obtained from the National Institutes of Health (NIH) and regularly maintained in DMEM supplemented with 10% FBS. MGL antibody was generated in our laboratory and used as in the previous studies (1-3). TNF-alpha, GAPDH, vinculin, IL-1β, p65, IκBα, HDAC2, and COX-2 antibodies were purchased from Santa Cruz Biotechnologies (Dallas, Texas). Phospho-IκBα (Ser32), IKKα, and IKKβ antibodies were from Cell Signaling Technology (Danvers, MA). NF-κB inhibitor DMAPT and SN50 were from Cayman Chemical Company (Ann Arbor, MI). Preparation of pDsRedN1-MGL vector (MGL-RFP) was described previously (1). Preparation of pSRα-HA-S-tagged MGL expression vector was also described in our previous publications (1-3). The MGL expression in cells was achieved through cell transfection.
using Lipofectamine 2000 following the protocol from the manufacturer (Invitrogen of Thermo Fisher Scientific, Waltham, MA).

**Luciferase assays**

Luciferase assays were performed as described in previously (35). Briefly, 293T or MEFs were transiently transfected with plasmid vector-alone or MGL-expression plasmid vector along with (kappaB) 3-IFN-LUC reporter plasmid vector containing the NF-kappa B responsive element (previously provided by laboratory of Dr. Baltimore, California Institute of Technology, CA). Following the protocol from Promega (Madison, WI), cells were collected 24 hours post-transfection and luciferase activity was analyzed using cell lysis buffer and luciferin substrate from Promega (Madison, WI).

**Lentivirus-mediated knockdown of MGL.**

Lentivirus-mediated MGL knockdown was performed as we have previously described (1-3). The scramble shRNA construct was obtained commercially from Addgene, Inc. (Cambridge, MA). All other shRNA constructs were purchased from Open Biosystems, Inc. (Huntsville, AL). Three nucleotides with different sequences targeting the human MGL were used in this study in different cell lines and they were as follows: shRNA1, 5’-ccaggacaagactctcaagat-3’; shRNA2, 5’-caactccgtcttccatgaaat-3’; shRNA3, 5’-ccaatcctgaatctgcaacaa-3’. Virus preparation, expansion and infection were performed following the Addgene’s protocol.
Western blot analysis and immunofluorescence staining

Western blotting and analyses were performed as previously described (1-3). For immunostaining of endogenous p65 protein, cells were blocked with 10% goat serum in PBS, and then incubated with p65 antibody (1:200 diluted in PBS with 1.5% goat serum), followed by FITC-labeled anti-rabbit antibodies (1:500 diluted in PBS with 1.5% goat serum). Cell nuclei were counter-stained with the DAPI nuclear dye, and the staining was analyzed under an Olympus fluorescent microscope.

Immunoprecipitation and S-tag pulldown assays

Immunoprecipitation was performed as described in the publications from our laboratory (2, 3). S-tag pulldown assays were performed using the commercially available S-protein agarose beads (EMD Millipore, Billerica, MA) to pull down the protein of interests as described previously (2, 3). Specifically, 50 µl of bead slurry was first washed with buffer and then mixed with cell lysate. Subsequently, the mixture was incubated at 4°C overnight with rotation. Following the incubation, the beads were washed three times and the proteins attached to the beads were denatured at 95°C in SDS-loading buffer for 5 min, and subsequently analyzed by western blot analyses. Complete triton lysis buffer [1% Triton-X 100, 20mM HEPES, 1mM EDTA, 150mM NaCl, 1mM PMSF, 20 mM NaF, 20 mM Na₃VO₄, 1% protease inhibitor cocktail (Sigma-Aldrich)] was used for preparing the cell lysate, incubation of mixture, and washing of beads.
In vitro kinase, phosphorylation, and protein interaction assays

*In vitro* phosphorylation of the recombinant IκB and IKKtide peptide was performed using the IKKβ kinase assay kit (Madison, WI) per company’s protocol. For the IKKβ kinase assay, recombinant IκBα protein from Rockland Immunochemicals (Limerick, PA) or IKKtide peptide provided by the IKKβ kinase assay kit were used as the kinase reaction substrates. The assays were performed at room the temperature. Step 1: the substrates (recombinant IκBα protein or IKKtide peptide) and MGL protein or S-tag only or BSA were added into each well (96-well plate) in the kinase buffer (40mM Tris, pH 7.5; 20mM MgCl2; 0.1mg/ml BSA; 50μM DTT) from the kit, then incubated for 60 minutes. Step 2: purified IKKβ kinase and 25μM ATP were then added into the reaction mix and incubated for 20 minutes. Step 3: for a 40μl reaction, 10μl of ADP-Glo Reagent from Promega (Madison, WI) was added for additional incubation of 40 minutes. Step 4: 20μl of Kinase Detection Reagent was added for further incubation of 30 minutes. Step 5: Reaction luminescence was recorded by a plate reader from BioTek (Winooski, VT) to reflect the IKKβ kinase activity. Results presented in the Fig. 8A were performed as above described. For *in vitro* IκBα phosphorylation detected by Western blot analyses (Fig. 8B), the steps 1 and 2 were performed in the same way as above described, and each of the reaction mixture yielded in the step 2 was then processed for Western blot analyses probed with IκBα serine 32 phosphor antibody from Cell Signaling (Danvers, MA). For studying *in vitro* protein interaction of IKKβ and IκBα in the presence or absence of MGL or control proteins, each protein (or controls) was sequentially added as above described in the Step 1 and Step 2 followed by immunoprecipitation assay using the IκBα antibody and Western blot analyses by indicated antibodies.
Statistical analysis.

Results are expressed as mean plus or minus SD. Two-tail Student’s t-test was used for statistical analyses.
Acknowledgements: This work is supported by NIH grants CA121850 (Ying Huang) and DK62136 (Ying Huang) and Michael Connolly Lung Cancer Research Grant, SUNY Upstate Medical University (Ying Huang).
References


Chapter 4

GENERAL DISCUSSION

The role of MGL in tumorigenesis

The study of MGL started in our laboratory about two decades ago when we independently cloned the human MGL gene. Our studies to date including the data presented in this dissertation, all support a tumor-suppressive role of MGL, not only in lung cancer but also in cancers of multiple tissue origins (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020). Our first piece of evidence was derived from the screening of human tumor samples, which showed that MGL mRNA or protein levels were either reduced or undetectable in the majority of tumor tissues when compared with matching normal tissues (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020). Furthermore, MGL overexpression suppresses cancer cell growth and induces cell death while MGL depletion promotes cell growth and tumor formation in multiple types of organs or tissues (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020). At the molecular level, we discovered significant suppression of the tumor-promoting EGFR, PI3K/ATK, and NF-κB signaling pathways and strong inhibition of the anti-apoptotic XIAP by MGL (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020; and chapter 3). These findings not only further confirmed the tumor-suppressive roles of MGL but also revealed specific therapeutic targets in MGL-deficient cancers as was already discussed in previous chapters.

Most importantly, we are not the only group that determined the tumor-suppressive
role of MGL. It was reported by another group that MGL was a tumor suppressor in the formation of hepatocellular carcinoma (HCC) (Rajasekaran et al., 2016). In addition to the studies of MGL in cancer cells, MGL deficiency in tumor-associated macrophages (TAMs) was also shown to play a positive role in tumor progression (Xiang et al., 2018), implying the tumor-suppressive characteristic of MGL.

Our studies of the role of MGL in tumorigenesis have been focusing on lung cancer since our initial discovery of lung cancer formation in MGL knockout mice.

The screening of human lung cancer tissues showed that there was loss or reduction of MGL in 65.5% of lung tumors as compared with normal tissues (Liu et al., 2018). Additional cellular and molecular studies further confirmed the tumor-suppressive role of MGL in lung adenocarcinoma (Liu et al., 2018; Liu et al., 2020).

