HHS Public Access

Author manuscript

J Cancer Res Forecast. Author manuscript; available in PMC 2019 February 19.

Published in final edited form as:

J Cancer Res Forecast. 2018 ; 1(2): .

Cell Specific Matrix Metalloproteinase-1 Regulates Lung Metastasis Synergistically with Smoke Exposure

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Abstract

MMP1, a matrix metalloproteinase that degrades the extracellular matrix, is produced not only by cancer cells but also synthesized in stromal and inflammatory cells during tumorigenesis, invasion and lung metastasis. However, the function of MMP1 expression from host cells, especially tumor-associated macrophages (TAMs), and cells in the lung parenchyma remains to be elucidated. Here we demonstrate that *in vitro* macrophages co-cultured with tumor cells drastically enhance MMP1 expression, which is further exacerbated upon cigarette smoke exposure. In addition, *in vivo*, macrophage specific MMP1 was found to have a causative role in primary tumor development and lung metastasis, which was enhanced under smoke exposure as demonstrated in a transgenic mouse model that expressed human MMP1 specifically in macrophages (Mac-MMP1). In contrast, MMP1 from lung cells (Lung-MMP1) reduced colonization to the lung despite the fact that collagen deposition decreased in the Lung-MMP1 mouse tumors. These results demonstrate that the varying cellular source of MMP1 in tumors leads to the complexity observed in the tumor microenvironment. Furthermore, macrophage-specific inhibition of MMP1 secretion may be a potential therapy to aid in the reduction of lung metastasis.

Keywords

Metalloproteinase; Macrophages; Metastasis; MMP1

Introduction

Metastatic tumor spread to secondary anatomic sites facilitated by invasion of cancerous cells into the circulatory system is typically the primary cause of mortality in solid tumor disease [1,2]. This process beings with destruction of the local tissue architecture by the

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primary tumor and is subsequently followed by invasion of tumor cells into the surrounding vasculature [2,3]. These cancerous cells are then free to establish secondary tumors throughout the body. Tumor induced extracellular matrix (ECM) destruction is critical to the development of a primary tumor and its metastatic progeny [4,5]. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptide proteases, are prominently associated with ECM destruction in tumorigenesis and cancer progression [5–7]. Of these, MMP-1 is a member of the lung metastatic gene signature (LMS) and is secreted from cancer cells. Notably, evidence also supports MMP secretion from tumor associated fibroblasts, myofibroblasts, inflammatory cells and endothelial cells [8]. Interestingly, MMP-1 derived from fibroblasts cleaves protease-activated receptor1 (PAR1) resulting in PAR1 dependent migration, this same activity is not observed in tumor derived MMP-1 [9]. These data suggest stromal-derived MMP-1 mediates tumor invasion via mechanisms independent of its role in ECM destruction. Nevertheless, the precise role for MMP1 in tumor metastasis has not been adequately defined and the specific interactions between various cell types expressing MMP1 in the tumor microenvironment needs further characterization.

Tumor-associated macrophages (TAMs) constitute the majority of tumor-infiltrating immune cells [10,11] with multiple studies suggesting their presence is a poor prognostic indicator for cancer progression [12,13]. Macrophages are thought to display different functional phenotypes dependent on their response to the microenvironment [14]. Mantovani et al. demonstrated two distinct phenotypes characterized by differential expression of macrophage surface markers in response to specific chemical stimuli; M1cells resulted from treatment with IFN-γ combined with LPS or TNF while M2 cells where created following exposure to IL-4, IL-10, and glucocorticoids [15]. TAMs exhibit an M2 phenotype and promote tumor invasion through high scavenging ability, promotion of angiogenesis, and matrix remodeling [16]. The TAMs express antigens specific for macrophages including high levels of scavenger receptor-A when compared to macrophages with the M1 phenotype [14,17] and once activated the M2 macrophages interact with cancer cells further up-regulating scavenger receptor-A expression [18].

Cigarette smoke stimulates the recruitment of inflammatory cells, cell death, and protease production within lung tissue [19]. Recent evidence demonstrates that cigarette smoke induces an M2 phenotype in macrophages via the JAK2/STAT3 pathway [20]. MMP-1 is detected in alveolar macrophages and lung epithelial cells, especially type II pneumocytes, in patients with emphysema [21]. Furthermore, increased serum levels of MMP-1 are observed in smokers [22] and studies have shown that patients with COPD have an increased risk of cancer [23]. Previously, our group reported that cigarette smoke induces MMP-1 expression by signaling through the TLR4 receptor [24] and promotes phosphorylation of ERK in lung epithelial cells and alveolar macrophages in humans and animal smoke exposure models [25]. Little is known regarding the mechanisms of cigarette smoke induced MMP-1 expression in primary tumor cells or the lung during cancer metastasis, although studies in humans have shown that cigarette smoke increases the risk of lung metastasis in esophageal cancer patients [26].

In the present study, we examined the effect of MMP-1 overexpressing TAMs on tumor progression and metastasis compared to tissue derived MMP-1 expression. To achieve these

aims, transgenic mice expressing human MMP-1 in macrophages using a scavenger-enhancer receptor A promoter [27] were compared to lung cell-derived MMP-1 expression in various pre-metastatic and metastatic tumor models. These studies provide novel insight into the relationship between MMP-1, lung metastasis and cigarette smoke.

Materials and Methods

Cell culture

The Lewis lung carcinoma cell line (LLC) (ATCC, Manassas, VA) was cultured at 37°C in Dulbecco's minimal essential medium (DMEM, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) containing penicillin (10,000IU/ mL) and streptomycin (10,000μg/mL). Monocytes were isolated by centrifugation of donated peripheral human blood with Ficoll. Cells were then placed into culture in RPMI containing 10% human serum and macrophage colony-stimulating factor (M-CSF, 100ng/mL) for 1 week. The M2 phenotype was then induced by overnight exposure to Interleukin-4 (20ng/mL IL-4). For co-culture studies, 2×10^5 of these M2 macrophages were plated on a 6-well plate with RPMI media containing 10% human AB serum. Adenocarcinoma human alveolar epithelial cells (A549) or human Caucasian esophageal carcinoma (OE33) cells were grown on an upper trans-well insert with the same medium. The upper and lower well were separated by a 0.4μm pore size of polycarbonate membrane filter (Millipore, Bedford, MA). Cells were co-cultured for 48 hours, and then harvested and medium collected.

