Rnase L Manipulates Macrophages in Innate Immunity and Tumor Growth

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RNASE L MANIPULATES MACROPHAGES IN INNATE IMMUNITY AND TUMOR GROWTH

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RNASE L MANIPULATES MACROPHAGES IN INNATE IMMUNITY AND TUMOR GROWTH

XIN YI

ABSTRACT

RNase L is one of the key enzymes in the 2-5A system of interferon (IFN) action against viral infection and cellular proliferation. Tissue distribution analysis has revealed that RNase L is highly expressed in the spleen, thymus, lung, testis, intestine and most of immune cells such as T, B cells and macrophages. However, the physiological role of RNase L in the immune system is largely unknown. My thesis thus focused on studying the possible physiological role of RNase L in macrophages.

By using bone marrow-derived macrophages (BMMs) from RNase L^+/+ and ^-/- mice, we demonstrated that RNase L is involved in macrophage functions and migration ability. RNase L deficient BMMs showed a significant reduction of endocytic activity to FITC-Dextran 40,000 compared to wild type cells. In addition, lack of RNase L remarkably decreased the migration of BMMs under both normal condition and condition induced by M-CSF, GM-CSF or CCL2. To determine the role of RNase L in tumor growth, P53^/- RL^+/- cancer cells were subcutaneously implanted on the back of RNase L null and wild type mice with C57BL/6 background, respectively. Surprisingly, the average tumor weight from RNase L^+/+ mice was 3-fold heavier than that from RNase L^--/ mice.
indicating that presence of RNase L was overtly favorite for tumor growth. Immunofluorescence staining revealed that the numbers of infiltrated macrophages were markedly higher in the tumor tissues from the wild type mice. Depletion of macrophages clearly inhibited tumor growth on RNase L\(^{+/+}\) mice, suggesting that RNase L may promote tumor growth through regulating the function of tumor-associated macrophages (TAMs). Taken together, our findings implicate that RNase L may play a dual role in innate immunity and tumor promotion.

Additionally, in a collaborated project, we successfully identified and investigated the molecular targets of an anti-cancer drug candidate. In this study, we performed protein pull down assays to purify the anti-cancer targets of the compound. Via proteomic approaches, the major proteins bound to the probe were identified to be tubulin and Hsp27, and the compound significantly inhibited tubulin polymerization and had the potential to be a class of new chemotherapeutic agents.
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1.1 2’-5’ oligoadenylate (2-5A) / RNase L system

The 2’-5’ oligoadenylate (2-5A)/ RNase L system was first discovered in the mid-1970s when researchers investigated how interferon (IFN) inhibits viral infection (1). Promising evidences showed that the 2-5A/RNase L system is an IFN-inducible RNA degradation pathway which is responsible for many of the antiviral and anti-proliferative effects of IFN. The 2-5A pathway comprises at least three important components, 2-5A synthetases (OAS), 2-5A degrading enzyme, and RNase L (Figure 1.1). The dsRNA generated from virus infection binds to and activates OAS. Once activatd, OAS converts ATP to PPi and a series of short 2’, 5’- linked oligoadenylates referred to as 2-5A molecules with the formula $[\text{ppp}\text{5’A}(2’\text{p5’A})n; , 2\leq n]$ (Figure.1.1) (2). At subnanomolar levels, 2-5A binds with high affinity to RNase L and converts RNase L from its monomeric, an inactive form to a dimeric, active state, with endonucleolytic activity (3).
Activated RNase L could cleave single-stranded RNA (ssRNA) in U-rich sequences, typically after UU or UA dinucleotides leaving a 5’-OH and 3’-monophosphate (4, 5), leading to the degradation of viral RNA and also host cell mRNA, which induces cell apoptosis (3, 6).
Figure 1. Diagram of 2-5A/RNase L system.