Most importantly, in the context of MGL deficiency, we identified a series of overly-activated tumor-promoting signaling pathways, including those of EGFR signaling, NF-κB signaling, and anti-apoptotic signals (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020). As mentioned in Chapter 1, inhibitors targeting these signaling pathways are used either in the clinic to treat cancer patients or in clinical trials for cancer therapy. Based on our findings, there is great potential for those inhibitors such as EGFR and NF-κB inhibitors to be developed for the targeted therapy of MGL-deficient lung adenocarcinoma. Our future studies will continue to test this hypothesis not only in lung cancer cells but also in our unique model of lung adenocarcinoma.
MGL-induced apoptosis in cancer

Since the beginning of our study of MGL, we noticed that MGL overexpression in cancer cells caused not only growth suppression but also cell death. Apoptosis is programmed cell death mediated by two major pathways including the extrinsic and intrinsic pathways (Elmore, 2007). Abnormal apoptosis underlie many diseases including some cancers (Elmore, 2007; Kerr, et al., 1994). Tumor cells have been found to express either anti-apoptotic proteins or lose the expression or normal function of pro-apoptotic proteins and thus become resistant to factors that induce apoptosis (Elmore, 2007). In our study, we determined that cell death induced by MGL was apoptosis by observing cell morphology and examining markers of apoptosis such as cleavage of PARP, caspase 8, caspase 9, and caspase 3. Since MGL levels are reduced in cancer cells or tissues as compared with non-transformed cells or normal tissues respectively (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020), it is possible that downregulation of MGL contributes to the evasion of apoptosis by cancer cells. Importantly, this downregulation of MGL appears to be a common mechanism by which cancer cells acquire resistance to apoptosis because MGL reduction is common in cancer tissues (Sun et al., 2013; Liu et al., 2018).

We further discovered that MGL inhibited the function of X-linked inhibitor of apoptosis protein (XIAP) by direct interaction and promoting its degradation by the proteasome (Liu et al., 2020). XIAP is a well-established negative regulatory protein
involved in the potent inhibition of apoptosis (Eckelman et al., 2006). In both cancer cells and cancer tissues, we found that there was a negative correlation between MGL and XIAP levels (Liu et al., 2020). Therefore, XIAP may be the major contributor of resistance to apoptosis in cancer cells that exhibit MGL deficiency and XIAP could be a target of cancer therapy for MGL-deficient cancers.

In addition, we demonstrated that N-terminus of MGL (MGL-1–105) mediated interaction with XIAP and was able to induce cell death as well as full-length MGL (FL-MGL). Furthermore, we constructed a even smaller N-terminus of MGL (MGL-1–60) and showed that it also induced cell death as potently as the full-length MGL (FL-MGL).

Previous studies have shown that two mitochondrial proteins, Smac (second mitochondrial-derived activator of caspases) and ARTS (Apoptosis Related protein in the TGF-β Signaling pathway), interact with and inhibits the function of XIAP and play a positive role in apoptosis (Abbas and Larisch, 2020). It is noteworthy that ARTS induces ubiquitination and degradation of XIAP (Garrison et al., 2011), which is similar to the effect of MGL on XIAP. Since induction of apoptosis is a major approach to the treatment of cancer, ARTS and Smac mimetics have been developed and tested for cancer therapy (Abbas and Larisch, 2020). For example, one of the Smac mimetics, LCL161, is under clinical trial for the treatment of lung adenocarcinoma in combination with PDR001 (checkpoint inhibitor targeting PD-1) (https://clinicaltrials.gov/ct2/results?cond=&term=LCL161&entry=&state=&city=&dist=)
Similarly based on our findings, MGL mimetics may also be developed as a cancer drug especially for the treatment of lung cancer.

**Inhibition of NF-κB signaling in cancer by MGL**

Through a large variety of tumor models, numerous studies have determined that inflammation plays an important role in tumorigenesis and cancer progression (Coussens and Werb, 2002; Greten and Grivennikov, 2019; Taniguchi and Karin, 2018). Here our study demonstrated that MGL deficiency in the lung led to tissue inflammation and formation of lung neoplasm, specifically lung adenocarcinoma. We further identified increase activity of NF-κB as the trigger of inflammation in the context of MGL deficiency.

Our initial incidental observation of lymphoid infiltration and lung adenocarcinoma formation in MGL-deficient lung tissues, and the well-established relationship between inflammation and cancer led us to hypothesize that MGL deficiency causes pro-tumor inflammation (PTI). We therefore performed more comprehensive examinations of the alterations in major inflammatory mediators upon changes in MGL expression in the current study (Chapter 3). Indeed, we found that MGL inhibited the expression of major inflammatory mediators such as COX-2, TNFα, and IL-1β to suppress inflammation. Our previous studies demonstrated that MGL is a tumor suppressor in the development of lung adenocarcinoma (Liu et al., 2018). Mechanistically, our data suggested that MGL
inhibited proliferative EGFR signaling and blocked the anti-apoptotic functions of XIAP in lung epithelial cells and finally resulted in lung cancer formation (Liu et al., 2018; Liu et al., 2020). Our new findings thus add to the complexity of the roles of MGL in lung tumorigenesis by implicating tumor microenvironment, specifically the inflammatory environment altered by MGL status in lung epithelium or cancer cells, in the development or progression of lung adenocarcinoma.

Consistently, another study showed that MGL deficiency promoted activation of tumor-associated macrophages (TAMs), and that TAMs subsequently suppressed the function of tumor-associated CD8+ T cells, thereby promoting tumor progression (Xiang et al., 2018). Furthermore, MGL deficiency in TAMs was induced by cancer cells (Xiang et al., 2019), implying the existence of interplay between cancer cells and macrophages. It is therefore possible that MGL deficiency in cancer cells not only promotes cell survival and growth by upregulating proliferative and anti-apoptotic signaling as shown by our previous studies (Liu et al., 2018; Liu et al., 2020), but also signals to tissue-resident blood cells involved in tumor inflammation and immunity to further promote tumor development, growth and progression. Since there is global knockout of MGL in our mouse model, it is likely that MGL depletion in lung epithelial cells initiated the dysplasia of the cells, and MGL deficiency in macrophages around the cells further supported the transformation of the precancerous cells into lung cancer cells. Further studies will be required to determine the degree of importance of the MGL
deficiency-induced inflammatory environment in tumorigenesis. It is also important to elucidate whether such an environment is a major determinant of lung tumorigenesis or it only facilitates the pathogenic process following epithelial cell transformation caused by enhanced and sustained intracellular oncogenic signaling upon MGL depletion.

In the quest of a link between MGL deficiency and multiple inflammatory mediators, we discovered the negative regulation of NF-κB activity by MGL, suggesting that enhanced NF-κB signaling resulted from MGL depletion is at least partially responsible for lung adenocarcinoma formation in our mouse model. Our findings are highly consistent with a previous study published in Nature in 2009 showing that NF-κB signaling is required in a mouse model of lung adenocarcinoma (Meylan et al., 2009) and therefore further underline the significance of NF-κB signaling in the development of lung adenocarcinoma. Moreover, we discovered a specific clinically-significant context in which NF-κB activity is enhanced. Such a context is MGL protein deficiency in lung tissues. Through patient tissue screening and comparison between lung tumors and matching normal lung tissues, we found a positive correlation between IκBα and MGL expression levels while there was a negative correlation between COX-2 and MGL expression levels (Chapter 3). Since IκBα is an endogenous inhibitor of NF-κB while COX-2 is an NF-κB target gene, our data strongly suggest a negative correlation between NF-κB activity and MGL expression levels in lung tissues or tumors. In addition, our previous studies showed that MGL expression is reduced in 65.5% of primary human...
l lung cancer cases (Liu et al., 2018). It is therefore likely that in at least 65.5% of primary human lung cancer cases NF-κB activity is elevated and NF-κB may serve as a major target for the treatment of lung cancer. Indeed, NF-κB inhibitors including bortezomib and Bay-117082 were tested in a previous study using a mouse model of lung adenocarcinoma [Kras(LSL-G12D/wt);p53(flox/flox) mice] and were shown to be efficacious although acquired resistance developed after repeated treatment. (Xue et al., 2011). Future work is needed to test NF-κB inhibitors in our mouse model of lung adenocarcinoma.