Micro invasion assay

Invasiveness of the cancer cells was assessed by the cancer migration rate of A549 and OE33 cells through an artificial basement membrane in a modified Boyden chamber. The membrane was made using Matrigel (ECM gel) diluted 1:3 in serum-free RPMI with polycarbonate (10μm pore diameter, Nucleopore, Pleasanton, CA). A549 and OE33 (1×10^6/ml) were seeded into the upper well of chamber, while the lower well was filled with human fibroblast conditioned medium. Ro32–3555 (TOCRIS bioscience, Ellisville, MO) was used as a MMP1 inhibitor at a concentration of 10nM. 5% CSE was also added to examine the effect of smoking. Human macrophages were isolated using a Ficoll-Hypaque density gradient and cultured with human M-CSF 100ng/ml (PeproTech, Inc. Rocky Hill, NJ) for 3 days. For co-culture experiments 2×10^5 macrophages/ ml were seeded in trans well inserts (Nunc, Wiesbaden, Germany). After 48 hours, cells in the lower chamber were detached and centrifuged. These cells were stained by DAPI (200ng/ml) on the slide and assessed the number by fluorescent microscope (Nikon Eclipse Ti, Melville, NY).

Quantitative real-time reverse transcription – PCR analysis

Messenger RNA (mRNA) was extracted and purified from cells using RNAse Mini Kit following the manufactures instructions (Qiagen, Hilden, Germany). The mRNA samples were further treated with DNase for 30 minutes (Promega, Madison, WI) to degrade genomic DNA contamination. cDNA was synthesized using 200ng of mRNA in 50uL reaction buffer by reverse transcription using the high-capacity cDNA transcription kit (Applied Biosystems, Foster City, CA) and random hexamer primers. The thermal cycling

conditions were 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 seconds. The products of cDNA were stored at −20 degrees C for future experimentation.

MMP-1 transgenic mice and smoke exposure protocol

The macrophages-specific and Lung cell-derived MMP1 transgenic mouse models were generated as described [4,27]. All of the studies and procedures were approved by the Columbia University Institutional Animal Care and Use Committee. Mice were exposed to cigarette smoke in a specially designed chamber for 6 hours/day 5 days/week for 3 weeks at a total particulate matter concentration of 100mg/m^3. Groups of 10 mice were used for these studies. A full description of the smoke exposure protocol has been described previously [28].

Skin Lewis Lung Cancer cells implantation and metastatic assay *in vivo*

Cultured LLC cells stably expressing GFP were harvested with 0.05mM Trypsin-EDTA in DMEM medium, washed three times with PBS, and re-suspended in PBS. The primary skin tumor was implanted by subcutaneously injecting 5×10^5 LLC cells stably expressing GFP in 200μl of PBS into the right lateral abdomen of the mouse. A cotton-tipped applicator was pressed onto the site of injection as the needle was withdrawn to stop any bleeding. For the metastasis assay, 2×10^5 LLC cells were intravenously injected 7 days after the skin LLC implantation. The size of the skin tumors was measured every 3 days in all injected mice. Tumor volume was calculated by the following formula: tumor volume (mm^3) = 1/2 x (long diameter) x (short diameter) [6,29].

Tissue isolation, measurement of the number of tumors and microscopic size evaluation

At the indicated time point, mice were sacrificed and organs (primary skin tumor, the lungs, and the liver) collected. Lung and liver were examined for metastatic nodules macroscopically. For each mouse, the left lung was fixed in formalin. The entire formalin-fixed left lungs were embedded in paraffin and sectioned at 6 micrometers. Five slides were randomly selected from the sections at each 200 micrometer interval for microscopic examination. Sections were dewaxed, hydrated through graded alcohols, stained with hematoxylin and eosin (H.E.), dehydrated, and mounted with cover slips. Two observers then independently evaluated the slides for counting tumor clusters and nodules using a digital camera. The analysis was performed blinded of all the mouse groups. The images of the tumors were captured and the cumulative tumor area (11) in each lung were calculated by the Pro-image-6 software.

Immunohistochemistry study

Formalin-fixed, paraffin-embedded sections were de-waxed, hydrated through decreasing grades of alcohol, and treated with 0.6% hydrogen peroxide in methanol (to destroy endogenous peroxidases). Sections were then incubated at high temperatures for 30 minutes for antigen retrieval and incubated overnight in blocking solution with horse anti-human MMP1 antibody (1:200 R&D Systems, Minneapolis, MN) at 4 degrees C. Sections were washed with TBST (150mmol/L NaCl, 10mmol/L Tris, and 0.05% Tween 20) and incubated with biotinylated anti-horse IgG (Vector Laboratories, Burlingame, CA) for 1 hour. Labeled

cells were visualized using an avidin-biotin peroxidase complex (Vectastain Avidin-Biotin Complex kit, Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO). Sections were counterstained with hematoxylin. Positive human MMP1 staining within lungs and tumors were assessed. Masson Trichrome Staining was performed using their manufacture's protocol (Thermo Scientific, Kalamazoo, MI).

Western blots

Freshly dissected mouse lungs were homogenized in protein lysis buffer (PBS containing Triton X-100 0.1%), and centrifuged. Forty μg of supernatant was separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Goat polyclonal antibody against MMP1 (1:1000) was detected with enhanced chemiluminescence reagents (Thermo, Rockford, IL).