(Chakrabarti A, Jha BK, Silverman RH. J Interferon Cytokine Res. 2011)
1.2 Properties of RNase L

RNase L is a highly regulated, latent endoribonuclease first cloned in 1993 (7). It is widely expressed in most mammalian tissues (8). The cDNA of human RNase L gene encodes an 84 kDa protein with 741 amino acids. The structural and functional analysis of RNase L has revealed that this enzyme is composed of 3 major domains: an N-terminal regulatory ankyrin repeat domain (ARD), a protein kinase (PK)-like domain, and a C-terminal ribonuclease domain (RNASE) (Figure 1.2). The N-terminal contains 9 ankyrin repeats, which are one of the most common amino acid motifs, typically functioning in mediating protein–protein interactions(1, 2), suggesting that RNase L may interact with other proteins. RNase L has 8 complete ankyrin repeats and 1 partial repeat appearing as a disordered segment in the crystal structure of the N-terminal (amino acid 1–333) of human RNase L (9). The unique feature of the ARD repeats in RNase L is that they interact with a nucleic acid, 2-5A. Repeats 2 and 4 in ARD are involved in 2-5A binding (Figure 1.2) (9). In its inactive state, the N terminal domain functions as a repressor of the ribonuclease domain in the C terminal domain (Figure 1.3). The minimum repressor function can be mediated by three ankyrin repeats, 7, 8, and 9 (10, 11).

The C-terminal part of RNase L has a cysteine rich region with a protein kinase homology although kinase activity of RNase L has not been demonstrated to date. The protein kinase-like and ribonuclease domains of RNase L [collectively referred to as the kinase-extensionuclease (KEN)] are homologous with Ire1, which is also a kinase and an
endoribonuclease that functions in the unfolded protein response (UPR) from yeast to humans (1, 11, 12). The KEN domain in RNase L functions in dimerization and catalysis. An isolated C-terminal domain of RNase L can cleave RNA in the absence of 2-5A (10, 13). The RNASE domain becomes constitutively active upon removal of the ARD (although at 6-fold reduced activity compared with activated full-length protein) (10). 2-5A binding to the N-terminal half probably induces a conformational change of the enzyme, causing the N-terminal repressor domain to release from the C terminal ribonuclease domain, and unmasks the dimerization domain (Figure 1.3).
Figure 1.2 The structure of RNase L.

(Chakrabarti A, Jha BK, Silverman RH. J Interferon Cytokine Res. 2011)
Figure 1. 3 Functional model for the activation of RNase L by 2-5A.

(Dong B, Niwa M, Walter P, Silverman RH. RNA. 2001)
1.3 Involvement of RNase L in the antiviral action

RNase L is one of the key enzymes in the 2-5A system of IFN action against viral infection and cellular proliferation. The 2-5A system mediates host defense against certain types of viral infections. The most compelling evidence is the accumulation of 2-5A and activated RNase L in virus-infected cells (14). Cells overexpressing RNase L overcome viral infection. In contrast, overexpression of a dominant negative mutant of RNase L results in increased susceptibility to viral infection, including picornaviruses, EMCV or Coxsackie virus B4, herpes simplex virus 1 (HSV-1), flavivirus, and West Nile virus (2, 15-18). In vivo studies show that mice containing targeted disruption of RNase L gene succumb to encephalomyocarditis (EMCV) infection more rapidly than infected wild type mice. Transfection of the 2-5A analog, CH3Sp(A2’p)2A2’pp3’OCH3, which binds to, but does not activate RNase L (19, 20) into IFN-treated, EMCV-infected murine L929 cells inhibited rRNA cleavage and increased virus production by up to 10 fold (21). Moreover, it is also notable that RNase L not only degrades RNA, but also regulates the expression of genes (22), which have antiviral function. It is believed that RNase L presents its antiviral effects through a combination of effect, including direct cleavage of viral RNA, inhibition of protein synthesis through the degradation of rRNA, induction of apoptosis, and induction of other antiviral gene expression (2).
1.4 Apoptotic activity of RNase L