To pinpoint the node where MGL directly exerts its effects on the NF-κB pathway, we identified IκBα as a direct binding partner of MGL, and MGL expression reduces the phosphorylation and degradation of IκBα. We further found that the interaction between IκBα and MGL prevented IKKβ from binding and phosphorylating IκBα. Not only did we detect the interaction between IκBα and MGL in cancer cells but also in the in vitro purified protein system, which strongly suggest that such an interaction exists universally in vivo. Overall, we described the regulation of IκBα or NF-κB by MGL in multiple cell lines including A549, H1299, MDA231, 293T, and MEFs. Therefore, the regulation of NF-κB pathway by MGL may be involved in a variety of physiological and pathological processes in different cells or tissues. It is noteworthy that in one of our previous studies, we detected follicular lymphoid hyperplasia (FLH) and lymphoma in MGL-deficient mouse tissues (Liu et al., 2018). It is well-established that NF-κB signaling is critical for
the growth and survival of B cells (Balaji et al., 2018) and is involved in the pathogenesis of mantle cell lymphoma, diffuse large B-cell lymphoma, multiple myeloma, and Hodgkin lymphoma (Balaji et al., 2018; Staudt, 2010). It is therefore reasonable to hypothesize that MGL depletion in B cells leads to activation of NF-κB signaling and finally the formation of lymphoma. Moreover, a variety of mutations have been described as the triggers of NF-κB signaling in those lymphomas (Balaji et al., 2018; Staudt, 2010). To our best knowledge, none of the mutations is related to MGL. Future studies of the roles of MGL in the formation of lymphomas will probably open a new venue for the development of therapeutics for lymphomas.

It is noteworthy that our previous studies showed that MGL suppressed cell proliferation, growth and survival, which may be the consequence from the inhibitory effect of MGL on AKT signaling, ERK signaling, and the function of XIAP (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020). NF-κB signaling has been known to play a positive role in cell proliferation and survival (Bassères & Baldwin, 2006), therefore, enhanced NF-κB activity due to MGL deficiency may be another mechanism by which MGL suppresses cell proliferation and survival. Since uncontrolled cell proliferation and survival are common characteristics of cancer cells, these findings altogether suggest that MGL is a potent tumor suppressor, at least in lung tumorigenesis as has been shown by our studies.

In summary, our recent findings have demonstrated a novel regulatory network of
NF-κB signaling in which MGL interacts with IκBα, prevents its phosphorylation by IKKβ, and maintains its level and function to sequester NF-κB in the cytosol, finally leading to reduced NF-κB activity. Deregulation of this network due to MGL depletion may underlie the formation of human lung adenocarcinoma. Our study here therefore present new targets for the development of potential therapeutics for lung adenocarcinoma.

The possible dual roles of MGL in tumorigenesis

Although the function of MGL in the context of cancer has been investigated by a number of groups, the study of MGL is still at its early stage, especially when compared with the decades of study of the typical tumor suppressor, p53. Since our group discovered that MGL was ubiquitously expressed in a large variety of tissues/organs, MGL loss/reduction was observed in numerous types of human cancers, and MGL depletion led to tumor formation in multiple tissues/organs, the question still remains as to whether MGL acts as a tumor suppressor in all types of human malignancies or only in some of them.

Not surprisingly, using different tumor models such as ovarian cancer and melanoma, one group demonstrated that MGL played an oncogenic role via its lipase activity (Nomura et al., 2011). Human ovarian cancer cell lines, OVCAR3 and SKOV3, and melanoma cells, MUM2C and C8161, were the major cell lines used in Nomura’s study.
to support their conclusion (Nomura et al., 2011). These cell lines are different from the common cancer cell lines used in our laboratory such as lung, colorectal, and breast cancer cells. Therefore, it is possible that MGL have different effects on tumorigenesis or cancer progression in different cell lines, cancers, or tissues. This is not surprising as our mouse model of MGL-knockout did not show tumor formation in all tissues but in certain specific tissues such as those of the lung, liver, and spleen (Liu et al., 2018). It is noteworthy that the MGL-knockout mouse model that we generated has global knockout of MGL. However, the types of organs/tissues that developed tumors are limited. Tumors did not develop in all organs/tissues although there was MGL knockout, suggesting that the tumor-suppressive role of MGL may be tissue-specific. This is similar to the function of TGF-β in that it also plays cell-specific or tissue specific roles in tumorigenesis (Elliott and Blobe, 2005). In addition, the online database (http://firebrowse.org/viewGene.html & https://www.oncomine.org/resource/) using information collected from human normal and tumor samples showed that MGL levels are lower in lung adenocarcinoma as compared with normal tissues while MGL levels are higher in pancreatic adenocarcinoma tissues than that in the normal tissues. These also suggest that MGL’s effects on tumor formation are tissue-specific.

Also in our mouse model, MGL knockout led to tumor formation in not more than 71% of the mice (age 16-24 months) while in younger mice (age 10 -15 months) the percentage is even smaller, being not more than 53% (Liu et al., 2018). Clearly,
tumorigenesis was not detected in 100% of the mice with MGL knockout (MGL-KO/+ or KO/KO). This suggests that additional factors such as random mutations of other tumor-related genes may be required for full tumor development in the context of MGL knockout. If that is the case, the loss of MGL alone may not be sufficient to cause tumor formation without the emergence of other genetic alterations. MGL deficiency alone may not be able initiate tumor formation but facilitate the development and progression of tumors. It is therefore likely that the function of MGL in tumorigenesis may depend on other genetic mutations. On the contrary, it is also possible that in the context of a different set of genetic mutations that either initiate tumor formation or promotes cancer metastasis, increased MGL expression may play a positive role cancer formation or metastasis. Lastly, the pro-tumorigenic genetic changes may result in altered MGL expression to further promote cancer progression.

During our screening of human tumor tissues, we observed that a small percentage of tumors have higher levels of MGL as compared with matching normal tissues, implicating a potential tumor-promoting role of MGL under specific conditions. Our investigation of different cancer cell lines also demonstrated differences in MGL’s effects on cell growth and death. For example, contrary to our findings in most of the cancer cell lines we examined, MGL depletion in C8161 and SKOV3 suppressed cell growth (Liu et al., 2020). In the future investigation of the function of MGL, we will need to expand our study to different tissues and cancer cell lines, and examine the potential dual function of
MGL in tumorigenesis as other genes such as tumor necrosis factor (TNF) (Balkwill F, 2009) and transforming growth factor-β (TGF-β) (Syed, 2016; Elliott and Blobe, 2005).