Active MMP1 fluorescent assay

MMP1 activity was detected in lung tissues of MMP1 transgenic mice using the Human Active MMP1 Fluorescent Assay kit (R&D Systems, Minneapolis, MN). For MMP1 activity analyses, lung tissues were put onto a pre-coated microplate in which MMP1 was bound by the immobilized antibody. After washing away any unbound substances, a fluorogenic substrate linked to a quencher molecule was added and any active enzyme present cleaved the peptide linker between the fluorophore and the quencher molecule. The fluorescent intensity was analyzed by the Spectra Max M2 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

All measurements were depicted as mean + standard deviation. The statistical analysis of the data was performed using two-tailed t-test with Prism software. In all cases, p-values less than 0.05 were considered significant. All experiments were repeated to verify accuracy. Representative experiments are shown in the figures.

Results

Expression of MMP-1 in human macrophages co-cultured with A549 cells exposed to cigarette smoke extract

In order to determine whether cigarette smoke exposure contributes to the induction of MMP-1 in TAMs, cigarette smoke extract (CSE) exposed A549 cells were co-cultured with peripheral blood derived human macrophages that were induced to an M2 phenotype via M-CSF and IL-4 exposure [14]. Macrophage MMP-1 expression levels increased significantly after 48 hours of co-culture with the most striking increase observed in CSE exposed M2 macrophages cultured with A549 cells. MMP-1 expression increased 40-fold in comparison to controls with significant increases in expression also observed in comparison to M2 macrophages and the A549 co-cultured cells without CSE (*P=0.0149*) (Figure 1A).

Interestingly, the increase was MMP-1 specific since other collagenases, such as MMP-8 and MMP-13, were not increased in the A549 co-culture with CSE (Figure 1B). Taken together, these data demonstrate CSE and cancer cells are sufficient to independently induce

an increase in macrophage MMP-1 mRNA expression but, in combination, act in a synergistic manner to cause an exponential and specific increase in MMP-1 expression.

Inhibition of tissue inhibitor of matrix metalloproteinase in human macrophages by cigarette smoke extract

The tissue inhibitor of matrix metalloproteinases (TIMPs) were examined to determine the effect of CSE in human macrophages co-cultured with A549 cells under the same conditions as described above. Interestingly, exposure of M2 macrophages to A549 cells increased TIMP-1, TIMP-2 and TIMP-3 mRNA expression while CSE substantially inhibited their induction. Notably, this effect was not observed in M1 macrophages (Figure 1C). These data suggest CSE downregulates TIMP expression potentially resulting in increased MMP-1 activity in tumor exposed M2 macrophages.

Cancerous cells enhance macrophage invasiveness via MMP-1

To evaluate whether cancer cells influence macrophage invasiveness via an MMP-1 related mechanism, micro invasion assays were performed on macrophages derived from peripheral blood mononuclear cells co-cultured with the invasive epithelial tumor cell lines A549 or OE33 (human esophageal adenocarcinoma cells) [30]. After 48 hours, macrophage invasiveness increased significantly (*P<0.05) in the co-culture system as compared to controls. This was further enhanced by treatment with CSE ($^{\#}$P<0.001) (Figure 1D). Notably, attenuation of macrophage invasiveness was observed with addition of the MMP-1 inhibitor Ro32–3555, consistent with the notion that MMP-1 mediates this process (Figure 2D).

Macrophage derived MMP-1 and smoke enhance primary tumor growth and macrophage invasiveness

Lewis Lung Carcinoma (LLC) cells were implanted subcutaneously into the left lateral abdomen of both wild type and macrophage specific MMP-1 transgenic mice (Mac-MMP-1) [27] to examine the role of MMP-1 expressing TAMs in primary tumor development. At 21 days post-implantation, average tumor volume in Mac-MMP1 was significantly larger as compared to wild type controls (1200mm^3 vs 1000mm^3, respectively, Figure 2A). Tumor volume was further increased in Mac-MMP-1 mice by smoke exposure as compared to their respectively controls (*P<0.05) (Figure 2A). Notably, macrophages in Mac-MMP-1 mice localized within the primary tumor stroma in smoke exposed mice (Figure 2B). Taken together, these data support the *in vitro* data and suggest that cancer cells and smoke exposure act synergistically to enhance primary tumor growth and macrophage invasiveness via an MMP-1 dependent mechanism.

Smoke exposure increases metastatic lesion associated MMP-1 expression in mac-mmp-1 mice that are characterized by increased tumor area and lung nodules *in vivo*

To determine the effects of smoke exposure on lung MMP-1 protein levels *in vivo*, western blot analysis was performed. Consistent with our hypothesis, MMP-1 levels in Mac-MMP-1 mice increased as compared to room air controls (Figure S2?). The increased levels of MMP-1 in Mac-MMP-1 mice led to increased growth of the primary LLC tumors *in vivo* as

well as increased macrophage invasiveness both *in vitro* and *in vivo*. Given these data, we next assessed if increased MMP-1 expression in macrophages from Mac-MMP-1 mice influenced metastatic spread via LLC tail vein injections 7-days post subcutaneous LLC implantation (Figure 3A).

Metastatic lung nodules were rarely detected in smoked exposed wild type mice (Figure 3) however, they were easily identified in the Mac-MMP-1 smoke exposed transgenic mice macroscopically 30 days following skin implantation (Figure 3B). As expected since there is no endogenous MMP-1 in wild-type mice [31–33], human MMP-1 was only detected in macrophages obtained from the lungs of the Mac-MMP-1 mice that had developed lung nodules (Figure 3C). Consistent with our previous data, MMP-1 mRNA expression from smoke exposed lungs displaying metastatic lesions was significantly higher than that observed in room air controls (Figure 3C). Furthermore, the lungs of smoke exposed Mac-MMP-1 mice were characterized by significantly greater tumor burden as assessed by total number of tumor nodules/clusters and tumor area as compared to room air controls and smoke exposed wild type mice (*P=0.029,*P=0.018, respectively, Figure 3D).