Several lines of studies have shown that activation of RNase L causes apoptosis. The 2-5A system is likely to contribute to the antiviral activity of IFN by inducing apoptosis of infected cells. Overexpression of dominant negative RNase L in cells reduced apoptosis whereas overexpression of wild-type RNase L enhanced the apoptosis in response to viral infection (21). However, evidences also shown that RNase L is involved in an apoptotic signaling pathway other than that induced by 2-5A. After down-regulation of RNase L by siRNA in the prostate cancer cell line DU 145, cells were resistant to induced apoptosis (23). RNase L null mice showed enlarged thymus resulted from a suppression level of apoptosis and there was a 2-fold decrease in apoptosis in the thymuses and spleens of RNase L null mice. Furthermore, thymocytes and lymphocytes isolated from spleen of RNase L-null mice were resistant to apoptosis induced by apoptotic agents (15, 24). These results suggest that RNase L contributes to apoptosis. RNase L mediating apoptosis is through activation of a JNK-dependent stress response pathway, leading to cytochrome c release from mitochondria and subsequently activation of the caspase cascade. Overexpression of Bcl-2 inhibits this process (25, 26).
1.5 The involvement of RNase L in cancer

RNase L was initially proposed to be a candidate tumor suppressor based on its involvement in the antiproliferative activity of IFN and on the location of \textit{RNASEL} at chromosome 1q25, a region deleted or rearranged in some breast cancers (2, 27-29). Studies have revealed that skin allograft rejection is suppressed in mice lacking RNase L, implicating the involvement of RNase L in T-cell immunity, particularly CD4$^+$ T-cell mediated immunity (30). In addition, alphavirus-based DNA vaccination against a non-mutated tumor-associate self-antigen (tyrosinase-related protein-1, TRP-1) is severely impaired in RNase L null mice, indicating that RNase L is involved in or required for host immune system against cancer (31). Moreover, RNase L was identified as a prostate cancer susceptibility gene by mapping hereditary prostate cancer 1 (HPC1) to the RNase L gene (2, 32). Recent studies in humans have demonstrated that mutations in \textit{RNASEL} can enhance the risk of prostate cancer by about 50% (33-36). However, not consistent with other group’s results, my research study found that RNase L would promote rather than suppress tumor growth, through its modulation on macrophage functions and cytokine profile in tumor tissues.
1.6 References


CHAPTER II

DEFECTIVE MACROPHAGE FUNCTIONS IN RNASE L DEFICIENCY

2.1 Introduction of macrophage endocytosis and migration ability

Macrophages are an essential part in innate immunity and adaptive immunity, as a frontline defense against invading disease or injury. Their roles include endocytosis to guard against microbial infections, migration to injury tissue, and production of an assortment of cytokines, which could regulate tissue remodeling and local tissue inflammatory response (1, 2).

2.1.1 Introduction of Macrophages
2.1.1.1 Macrophage generation

Blood monocytes are known to arise in the bone marrow from precursor cells (monoblasts) that are derived from the differentiation of multipotential progenitors. Blood monocytes are then released into the peripheral blood, and circulate for several days before entering into inflamed or infected tissues, where they can mature into macrophages and substantially augment resident macrophage populations throughout the body, as well as give rise to specialized cells as dendritic cells (DCs) and osteoclasts. Dendritic cells could efficiently present antigen to T cells and osteoclasts are the cells that resorb, or break down and absorb, bone tissue back into the body (Figure 2.1) (3, 4, 5).