Lastly, the function of MGL may differ at various stages of tumor development and progression. Our studies demonstrated that increased MGL expression inhibits AKT and ERK phosphorylation and reduces the levels of XIAP, by which MGL suppresses their activities in promoting tumor cell growth, proliferation, and survival (Sun et al., 2013; Liu et al., 2018; Liu et al., 2020). We further found that these functions of MGL are independent of its lipase activity (Liu et al., 2020). Therefore, it is likely that MGL deficiency facilitates tumor cell growth, survival, and proliferation and it works together with other tumor-promoting genetic changes to initiate tumor formation. After the tumor is well developed, MGL expression may be up-regulated to promote cancer metastasis via its lipase activity. It is possible that after the tumor is formed from normal tissues, MGL via its lipase activity may increase the production of pro-tumorigenic lipid metabolites, which then contribute to the development of more aggressive tumors as reported previously (Nomura et al., 2010). As for this function of MGL, its lipase activity appears to be important, since MGL inhibitor, JZL184, was shown to block the production of pro-tumorigenic lipid metabolites and suppress cancer cell migration and invasion (Nomura et al., 2010). Therefore, it is possible that the lipase activity of MGL plays a positive role in later-stage tumors while other functional domains of MGL are active in the earlier stages of tumor formation and they work to suppress cell growth and
proliferation to prevent the initial tumor development. As mentioned previously, N-terminus of MGL (MGL-1–60) induced cell death as potently as the full-length MGL (FL-MGL). Since MGL lipase catalytic triad is located outside of MGL-1-60 (Karlsson et al., 1997), MGL-1-60 may contain a non-lipase functional domain that inhibits XIAP. Future studies are needed to fully characterize those functional domains of MGL.

In summary, so far the studies of MGL’s role in tumorigenesis are still not comprehensive although they have shed light on the directions of further investigation. Accumulating evidences support the notion that MGL has dual function in tumor development and progression. However, the detailed molecular mechanisms for MGL’s dual role in tumorigenesis remain to be studied. The functional studies of MGL from our and other laboratories so far suggest that MGL’s biological activities may be different in different types of tissues or cells and may also be different at various stages of tumorigenesis. It also appears that MGL alone does not initiate tumor formation but a certain genetic background determines the consequences of MGL deficiency in tumorigenesis. As we know, differences in tissue origin, evolving tumor stages, and sporadic mutations all contribute to the distinct genetic backgrounds. Thus they may all determine the function of MGL. Although my entire study of MGL revealed the tumor-suppressive function of MGL in lung adenocarcinoma, it is also important for us to identify the genetic factors that determine the tumor-suppressive or potentially tumor-promoting function of MGL in other types of cancers in our future studies.
The lipase activity of MGL in tumorigenesis

As mentioned in the introduction, MGL is essentially a lipase. One intriguing question about the function of MGL in tumorigenesis is whether its lipase activity plays a major role. In fact, MGL inhibitors inhibiting its lipase activity were used in several studies to demonstrate the tumor-promoting effects of MGL (Nomura et al., 2011; Pagano et al., 2017; Yin et al., 2020). We therefore tested the commonly-used MGL inhibitor, JZL184, in multiple cancer cell lines at the concentration of 1 μM as was used in Nomura and Yin’s studies (Nomura et al., 2011; Yin et al., 2020) and found that JZL184 did not significantly reduce cell viability (Liu et al., 2020). At high concentration of 10 μM, however, JZL184 significantly reduced cell viability (Liu et al., 2020). It was reported that the IC50 for JZL 184 to block 2-Arachidonoylglycerol (2-AG) hydrolysis is 8 nM while the IC50 is 4 μM for blocking the hydrolysis of oleamide, a substrate of fatty acid amide hydrolase (FAAH) (Long, 2009). Thus JZL184 at 10 μM may not be specific in inhibiting MGL but other enzymes such as FAAH, by which it reduces cell viability. 1 μM of JZL184 is also a high concentration considering its IC50 of 8 nM in the blockade of 2-AG hydrolysis and may also have non-specific effects. In that case, studies using JZL184 at 1 μM to inhibit MGL may inhibit not only the lipase activity of MGL but also that of FAAH or possibly other enzymes. Therefore, the results from those studies are debatable pending further studies to confirm the specificity of MGL inhibitors. Interestingly, we generated a lipase-dead mutant MGL, MGL-S132A, and found that it
also induced cell death to a similar degree as wild-type MGL does (Liu et al., 2020). In addition, we have identified the interactions between MGL and XIAP, and showed that MGL induced cell death through the protein interaction (Liu et al., 2020). These findings strongly suggest that MGL contains other non-lipase functional domains that confer its effect on cell death. It is noteworthy that the aforementioned MGL inhibitor may not inhibit these functional domains and thus the experimental results obtained from using MGL inhibitors may not reveal the full functional profile of MGL. Overall, the lipase activity of MGL is dispensable for it to induce apoptosis; however, it remains to be studied as to whether the lipase activity of MGL is critical for tumorigenesis in certain types of tissues and future studies are needed to fully delineate the functional domains of MGL and determine their significance in tumorigenesis. It is likely that the functional domains of MGL become conditionally active or inactivated under different cellular conditions.

In summary, the new data presented in this dissertation combined with the data previously published by our laboratory all support a tumor-suppressive role of MGL and the findings are especially relevant to human lung cancer. Importantly, we further explored the molecular mechanisms by which MGL works as a tumor suppressor. We found that MGL appears to prevent tumor development through its inhibitory regulation of EGFR signaling, XIAP function, and NF-κB activity. Previous studies have shown that XIAP promotes EGFR protein translation and also induces NF-κB activation (Huang et
al., 2017; Lu et al., 2007). It will thus be interesting to explore whether MGL regulates EGFR and NF-κB via its interaction with XIAP. Finally, as discussed above, we cannot rule out the oncogenic activities of MGL under a cell or tissue-specific circumstance. Future studies are needed to clarify this.
References


Appendix

Publication:

Monoglyceride lipase gene knockout in mice leads to increased incidence of lung adenocarcinoma

Renyan Liu1, Xin Wang1, Christopher Curtiss2, Steve Landas2, Rong Rong3, M. Saeed Sheikh1 and Ying Huang1

Abstract
Monoglyceride lipase (MGL) is a recently discovered cancer-related protein. The role of MGL in tumorigenesis remains to be fully elucidated. We have previously shown that MGL expression was reduced or absent in multiple human malignancies, and overexpression of MGL inhibited cancer cell growth. Here, we have generated the MGL knockout mice to further investigate the role of MGL in tumorigenesis in vivo. Our results indicate that MGL-deficient (MGL+/−, MGL−/−) mice exhibited a higher incidence of neoplasia in multiple organs, including the lung, spleen, liver and lymphoid tissues. Interestingly, lung neoplasms were the most common neoplastic changes in the MGL-deficient mice. Importantly, MGL-deficient animals developed premalignant high-grade dysplasia and adenocarcinomas in their lungs. Investigation of the MGL expression status in lung cancer specimens from patients also revealed that MGL expression was significantly reduced in the majority of primary human lung cancers when compared to corresponding matched normal tissues. Furthermore, mouse embryonic fibroblasts (MEFs) from MGL-deficient animals showed characteristics of cellular transformation including increased cell proliferation, foci formation and anchorage-independent growth. Our results also indicate that MGL deficiency was associated with activation of EGFR and ERK. In addition, pro-inflammatory molecules COX-2 and TNF-α were also activated in the MGL-deficient lung tissues. Thus, our results provide new insights into the novel role of MGL as an important negative regulator of EGFR, COX-2 and TNF-α. Accordingly, EGFR and COX-2/TNF-α activation/induction is expected to play important roles in MGL deficiency-driven lung tumors. Collectively, our results implicate the tumor suppressive role of MGL in preventing tumor development in vivo, particularly in context to the lung cancer, and highlight its role as a potential tumor suppressor.

Introduction
Lung cancer is the leading cause of cancer mortality for both men and women worldwide1. From both histologic and therapeutic perspectives, lung carcinomas are traditionally divided into small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). NSCLC represents the majority (>85%) of lung carcinomas that is further subclassified as adenocarcinoma (50%), squamous cell carcinoma (~40%) and large cell carcinoma (~10%).2. Squamous cell carcinoma often arises in the proximal airways and is largely associated with smoking. Adenocarcinoma often arises in the peripheral lung, and while adenocarcinoma does occur in smokers, an increasing proportion of adenocarcinomas are diagnosed in non-smokers and women2,3.