Next the localization of MMP-1 expression was examined via immunohistochemistry. As expected, MMP-1 was not visualized in the lungs of wild type mice (Figure 3E). Interestingly, MMP-1 was mainly expressed at the edge or surrounding metastatic tumors in the Mac-MMP-1 transgenic mice, this was most prominent in large tumors (Figure 3E). Consistent with the notion that MMP-1 was associated with metastatic spread it was detected only in the proximity of these lesions and was primarily expressed around the terminal bronchiole and pulmonary vessels transgenic mice without smoke exposure (Figure 3F). Lesions from smoke exposed Mac-MMP-1 transgenic mice were also characterized by the presence of MMP-1 expression however, additional MMP-1 was detected only at the periphery of the lung (Figure 3F). These data demonstrate that smoking induces an additional macrophage inflammatory response that exacerbates the up-regulation of MMP1 in TAMs, hence increasing the susceptibility to further metastasis.

Lewis lung cancer (LLC) cells stably expressing GFP were implanted subcutaneously in the mice to induce primary tumors and increase circulatory TAMs in the pre-metastatic phase (Figure 3A). One week later, LLC cells were injected into the tail vein to examine the lung localization or metastasis in tumor-bearing mice (Figure 3A). Most interestingly, in these studies GFP was detected by western blotting in the lungs of forty percent of the smoke exposed Mac-MMP-1 mice, but not in the wild type and non-smoke Mac-MMP-1 mice (Figure 4A, 4B). In addition, GFP positive cells were detected as a mosaic pattern in the metastatic lesions of Mac-MMP-1 mice by immunohistochemistry (Figure 4C). These results suggest that MMP-1 derived from macrophages and the stimulus of smoking synergistically induced lung localization of the cancer cells within a 1–3 week time frame. Altogether, these data are in agreement with the cell culture experiments demonstrating both smoke exposure and macrophage MMP-1 expression are important contributors to tumor size and influence metastatic spread within the lung.

TAM specific expression of active MMP-1 is required for enhanced development of lung tumors and metastasis in smoke exposed animals

Macrophage specific MMP-1 expression in our transgenic mice significantly influenced development of pulmonary tumors following smoke exposure. In order to ascertain if these effects were mediated by TAM derived MMP-1, tumor development in Mac-MMP-1 mice was compared to transgenic mice in which MMP-1 expression was restricted to the lungs [4]. As previously described, LLC cells were injected into the tail vein and lungs were examined after 30 days (Figure 5A). Interestingly, MMP-1 mRNA expression in the lungs of smoke exposed Lung-MMP-1 mice was not increased as compared to room air controls, suggesting TAM's are primarily responsible for the observed increase of MMP-1 in the smoke exposed lung. This is expected as the haptoglobin promoter driving the MMP-1 transgenic is not regulated by smoke exposure [4]. Fewer tumors and decreased total tumor area were also noted in the smoke exposedLung-MMP1 transgenic group as compared to wild-type mice (Figure 5C, left).

Since MMP1 is secreted as a zymogen, it is essential to determine the activation state *in vivo* in addition to mRNA expression levels. Consistent with the observed mRNA levels, activity assays revealed MMP-1 was activated in the smoke exposed as compared to non-smoke exposedMac-MMP-1 transgenic mice (*P=0.0005*) (Figure 6A), similar results were observed in Lung-MMP-1 transgenic mice (Figure 6B). Furthermore, mRNA transcripts of the MMP-1 inhibitors *Timp2* and *Timp3* were decreased in both smoke exposed Mac-MMP-1 and Lung-MMP-1 mice as compared to room air controls, consistent with our *in vitro* data. Masson Trichrome Stain (specific for collagen) revealed a diffuse decrease in collagen deposition, including within metastatic tumors, in Lung-MMP-1 mice that was exacerbated by smoke exposure. Notably, decreased collagen deposition was not observed in metastatic lung tumors of Mac-MMP-1 mice even with smoke exposure however, it was observed within the primary tumors (Figure 7A, B). Taken together, the above data suggest that MMP-1 derived from lung host cells does not facilitate colonization and metastasis of cancer cells (Figure 5C) despite being upregulated and cleaving collagen within the tumor in response to cigarette smoke (Figure 7A, B), rather it appears to be protective in nature. In contrast, TAM specific MMP-1 expression is increased following exposure to cigarette smoke with a simultaneous decrease in TIMP expression thereby enhancing MMP-1 activity and promoting tumor growth and metastatic spread (Figure 8A, B).

Discussion

In the present study, our data indicate that TAM derived MMP-1 plays a significant role in primary tumor development and lung metastasis *in vivo*. Furthermore, the observed effects are amplified by exposure to cigarette smoke. In contrast, lung cell derived MMP-1 reduced colonization of the lung by cancer cells; this effect was enhanced by exposure of mice to cigarette smoke. These results suggest a complex interplay between the source of MMP-1 production and its physiological effects on lung tumor growth and metastasis.

Due to their involvement in cancer growth and metastasis, MMPs were considered a promising therapeutic target. However, clinical trials were largely disappointing with questionable benefit only observed in patients with early stage disease [34]. This observation

maybe explained by a lack of understanding of temporal and source specific effects of MMP expression throughout the tumor life cycle. Consistent with this finding, our data demonstrate early MMP-1 expression significantly influences early primary tumor growth.