2.1.1.2 Macrophage heterogeneity

In the absence of inflammation, monocytes are found principally in the marrow and blood. In 1939, Ebert and Florey firstly reported the observation that monocytes emigrated from blood vessels and developed into macrophages in the tissues, where they differentiate into typical tissue macrophages and assume different morphologic and functional properties depending on their location and environment in organs and tissues (4, 6-8). In the adult, large populations of tissue macrophages exist in the small intestine, liver (Kupffer cells), and lungs (alveolar and interstitial macrophages), but they are also found in the spleen, lymph nodes, bone marrow, peritoneal and pleural serosal cavities, kidneys and endocrine glands, and brain (microglia) (3). Studies of the mononuclear-
phagocyte system, using monoclonal antibodies specific for various cell-surface receptors and differentiation antigens, have shown that there is substantial heterogeneity of phenotype, which most probably reflects the specialization of individual macrophage populations within their microenvironments and result in different physiological roles of the subsets in vivo. However, the determinants of this tissue-specific differentiation remain unrevealed (4).

Figure 2. Development and function of monocyte subsets.
2.1.1.3 Macrophage functions and its relevance in diseases

Macrophages play a central role in the immune system through presenting antigens to directly activate T-lymphocytes, participating in ingestion and killing of various invading or infectious microorganisms (9). In addition, macrophages have cell surface receptors for a variety of substances (e.g., IgG, complement components, fibronectin, and sugars) and are capable of secreting a great number of mediators involved in host defense and inflammation response (10, 11, 12). Tissue specific macrophages also express specific functions in particular anatomic sites and organs. During infection, macrophages have the capacity to become activated by both specific and nonspecific immunologic stimuli such as lymphocyte cytokines and different bacterial products, and the activated macrophage has enhanced functional capabilities in inflammatory responses, antigen presentation, and immunoregulatory networks (10). Because of the central role in host defense, abnormal monocyte/macrophage function may result in pathophysiologic consequences and have been described in various disorders such as deficiencies in the clearance of physiologic substrates in lysosomal diseases, decreased secretion of mediators (complement component deficiencies), deficiencies in the clearance of physiologic substrates in lysosomal diseases, defects in microbicidal activity (chronic granulomatous disease) and defects which are acquired following infection and during chemotherapy (e.g., HIV) (10,12, 13).
2.1.2 Macrophage endocytosis and phagocytosis

Macrophages are cells that function in both innate and adaptive immunity and can exert protective and pathogenic activity through ingesting specific matter efficiently by phagocytosis and endocytosis. These processes are essential for the removal of pathogens and macrophage antigen presentation (14, 15). During endocytosis, the plasma membrane of the cell forms a pocket (endosome or phagosome) around the material to be internalized including fluid, large and small molecules, and even other cells from their surroundings. Endocytosis includes the processes of phagocytosis, receptor-mediated endocytosis, and fluid phase endocytosis/pinocytosis, contributing to multiple pathways of cell homeostasis, development of immune responses to soluble antigens, and infection by intracellular pathogens (Figure 2.2) (16).

2.1.2.1 Pinocytosis and Receptor-mediated endocytosis

Pinocytosis usually refers to the uptake of fluid and solutes, which brings fluid and material into the cell and removes membrane from the plasma membrane with a continuous stream of vesicles budding. Receptor-mediated endocytosis selectively internalizes specific molecules that are bound to receptors on the outside surface of the cell, such as hormones, growth factors, antibodies, iron, enzymes, vitamins, and cholesterol. Once molecules bind to their receptors, the receptors move within the plasma membrane and the inside surface of the plasma membrane then progressively invaginate, or form inward, form a membrane-enclosed bubble, or vesicle, containing the ingested
material. The basketlike structure vesicles pinch off the plasma membrane into the cytoplasm and fuses with another membranous organelle called endosome (17, 18). In 1985, Brown and Goldstein won the Nobel Prize in medicine for their discovery of the endocytosis of cholesterol and lipoprotein (LDL) by cells from the bloodstream (19). Moreover, endocytosis is an important step in presenting antigens to generate acquired immunity and more recently, studies also found that endocytosis can be utilized by viruses to enter cells (20-22).