Despite advances in chemotherapy, NSCLC remains difficult to cure because of poor understanding of the pathological mechanisms underlying this disease. Studies have demonstrated that epidermal growth factor receptor (EGFR) is important in lung cancer development. EGFR
gene mutation, amplification and overexpression have been found in a significant portion of NSCLCs. Increased EGFR activity is associated with frequent lymph node metastases, insensitivity to chemotherapy and poor survival . Activating mutations or overexpression of EGFR leads to activation of downstream oncogenic signals, i.e., the extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase/Akt pathways. Thus, EGFR has become an important therapeutic target for lung cancer treatment. The molecular mechanisms responsible for NSCLC remain to be fully elucidated. Accordingly, a better understanding of the molecular alterations underlying this disease is critical for accurate pathological diagnosis, and development of effective targeted therapies to improve patient survival.

Our previous study had suggested monoglyceride lipase (MGL) to be a potential tumor suppressor that could play an important role in tumorigenesis . We had shown that MGL messenger RNA (mRNA) expression was reduced or absent in multiple human malignancies. For example, 16 out of 21 (76%) lung cancer patients showed reduced or absent MGL mRNA levels in their tumor tissues compared to matching normal tissues . MGL mRNA reduction was also noted in cancers of the colon (59.4%), rectum (50%), stomach (61%) and breast (50%) . Expression of exogenous MGL in colon (HCT116) and lung (H1299) cancer cells, having inherently undetectable or low MGL levels, suppressed their growth . Thus, in vitro findings of our previous study indicated that MGL appeared to exhibit important growth regulatory functions of a potential tumor suppressor.

We undertook this study to further investigate the role of MGL in tumorigenesis in animals and to that end generated MGL knockout mice. Our results show that MGL-deficient (MGL+/−, MGL−/−) mice exhibit a significantly higher incidence of neoplasmia in multiple organs, especially in lung, spleen and liver. Our mechanistic studies demonstrate that MGL depletion activates several important oncogenic signals. Importantly, MGL appears to act as a suppressor of lung tumorigenesis that could also serve as a target for developing newer therapeutics to manage this malignancy.

**Results**

**MGL-deficient mice exhibit higher tumor incidence in multiple organs**

Using the germline MGL gene targeting approach, we generated the MGL-deficient mice lacking one MGL allele (MGL+/−) or both (MGL−/−) in all tissues. The strategy used for MGL gene targeting is outlined in Supplementary Figure 1A and Materials and methods. Mice of all MGL genotypes used in our study have a mix genetic background (Supplementary Figure 1A). Genotypes of all mice used in our studies were confirmed via DNA-PCR and/or protein analyses performed on tail, lung or embryonic fibroblasts. The representative results of the genomic DNA-PCR, shown in Supplementary Figure 1B, highlight the expected PCR products from the DNAs corresponding to the MGL+/−, MGL+/− and MGL−/− genotypes. Supplementary Figure 1C shows that MGL protein levels were absent in tissues from MGL−/− mice and lower in the tissues from MGL+/− mice compared to those in the wild-type animals. MGL protein expression was noted in all tissues of the wild-type mice (MGL+/+) (Supplementary Figure 1D).

MGL+/− and MGL−/− mice were viable, fertile and did not show significant differences in terms of overall appearance and movement compared to their wild-type littermates. Crossing MGL+/− mice produced MGL+/+, MGL−/− and MGL+/+ pups with expected Mendelian ratios. Initially, we did not observe visible tumors in animals under 6 months of age; accordingly, in the later experiments, older animals (>10 months) were analyzed. We defined a visible tumor-like nodule as a solid (not fluid-filled) mass, white or brown in color, protruding from the surrounding tissue. Tissues (lung, liver, kidney, intestine, spleen, skin, muscle and brain) from euthanized animals of all MGL genotypes at the comparable age were assessed blindly for tumor formation microscopically by at least two pathologists, regardless of whether tumor nodules were observed. As shown in Fig. 1a, between the ages of 10–15 months, none (0/10) of the MGL−/− mice had any grossly visible tumor, whereas 53% (9/17) (p < 0.05) of MGL+/− mice and 43% (6/14) (p < 0.05) of MGL−/− mice had visible tumor nodules in several different organs (Fig. 1a and Table 1). In the older mice (15 to 24 months), visible tumor nodules were seen in 57% (16/28) (p < 0.001) and 71% (15/21) (p < 0.001) of MGL+/− and MGL−/− mice, respectively, whereas only 3 out 20 (12%) wild-type (MGL+/+) animals had visible tumor nodules (Fig. 1a). These results indicate that MGL deficiency leads to significantly higher incidence of tumor formation in animals.

In addition to increased incidence of tumorigenesis, the MGL+/− and MGL−/− animals developed tumors in multiple organs (Table 1). Figure 1b, left panel, shows a normal (MGL+/+) mouse without any visible tumor and the right panel highlights an MGL−/− mouse with multiple tumor nodules in the liver, spleen and abdominal tissues. Figure 1c shows representative photographs of tumor nodules derived from lung, abdomen, liver and spleen of the MGL-deficient mice. Pathological examinations were also subsequently performed (Fig. 2). In general, normal MGL+/+ mice had spleens that measured 1.2–1.7 cm in length. On the other hand, MGL-deficient mice displayed significantly higher incidence of splenomegaly with spleens measuring 2–4 cm. For example, 10/45 (22%) MGL+/− and 10/35 (29%) MGL−/− mice
A. Neoplastic changes observed in mice

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<th>Age group:</th>
<th>10-15 months</th>
<th>16-24 months</th>
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<td>% Neoplastic changes observed in mice</td>
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<tr>
<td>0% (0/10)</td>
<td>53% (9/17)</td>
<td>57% (16/28)</td>
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<td>12% (3/20)</td>
<td>43% (6/14)</td>
<td>71% (15/21)</td>
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B. MGL +/+ MGL +/− MGL −/−

C. MGL +/− (#132), liver  MGL +/− (#154), liver  MGL −/− (#188), lung  MGL −/− (#215), lung

D. MGL +/+ MGL +/− MGL −/−

Fig. 1 (See legend on next page.)
displayed splenomegaly, whereas only 1/30 (3%) wild-type (MGL\(^{+/+}\)) mice had splenomegaly (Table 1 and Fig. 1d).

Initial evaluation of the animals exhibiting splenomegaly revealed that the common abnormality in these animals was follicular lymphoid hyperplasia. Although some animals with splenomegaly also had only hyperproliferative changes with no tumors, splenic sarcoma and lymphoma were also noted in some animals. The detailed pathological evaluations of the animals exhibiting splenomegaly are currently ongoing and the outcome would provide more information about the role of MGL in hematological changes.

Figure 2a shows the photographs of the tumors derived from different organs of several MGL-deficient mice with gross appearance (top panels) and corresponding histology (lower panels). Histopathological examinations confirmed these tumors to be splenic sarcoma (a-a\(^*\)), ameloblastoma (b-b\(^*\)), soft-tissue sarcoma (c-c\(^*\)) and hepatoma (d-d\(^*\)).