Supporting the hypothesis that site specific expression of MMP-1 differentially influences tumor signaling pathways, our data indicate TAM specific expression of MMP-1 facilitated primary tumor growth and metastasis without effecting collagen deposition while MMP-1 derived from host lung inhibited colonization and reduced collagen deposition. These data are in agreement with several studies demonstrating variable effects of MMP expression on cancer growth depending on location and type of MMP expressed. For instance, a study by Gupta et al. demonstrated breast cancer specific expression of MMP −1 and −2 enhanced primary tumor growth and metastatic potential [35] while several other studies report protective effects of various MMPs when expressed by host tissue in murine cancer models [36,37]. Furthermore, the observation that TAM specific expression of MMP-1 did not influence collagen deposition supports prior studies demonstrating that fibrillar collagen types I and III accumulate and facilitate metastasis of cancer cells to remote sites [38–41]. Taken together, these data suggest that MMP-1 expression exerts specific effects on cancer development that are determined by the cell type and location of enzyme productions. MMP-1 expressed by native lung cells results in collagen cleavage induces a protective effect against invasion of cancer cells, while its expression by TAMs at the primary tumor periphery promotes cancer cell invasion into the stroma and subsequent metastasis to distant sites.

Although prior studies report that MMP-1 secreted primarily from cancer cells induces invasion and tumor development [35], we propose that MMP-1 produced by the stroma, and specifically TAMs, also plays a prominent role in the process of lung invasion and metastasis. Macrophages are released from bone marrow as monocytes, circulate throughout the blood stream, and migrate into tissues where they differentiate into tissue macrophages [10] and are subsequently recruited to tumors by growth factors and chemokines released by cancer cells [42]. This process is enhanced by smoking which stimulates secretion of additional cytokines and pro-inflammatory compounds. Recruitment of macrophages by a tumor results in their conversion to an M2 phenotype and is characterized by their congregation at the invasive front of the tumor where they are shed along with cancer cells into the venous blood supply [43]. Data from the present study support the concept that MMP-1 expression by M2 macrophages is a distinguishing feature of TAM activated by cancer cells and cigarette smoke. This is exemplified by our *in vitro* data demonstrating a synergistic induction of MMP-1 production following exposure of M2 macrophage to adenocarcinoma cells and cigarette smoke extract. In agreement with our *in vitro* data, similar increases in MMP-1 activity were detected in Mac-MMP-1 mice *in vivo* and, furthermore, they displayed increased number and size of lung metastasis with smoking. Unlike in lung-MMP-1 mice, a global decrease in collagen deposition was not observed in metastatic lung of Mac-MMP-1 mice. Since TAMs in these mice were detected at the tumor periphery (Figure 3E), this observation suggests the tissue and site-specific expression of MMP-1 by TAMs may serve to restrict collagen degradation to the invading tumor front and facilitate cancerous invasion of the stroma. In fact, this finding is consistent with the

observation that overexpression of MMP-1 by breast cancer cells results in enhanced tumor invasiveness and metastatic spread (8).

In the present study, smoke exposure increased MMP-1 expression and activation in the lungs and TAMs of transgenic mice. Critically, the presence of cancer cells and smoke exposure had a synergistic effect on TAM specific production of MMP-1 both *in vitro* and *in vivo*. In prior studies from our laboratory and others, it has been shown that MMP-1 was increased in the lungs of smokers [44]. These studies proposed that cigarette smoke allowed the activation of MMP-1 through increased expression of activating enzymes via activation of the ERK1/2 signal transduction pathways in human small airway epithelial cells [25]. This pathway has been additionally confirmed in subsequent studies utilizing human breast cancer cell lines [44]. This up-regulation may stem from two sources: increased activation of macrophages to secrete more MMP-1 or increased MMP-1 expression from lung epithelial cells [21]. Several studies show that increased expression of MMP-1 in the tumor stroma correlates with aggressiveness in several cancers [45,46]. Altogether, our *in vitro* and *in vivo* data provide direct evidence that MMP-1 secreted from macrophages contributes to lung metastasis and are consistent with correlative clinical data. Furthermore, these studies suggest that macrophage MMP-1 is a possible therapeutic target to decrease the dissemination and metastasis of cancer.

In summary, the present study demonstrates the importance of smoke induced macrophage-specific MMP-1 expression in promoting the ability of cancer cells to invade into the stroma adjacent to the primary tumor and the subsequent establishment of lung metastasis. Conversely, when MMP1 is produced by lung cells at the site of metastasis and disrupts the collagen network, lung colonization is inhibited. Through the cell specific expression of MMP-1 in these transgenic models we have documented the long understood concept that MMPs are neither “bad nor good” players in cancer. What is most important in MMP pathogenesis is the cell type of expression and the condition under which the MMP is produced. The enzymes serve to protect the organism from damage or alternatively contribute to the pathology depending on the where and how they are produced. Therefore, tumor metastasis may be reduced through inhibition of the macrophage-specific secretion of MMP-1, but therapy would need to directly target this specific cell type as MMP-1 inhibition within the lung would potentially worsen metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Support for the present study was provided by grants from the Columbia University LAM Center and the National Heart, Lung, and Blood Institute (NHLBI), contracts R01-HL086936 and RC1HL100384 (JMD). We thank Arjun Sehgal and Peter Rauschkolb and for technical assistance.

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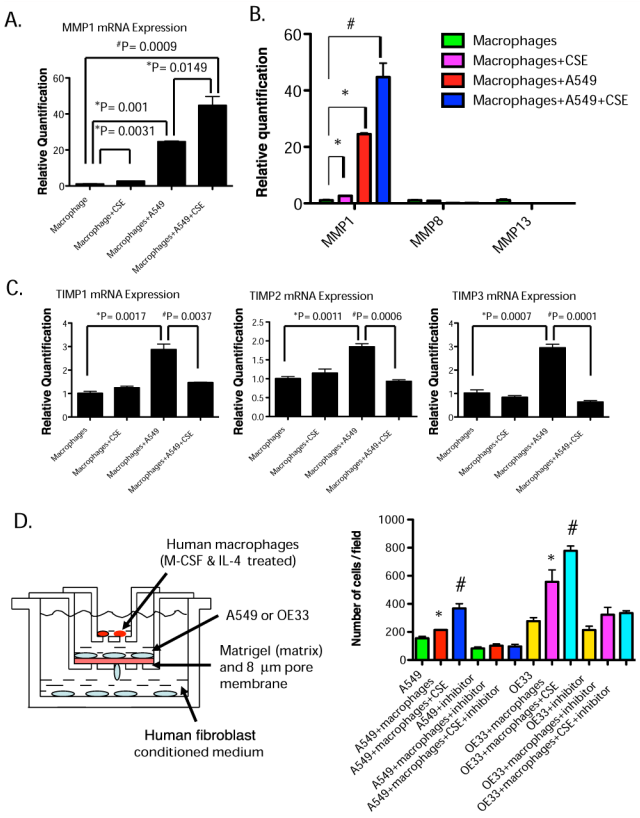

Figure 1: *MMP1* and *TIMP* mRNA regulation and cell invasion in macrophages co-cultured with cancer cell lines and CSE.

(A) *MMP1* mRNA expression in macrophages with A549 cells and CSE. (B) Co-culture of cancer cells with macrophages and treatment with CSE up-regulates *MMP1* mRNA more potently than other collagenases. (C) CSE inhibits co-culture-derived *TIMP* mRNA expression. (D) A Matrigel invasion assay co-cultured with human macrophages and CSE. Ro32–3555 inhibited the invasiveness of A549 and OE33 cells co-cultured with human macrophages and CSE. *, <0.05; #<0.01.

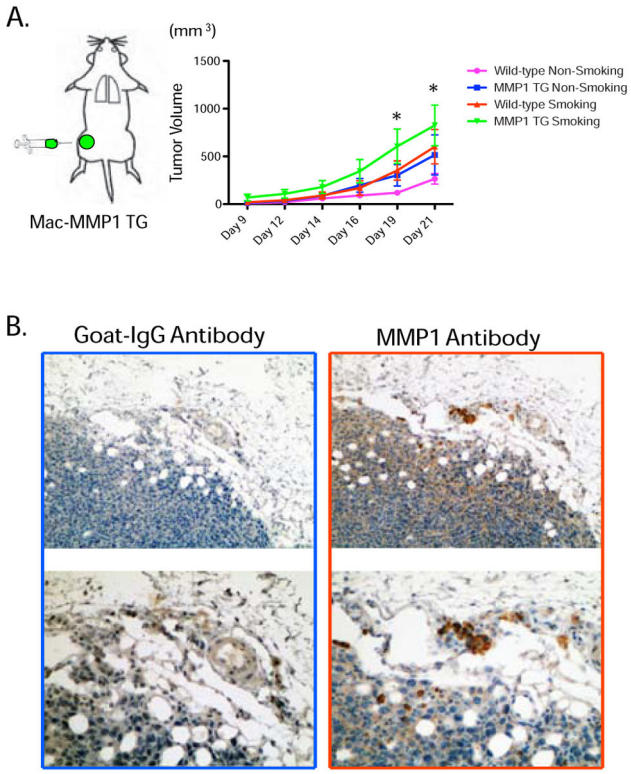

Figure 2: Primary tumor development in mouse allograft model of macrophage MMP1 transgenic mice.

(A) GFP-labeled LLC was implanted in the right fourth mammary gland. The primary tumor in Macrophage-specific MMP1 smoking transgenic mice developed more rapidly than in wild-type non-smoking mice*, <0.05.(B) Immunohistochemistry of the primary tumors. MMP1 expressed cells were detected at the edge of the tumor.

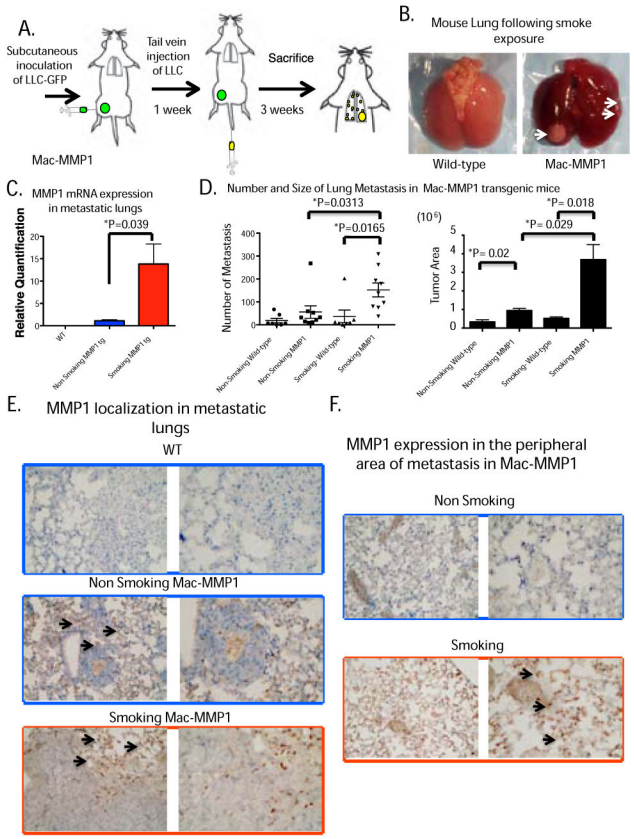

Figure 3: Lung colonization in macrophage specific-MMP1 mouse model.

(A) Scheme of the lung colonization model in macrophage-specific human MMP1 expressed mice. Primary tumor labeled with GFP was generated prior to tail vein injection to induce TAMs. (B) Lung metastasis was detected macroscopically in Mac-MMP1 mice. (C) The integrity of the experimental model. *MMP1*mRNA expression in smoking Mac-MMP1 mice was up regulated as compared to non-smoking Mac-MMP1 mice. (D) Number and size of lung metastasis in Mac-MMP1 mice. MMP1 and smoking synergistically increased the number and tumor area of lung metastasis. (E) Immunohistochemistry of metastatic tumor. MMP1 expressed cells were mainly located at the edge of tumor. (F) MMP1 expressed cells were detected even in the peripheral area of metastasis. *, <0.05.

A.