Lung neoplasia is the predominant abnormality in MGL-deficient mice
Interestingly, we found that MGL\(^{+/−}\) and MGL\(^{−/−}\) mice exhibited a significantly higher incidence of neoplastic changes in the lungs when compared to their wild-type littermates. For example, only 1/30 (3%) MGL\(^{+/+}\) mice exhibited lung tumor nodules, whereas 11/45 (24%) MGL\(^{+/−}\) and 9/35 (26%) MGL\(^{−/−}\) animals had lung tumor nodules (Table 1). Lung tissue sections from all euthanized animals with or without visible nodules were evaluated by two pathologists. Analyses of animals in the 10–24-month age group revealed that only 1 out of 26

<table>
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<th>Table 1</th>
<th>Nodules and splenomegaly observed grossly in all mice examined</th>
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<tr>
<td><strong>MGL status</strong></td>
<td><strong>No. of mice examined</strong></td>
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<td>KO/KO</td>
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\(^*\) Some animals exhibited tumor nodules in multiple organs. *\(P<0.001\)
(3.8%) wild-type (MGL+/-) animals had adenocarcinoma and none had dysplastic epithelial changes. By contrast, 7 out of 39 (17.9%) MGL+/- animals (p < 0.05) and 5 out of 31 (16.1%) MGL-/- animals (p < 0.07) in the same age group exhibited adenocarcinoma (Fig. 2b) or high-grade glandular dysplasia in the bronchiolar epithelium (data not shown). In the 16–24-month age group, the numbers of MGL-deficient animals with lung neoplasia were significantly higher. For example, 7 out of 24 (29.2%) MGL+/- animals developed lung neoplasia (2 high-grade glandular dysplasia and 5 adenocarcinoma) and 5 out of 14 (35.7%).

MGL-/- animals exhibited lung neoplasia (1 high-grade glandular dysplasia and 4 adenocarcinoma). By contrast, only 1 out of 17 (5.9%) wild-type (MGL+/+) animal was found to have lung lesion (adenocarcinoma) (Fig. 2c). Thus, the higher proportion of the older MGL-deficient animals exhibited lung neoplasia. Of all adenocarcinomas seen in MGL-deficient animals (5 from MGL+/- and 4 from MGL-/- animals), 4 were papillary predominant adenocarcinomas and 5 were solid predominant adenocarcinomas. Thus, our studies, using animals as a model, demonstrate for the first time that MGL deficiency is an important contributing factor in the development of lung adenocarcinomas.

We also investigated MGL status in tissues from lung cancer patients including their matching normal tissues. Figure 3a shows the representative results of MGL protein expression analyzed by western blotting and, as is shown, MGL expression was reduced (marked with *) in the majority of lung tumors compared to their matched normal lung tissues. Figure 3b summarizes the results of our two MGL expression studies performed on primary human tumors. The combined analysis of these studies indicates that MGL expression is reduced in 65.5% (38 out of 58) of the primary human lung cancer cases. Tumor histology for both studies is shown in Supplementary Tables 1 and 2. We also analyzed the Firehose/RSEM7 and Oncomine8 databases that curate data from a number of complementary DNA (cDNA) microarray studies, and our analyses revealed that indeed MGL mRNA reduction occurred in the majority of multiple cancers including lung cancers. Thus, findings from these databases confirm our results.

MGL-deficient MEFs show oncogenic transformation and increased cell proliferation

Figure 4a shows that MGL-deficient mouse embryonic fibroblasts (MEFs) exhibited significantly increased cell proliferation compared to MGL wild-type MEFs. Cell doubling time in MGL-deficient cells was significantly shortened compared to that in MGL-proficient cells (Fig. 4a). In addition, MGL+/- and MGL-/- MEFs, but not the MGL+/+ MEFs, exhibited phenotypic changes of oncogenic transformation. For example, the MGL-deficient MEFs exhibited spindle-like arrangements and formed large numbers of foci under regular culture conditions (Fig. 4b) and also on soft agar (anchorage-independent growth) (Fig. 4c). These results indicate that absence or diminished levels of MGL promote cellular transformation in MGL-deficient MEFs, and the presence of MGL inhibits such oncogenic transformation.

MGL deficiency activates EGFR-ERK pathway in mouse MEFs and lung tissues

We also investigated EGFR expression status in MGL-deficient cells and tissues. The EGFR expression and phosphorylation were elevated in MGL+/- and MGL-/- MEFs (Fig. 5a). Furthermore, EGFR levels were also significantly higher in the lung tissues (both tumor (T) and non-tumor (NT)) of MGL-deficient mice (Fig. 5b, lanes 2–5 and 7–10) compared to those in the MGL wild-type animals (lanes 1 and 6). Phosphorylation of ERK and Akt was also increased in the MGL-deficient (+/-) MEFs, while the levels of total ERK and Akt proteins were not similarly changed (Fig. 5c). Similarly, ERK phosphorylation was increased in lung tissues (T and NT) of MGL-deficient mice (Fig. 5d, lanes 2–5 compared with lane 1). Figure 5e shows ERK status from tissue lysates extracted from an additional group of animals; as is shown, compared to that of the wild-type littermate (lane 4), ERK phosphorylation was modestly increased in the lungs of the MGL+/- animal (top panel, lane 5) and strongly increased in the lungs of the MGL-/- animals (lane 6). Surprisingly, MGL deficiency did not lead to increased phospho-ERK levels in several other tissues in MGL-deficient animals (Fig. 5e and Supplementary Figure 2). These results suggest that MGL deficiency in mice affects ERK activity in a tissue-specific manner. In the case of lung tissues, ERK activation due to MGL deficiency likely promotes cell proliferation in animals.

Using human NSCLC cell lines, we further investigated MGL-mediated regulation of EGFR and ERK. MGL knockdown (KD) in A549 cells (cells with detectable MGL) increased EGFR protein and phosphorylation levels (Fig. 6a), and also ERK phosphorylation level (Fig. 6b). Conversely, overexpression of MGL in H1299 cells (with no detectable MGL) reduced EGFR protein and phosphorylation levels (Fig. 6c, d), and also levels of ERK phosphorylation (Fig. 6e). MGL KD in human colon (HT29) and breast (MDA-MB-231) cancer cells also led to enhanced ERK phosphorylation (Supplementary Fig. 3). Interestingly, MGL KD also significantly increased phosphorylated mammalian target of rapamycin (mTOR; Akt substrate) levels in A549 cells (Fig. 6f). Together, these results highlight the important role of MGL in regulation of EGFR/ERK and Akt signaling in lung cancer.
**A**

- MGL +/- (#162)
- MGL +/- (#14)
- MGL +/- (#150)
- MGL +/- (#98)

**B**

- MGL+/+ (#006)
- MGL+/+ (#105)
- MGL-/-(#89)
- MGL-/-(#92)

**C**

- % Lung neoplastic changes in aged mice (16-24 months)

- WT
- Ko/+ (5/14)
- Ko/ko

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**Fig. 2** (See legend on next page.)

Splenic histiocytic sarcoma

Ameloblastoma

Soft-tissue sarcoma

Hepatoma

Normal lung tissue

Lung adenocarcinoma

MGL deficiency increases the levels of inflammatory molecules COX-2 and TNF-α

Figure 7a shows that cyclooxygenase-2 (COX-2) expression was also elevated in 3 out of 4 NT and 4 out of 4 T lung tissues from MGL+/− and MGL−/− mice (lanes 2–5 and 8–10) compared to those of MGL wild-type (MGL+/+) mice (lanes 1 and 6). Strong COX-2 induction was also noted in MGL-deficient MEFs (Fig. 7b). Real-time reverse transcription PCR (RT-PCR) analyses revealed that COX-2 mRNA was also elevated in the MGL-deficient MEFs, especially in the MGL−/− MEFs (Fig. 7c). Conversely, re-introduction of MGL into the MGL-deficient MEFs (MGL+/−) resulted in reduced COX-2 levels (Fig. 7d). In A549 cells, MGL knockdown also led to increased COX-2 protein and mRNA levels (Fig. 7e, f). Levels of another pro-inflammatory molecule tumor necrosis factor-α (TNF-α) were also elevated in MGL-deficient lung tissues (Fig. 7g). Together, these results indicate that the activation of pro-inflammatory state in the lung tissues may be important for lung tumorigenesis in MGL-deficient animals.