Group	GFP positive in PT	GFP positive in Lung Metastasis
Non-Smoking WT	10/10	0/10
Non-Smoking TG	10/10	0/10
Smoking WT	10/10	0/10
Smoking TG	10/10	4/10

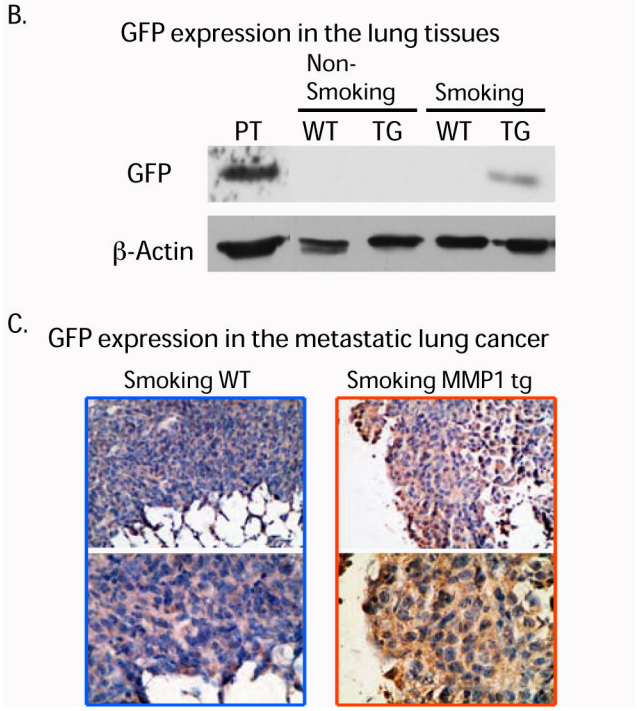

Figure 4: Macrophage-specific MMP1 directly induced lung metastasis from primary tumor.
(A) Number of GFP positive metastatic lung. Four of ten samples are GFP positive in smoking Mac-MMP1 mice. (B) Western blotting of metastatic lung. Primary tumor (PT) was positive control. (C) Immunohistochemistry of GFP. GFP positive cells were detected in smoking Mac-MMP1 mice.

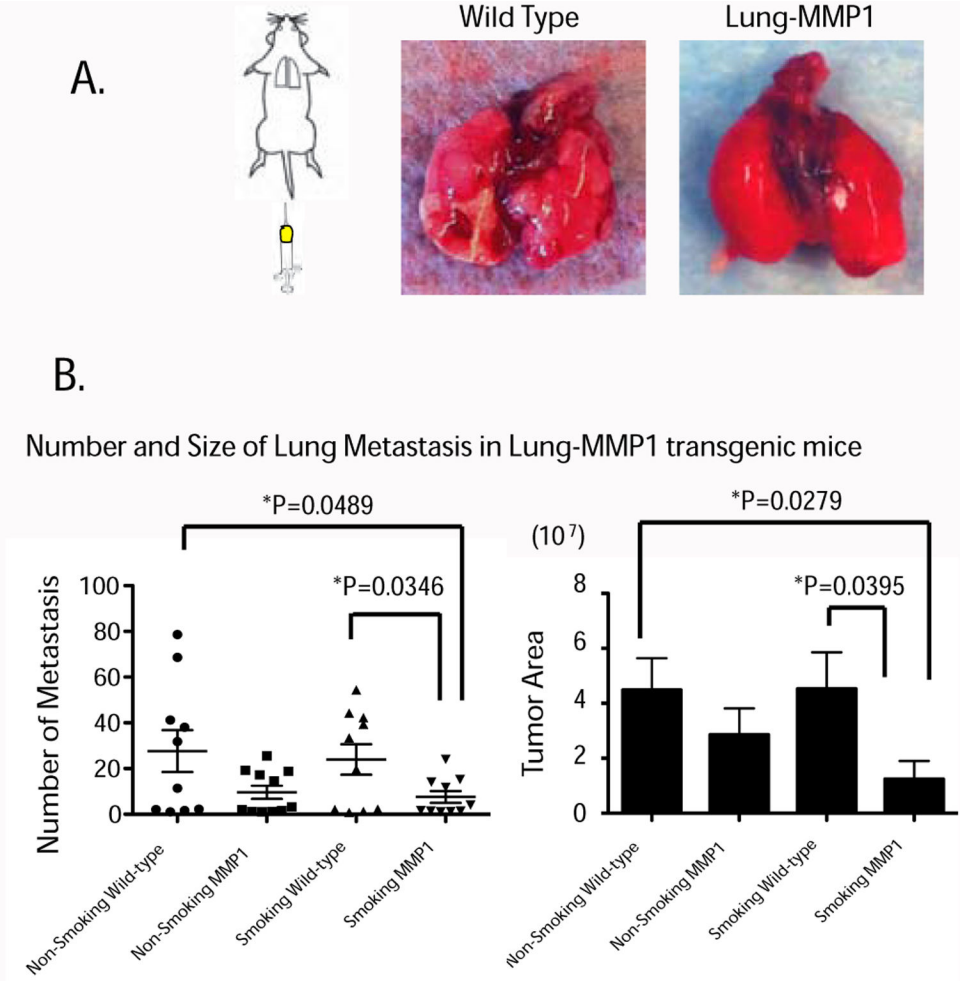

Figure 5: MMP1 expression in the lung inhibited lung colonization.

(A) Tail vein injection of LLC in Lung-MMP1 mouse model. Lung metastasis was detected macroscopically in Lung-MMP1 mice. (B) *MMP1* mRNA was not significantly up regulated in the metastatic lung of smoking Lung-MMP1 mice. (C) The number and size of lung metastasis in the Lung-MMP1 mice. MMP1 and smoking synergistically decreased the number and tumor area of lung metastasis. *, <0.05.