Discussion

In this study, we demonstrate that MGL deficiency in mice leads to tumor development in several organs including lungs, lymphoid tissues, liver and soft tissues. Although tumors were noted in various organs, lungs were the most frequent site of neoplastic changes. In the older animals (16–24 months old), neoplastic changes in lungs were noted in 29.2% of MGL+/− and 35.7% of MGL−/− mice compared to only 5.9% in MGL+/+ animals (Fig. 2c). These novel findings indicate that MGL plays an important role in lung tumorigenesis in animals. Currently, the mechanisms underlying lung tumorigenesis are not fully understood, especially lung cancer development in non-smokers. Our results show that MGL-deficient (MGL+/− and MGL−/−) animals, without any exposure to carcinogenic/inflammatory substances, developed pre-malignant and malignant changes (adenocarcinoma) in their lung tissues. Abnormality in lung tissues due to MGL deficiency noted in animals is also recapitulated in humans. For example, our results demonstrate that insufficient MGL expression (either absent or decreased) is common (~65%) in human primary lung cancers compared to matched normal lung tissues (Fig. 3b). Searches in the Oncomine Cancer Microarray database (https://www.oncomine.org) reveal that in several studies of large-scale microarray analyses, MGL expression is found to be reduced in the majority of lung cancers compared to normal tissues. MGL reduction is not
restricted to one cell type but involved in many types of human lung cancers, i.e., squamous cell lung cancer, adenocarcinoma, small-cell and large-cell lung cancers (see ref. 8, and Supplementary Tables 1 and 2). Currently, it is not clear why the MGL-deficient animals develop adenocarcinomas but not the other types such as squamous cell carcinomas. It is possible that for squamous cell cancer or other types of lung cancer to develop in MGL-deficient animals, additional genetic changes and/or smoking-like environment may be required.

We also identified important molecular changes associated with MGL deficiency including increased expression and phosphorylation of EGFR (Figs. 5 and 6), and induction of COX-2 and TNF-α (Fig. 7). EGFR activation

Fig. 4 MGL-deficient MEFs display features of oncogenic transformation. a Left panel: MGL-deficient MEFs show increased cell proliferation and shortened cell doubling times. Equal numbers of MGL+/+, MGL+/− and MGL−/− MEFs (passage 15) (isolated from mice embryos of ~day 14 as described in Materials and methods) were cultured and counted at the indicated times. The presented data are based on experimental results collected from two independent experiments performed in triplicate. *P-value <0.1, **P-value <0.001. Right panel: MGL status of MEFs was confirmed by genomic DNA-PCR (upper panel) and western blot analyses (lower panel). b, c MGL-deficient (MGL+/− and MGL−/−) MEFs but not MGL-proficient (MGL+/+) MEFs form foci on cell culture plates (b) and exhibit anchorage-independent growth on soft agar (c)
Fig. 5 (See legend on next page.)

A. MEFs

- **MEFs**
  - MGL: +/+ +/− −/−
  - kDa: 130, 55

- **MEFs, EGF 5 min**
  - MGL: +/+ +/− −/−
  - kDa: 130, 55

Lane: 1 2 3

B. Mice group 1

- MGL: +/+ +/− −/− −/−
- Lung tissue: NT NT T NT T
- kDa: 130

- Fold difference: 1 1.5 1.4 1.7 1.8

- Ponceau staining

- Lanes: 1 2 3 4 5

C. MEFs

- MGL: +/+ +/− −/−
- kDa: 40, 40, 55

- **P-ERK**

- **ERK**

- **P-AKT**

- **AKT**

- **α-tubulin**

Lane: 1 2 3

D. Mouse lung tissues:

- MGL: +/+ +/− −/− −/−
- Animal #: 112 77 75 132 79 75 134 77 79 75 134 77 79 75

- kDa: 40

- **P-ERK**

- **ERK**

Lane: 1 2 3 4 5 6 7 8 9 10

E. Mouse tissues:

- MGL: +/+ +/− −/− −/−
- Animal #: 77 79 75 77 79 75 77 79 75 77 79 75

- kDa: 40

- **p-ERK**

- **ERK**

- **MGL**

- **β-actin**

Lane: 1 2 3 4 5 6 7 8 9 10 11 12
due to EGFR mutation is considered as one of the important oncogenic events in lung tumorigenesis. EGFR is reported to be overexpressed in 22–81% of NSCLC12–14; thus, EGFR overexpression has also been implicated in NSCLC pathogenesis5. Accordingly, EGFR is now an important target for lung cancer therapy24,25. Our present study has identified MGL to be a novel negative regulator of EGFR. For example, MGL depletion led to induction of EGFR in MGL-deficient MEFs and lung tissues as well as in MGL KD lung cancer cells (Figs. 5 and 6). Although the exact mechanisms via which MGL suppresses EGFR remain unclear, our preliminary results indicate that EGFR mRNA levels are elevated in MGL-deficient MEFS (data not shown). Thus, MGL appears to modulate EGFR expression, at least in part, at the mRNA levels. We also show that MGL negatively regulates both ERK and Akt signals (Figs. 5 and 6); both of these pathways are known to positively modulate EGFR expression. For example, EGFR promoter is known to be regulated by oncoproteins, such as AP1 (c-Jun/Fos) and β-catenin15–17 that are downstream targets of the ERK and Akt pathways18,19. It is therefore possible that MGL may modulate EGFR expression via its negative regulation of ERK and Akt pathways. It is also possible that MGL may modulate EGFR via a transcription-independent mechanism, since EGFR levels are also modulated by protein–protein interaction, endocytosis and protein turnover/degradation (reviewed in ref. 20). Further in-depth studies are needed to elucidate the exact mechanisms via which MGL regulates EGFR.

We also found that elevated COX-2 expression is one of the important molecular changes identified in MGL-deficient cells. COX-2 protein expression was significantly elevated in the lung tissues and the MEFS of the MGL-deficient animals as well as in the MGL KD human lung cancer cells (Fig. 7). COX-2 mRNA levels were also significantly increased in MGL-deficient MEFS and MGL KD lung cancer cells. These findings suggest that MGL regulation of COX-2 occurs, at least in part, at the mRNA level. However, we also note that the extent of changes in COX-2 mRNA and protein levels are not always congruent, thus raising the possibility of both transcriptional and post-transcriptional mechanisms. Future studies are expected to delineate these possibilities.

COX-2 is known to be a critical modulator of inflammatory response and its expression is commonly upregulated during inflammation; constitutive overexpression of COX-2 has also been implicated in a variety of human malignancies, including lung cancer21,22. Studies have shown that the use of COX-2 inhibitors for more than 1 year significantly reduced lung cancer risks23. Thus, elevation of COX-2 levels appears to be one of the important events in lung cancer development. Likewise, induction of COX-2 due to MGL insufficiency, as noted in our study, could also be one of the important events leading to lung cancer development in the MGL-deficient animals. In agreement with this notion, we found that the expression of pro-inflammatory cytokine TNF-α was also significantly elevated in MGL-deficient lung tissues (Fig. 7g). Inflammation is one of the common features underlying the pathogenesis of cigarette smoke-associated disease, including lung cancer. Conceivably, the prolonged and consistent induction of pro-inflammatory molecules such as COX-2 and TNF-α could lead to chronic inflammation and damage in the lung tissues and such condition, mimicking smoking, could favor tumor development in MGL-deficient lung tissues.