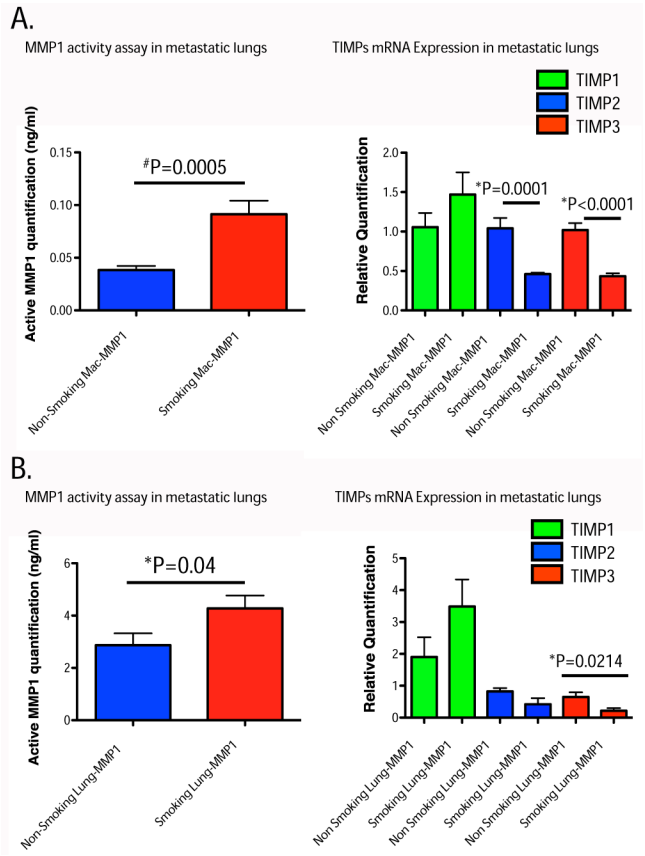

Figure 6: Smoking activated MMP1 and inhibited TIMP expression metastatic lungs.

MMP1 activation was identified with a marked reduction of *Timp2* and *Timp3* mRNA expression in smoking (A) Mac-MMP1 mice and (B) Lung-MMP1 mice. *, <0.05; #<0.01.

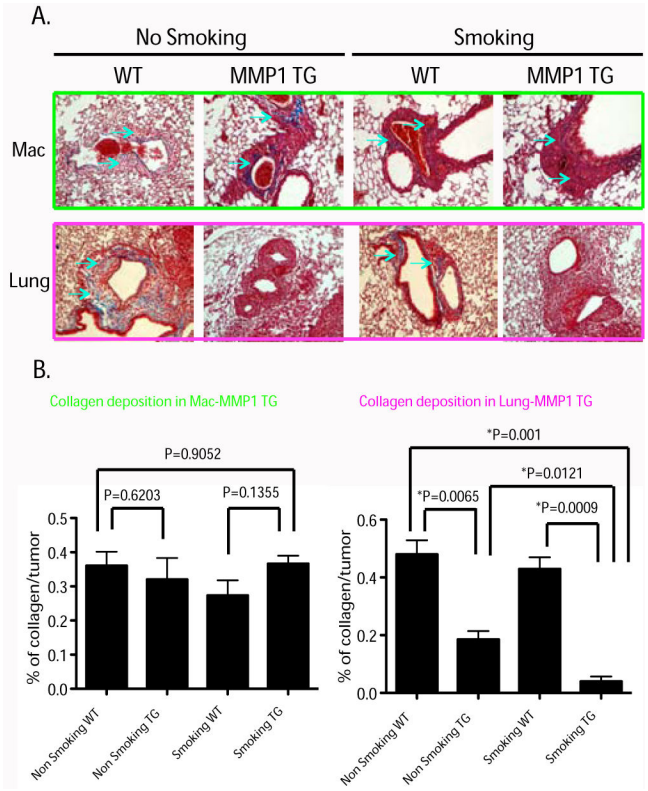

Figure 7: Collagen deposition was decreased in Lung-MMP1 mice, but not in Mac-MMP1 mice.

Collagen deposition was analyzed using Masson Trichrome Staining. (A) Collagen deposition was decreased in each of the metastatic tumors of the Lung-MMP1 mice, but not in Mac-MMP1 mice. (B) The area of collagen deposition in each metastatic tumor was decreased in Lung-MMP1 mice. MMP1 secreted from the lung and smoking synergistically decreased collagen deposition in the metastatic lungs of Lung-MMP1 mice. *, <0.05.

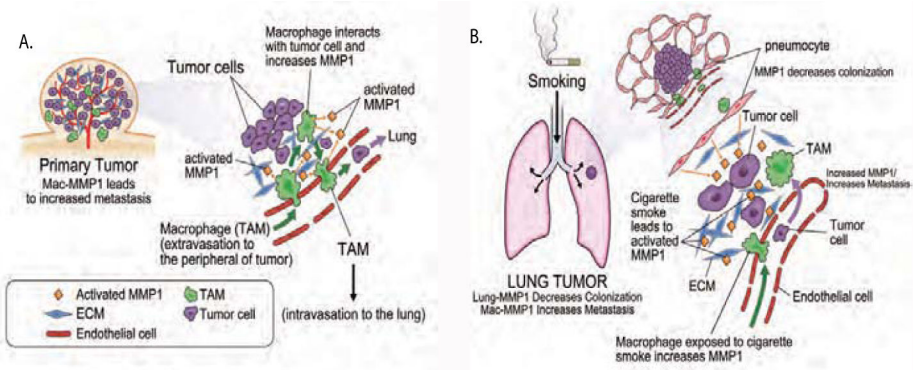

Figure 8: Cell specific role of MMP1 in lung tumorigenesis.

(A) Primary Tumor: Mac-MMP1 leads to an increase in lung metastasis degrading the ECM in the primary tumor allowing extravasation of cells. (B) Lung Tumor: Lung-MMP1 decreases colonization through loss of the ECM scaffold, while Mac-MMP1 leads to an increase in metastasis synergistically with cigarette smoke exposure.