Based on our current findings, we propose a model depicting the MGL involvement in tumorigenesis (Fig. 7h). As is shown, in normal lung cells, MGL levels are in abundance; accordingly, MGL tends to reduce EGFR expression, suppress ERK and Akt/mTOR phosphorylation and keep cell proliferation/survival under control. In addition, MGL suppresses COX-2 and TNF-α and inhibits inflammatory responses. In MGL-deficient cells (i.e., in lung and other tissues), MGL deficiency would lead to higher activities of EGFR, ERK and Akt/mTOR, as well as induction of pro-inflammatory molecules COX-2 and TNF-α. Prolonged and cumulative effects of these overly active signaling molecules would eventually lead to tumor initiation/progression.
Fig. 6 MGL negatively regulates EGFR-ERK and Akt pathway in human lung cancer cells. a MGL knockdown increases the levels of total EGFR and phosphorylated-EGFR in A549 lung cancer cells. Two different MGL shRNAs targeting different regions of MGL mRNA were used. Cells were treated with EGF for 5 min before harvesting for western blot analysis. b MGL knockdown increases the levels of phosphorylated-ERK (p-ERK) in A549 cells. c Overexpression of MGL reduces EGFR levels in H1299 human lung cancer cells. Lane 1: vector alone; lanes 2 and 3: cells transfected with 1 and 3 µg of MGL expression vector, respectively. d Overexpression of MGL in H1299 cells negatively regulates the EGFR phosphorylation (p-EGFR) and EGFR protein levels. Vec vector transfected, MGL MGL transfected. Cells were treated with or without EGF (10 ng/ml) for the indicated times. e ERK phosphorylation is also inhibited in the H1299 cells overexpressing MGL. Cells were treated with or without EGF (10 ng/ml) for the indicated times. f MGL knockdown leads to increased mTOR phosphorylation (p-mTOR) in A549 human lung cancer cells. Two different MGL shRNAs targeting different regions of MGL mRNA were used.
There are different views about MGL role in tumorigenesis; for example, a report by Nomura et al.\(^{24}\) suggested MGL to be an oncogenic protein involved in tumor metastasis due to its lipase activity. Interestingly, according to the Firehose/RSEM and Oncomine public databases, MGL expression is significantly lower in the lung tumor tissues, but higher in some kidney cancers.\(^{7,8}\) Therefore, it is possible that MGL may have dual roles as a tumor suppressor in lung cancer.
suppress or oncogene, depending on the tissue types. Several lines of published evidence indicate that certain proteins, such as Notch, Spleen Tyrosine Kinase, Sirtuins, WT-1 and transforming growth factor-β, exhibit both tumor suppressive and oncogenic functions that depend on cell/tissue types or developmental stages of tumors. In our MGL knockout (MGL−/−) animals, MGL lipase activity is expected to be completely abolished due to the gene deletion; however, 60% (21 out of 35) of MGL−/− mice developed tumors in various tissues (Table 1). Interestingly, a recent study by Rajasekaran et al. showed that MGL expression was undetectable in primary human hepatocellular carcinoma (HCC) samples (Oncomine database also shows similar findings) and overexpression of MGL inhibited HCC cell growth in vitro and in xenografted tumors. These findings are consistent with the tumor suppressor role for MGL, and in line with our previous study and current findings indicating that MGL-deficient mice also developed liver tumors.

In summary, using the MGL-deficient animal model, we report for the first time that MGL functions as a tumor suppressor particularly in context to lung cancer. Based on our results, we propose that absent or reduced MGL expression is one of the important molecular alterations underlying human lung cancer development. Our results also suggest that the MGL-deficient animals are a unique mouse model that can be used, in the future, to develop newer targeted therapies for human malignancies particularly lung adenocarcinomas.

Materials and methods

Animal studies

MGL knockout mice were generated commercially by the Gene Targeting and Transgenic Facility (GTTF) at the University of Connecticut Health Center (Farmington, CT). The details of MGL gene targeting strategy are outlined in Supplementary Figure 1. Studies involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the State University of New York (SUNY) Upstate Medical University. Male and female C57BL/6 mice of mixed background were used in these studies. For histological studies, the tissues were fixed in 4% paraformaldehyde and hematoxylin and eosin (H&E) stained as previously described and reviewed by two or three pathologists (SL, CC and RR). For genotyping of mice and MEFs, genomic DNAs extracted from tails of mice (<20 days) or MEFs were subjected to PCR reactions using the following primers: Lox gtF 5′-AGCTGAGGTCCATGCTTAG-3′; FrtgtR 5′-CTAAGAGTGTATTACAGCAC-3′; FrtgtF: 5′-AGCTGAGGTCCATGCTTAG-3′; FrtgtR 5′-AGCTGAGGTCCATGCTTAG-3′. The PCR products of 354 and 540 bp fragments indicated the presence of the MGL wild-type and MGL knockout alleles, respectively. For tissue studies, animal organs were collected from the euthanized animals and protein analyses were performed as described previously.

Antibodies, expression vectors and reagents

The phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-AKT (Ser473), Akt, phospho-EGFR (Tyr1068) phosphor-mTOR (Ser2248) antibodies were from Cell Signaling (Danvers, MA); EGFR antibody was from Abcam (Cambridge, MA). MGL antibody was generated in our laboratory as previously described and reviewed by two or three pathologists (SL, MO). GAPDH, vinculin, TNF-α and COX-2 antibodies were from Santa Cruz Biotechnologies (Dallas, TX). pDsRedN1-MGL vector (MGL-RFP) has been previously described. For pSRα-HA-S-tagged MGL expression vector, complete human MGL ORF was subcloned into mammalian pSRα-HA-S vector (contains HA and S tags) and DNA sequencing was performed to confirm the integrity of MGL sequence.
Cell culture and cell doubling time assay

MEFs were prepared from mice embryos between days 12.5 and 14.5 of gestation according to the procedures described\(^3\). MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro/Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Gemeni Bioproducts Inc., Calabasas, CA). Human cancer cell lines A549 (NSCLC), H1299 (NSCLC) and HeLa (cervical cancer) were obtained from the National Institutes of Health (NIH) and regularly maintained in DMEM supplemented with 10% FBS. To perform cell doubling time assay, MEFs of all MGL genotypes were seeded at equal numbers, harvested at the indicated times (up to 5 days) and cell numbers were then counted.

Soft agar assay

The assays were performed in 6-well plates using the low-melt agarose (Bio-Rad, Hercules, CA). The bottom layer was 0.8% agar diluted in DMEM with 20% FBS. For the top layer, cells were first suspended in 0.48% agar in DMEM with 20% FBS and then plated on top of the bottom layer. Additional 1 ml of DMEM was added to each well. Cells were incubated in humidified 5% CO\(_2\) incubator at 37°C for 2–3 weeks.

Lentivirus-mediated shRNA silencing

MGL knockdown was achieved by the lentivirus-mediated small hairpin RNA (shRNA) expression as we previously described\(^6\). Three different nucleotide sequences targeting the human MGL were as follows: shRNA1 5′-ccaggacaagactctcaagat-3′; shRNA2 5′-caacctgcttcagtaaat-3′; and shRNA3 5′-caaacctctgaatctgcaacaa-3′. The scramble shRNA construct was commercially obtained from Addgene, Inc. (Cambridge, MA). Lentivirus preparation, expansion and infection were performed per protocol provided by Addgene.

Western blot

Western blot (WB) analyses were performed as previously described\(^6\). Densitometric analyses were performed for all WB membranes stained with S-Ponceau using total amount of stained protein in each lane. The levels of a specific protein (i.e., EGFR, COX-2 and MGL) are normalized with respect to corresponding S-Ponceau levels. The fold differences are given under the relevant WB images.

Quantitative RT-PCR for analyzing mRNA expression

Quantitative real-time PCR (qRT-PCR) assays were performed as previously described\(^3\) using the iScript cDNA synthesis kit and iQ SYBR Green super-mix from BIO-RAD (Hercules, CA). C(T) values for COX-2 were normalized to the C(T) values of GAPDH mRNA in the same sample from either human or mouse cells. The detailed information about primers used for qRT-PCR studies is listed in Supplementary Fig. 4.

Statistical analysis

Results are expressed as mean±s.e. Student’s t-test and one-way analysis of variance were used for statistical analysis in continuous data. Logistic regression and z-test were used for statistical analysis in categorical data. Statistical powers were calculated by general linear model in SPSS.

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Supplementary information

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