### 2.1.2.2 Phagocytosis

Phagocytosis is also important in processing and presenting antigens to T cells, and moreover, it could defend the body against infection by engulfing invading microorganisms and to remove cell debris from the body by ingesting damaged or old cells. In phagocytosis, cell membrane extend and project large vesicles surrounding the particle and fuse together so that the particle is completely engulfed in large vesicles within the cell, which are called phagosome (24). Inside the cell, the phagosome fuses with another membranous organelle called lysosome, forming a single membranous organelle and mixing their contents and breaking down the ingested materials into small molecules in the process (3). In phagocytosis and receptor mediated endocytosis, macrophages have evolved a restricted number of receptors, like the mannose receptor, that recognize conserved motifs on pathogens (25).
However, in phagocytosis and receptor-mediated endocytosis, different receptors and underlying molecular mechanisms are utilized for internalizing different agents (25). These differences include the cytoskeletal elements that mediate ingestion, vacuole maturation, and inflammatory responses (25, 26). Infectious agents, such as M. tuberculosis, Legionella pneumophila, and Salmonella typhimurium, enter macrophages via heterogeneous pathways and modify vacuolar maturation in a manner that favors their survival (26).
Figure 2. Endocytosis includes the processes of phagocytosis, receptor-mediated endocytosis, and fluid phase endocytosis/pinocytosis.
2.1.3 Macrophage migration

When monocytes enter damaged tissue through the endothelium of a blood vessel, it undergoes a series of changes to become tissue specific macrophages. Monocytes are attracted to a damaged site by chemical substances through chemotaxis, triggered by a range of stimuli including damaged cells, pathogens and cytokines released by macrophages already at the site. The recruitment of macrophages to an inflammatory site is a complex process involving their adhesion to endothelial cells, passage into the perivascular connective tissue, and migration toward a chemotactic gradient (27, 28). Chemokine C-C motif ligand (CCL) 2, also known as monocyte chemoattractant protein 1 (MCP-1) is a member of the cytokine/chemokine superfamily and is revealed to promote the migration of monocytes and macrophages to sites of inflammation (29-32), as mice deficient in CCL2 have decreased recruitment of macrophages in response to infection (33). Furthermore, macrophages from CCL2 deficient mice with impaired migration activity also produced lower amounts of IL-10 and tumor necrosis factor compared with wild-type mice (34). In addition to being a cytokine synthesis-inhibitory factor, IL-10 was also reported to facilitate macrophage migration through its inhibitory effect on macrophage migration inhibitory factor (MIF) (35). Moreover, TGF-β and M-CSF are also important factors known to attract macrophage recruitment into inflammation sites and tumor tissues (36, 37). Macrophages, in particular, secrete TGF-β, which in turn stimulates the migration of macrophages. It was reported that TGF-β induced mRNA and protein level of CCL2 in macrophages, and this induction was mediated by RhoA (36).
2.1.4 Highly expressed RNase L in macrophages

RNase L is present at basal levels in most mammalian cells. Tissue distribution analysis has revealed that RNase L is highly expressed in the spleen, thymus, lung, testis, intestine and most of immune cells such as T, B cells and macrophages. However, the physiological role of RNase L in the immune system, especially in macrophages, is largely unknown. In the present study, we demonstrated that RNase L contributes to macrophage endocytosis and migration ability by using BMMs from RNase L wild type and knocked out mice. Furthermore, significantly higher expression of CCL2, TGF-β, IL-10 and COX-2 was observed in cells in the presence of RNase L, suggesting that RNase L may regulate the expression of cytokines and chemokines in macrophages to modulate their immune functions. Taken together, our findings provide new insight into how RNase L regulates macrophage function, and suggest a novel role of RNase L in macrophage-dependent immune response.
2.2 Materials and Methods

2.2.1 Culture and activation of macrophages from mouse bone marrow

Bone marrow-derived macrophages (BMMs) were generated from the bone marrow of RNase L wild type and deficient C57BL/6 mice by a modification of a previously reported method. Bone marrow cells were isolated from femurs by repeated flushing of the bone shaft with cold medium and cultured in RPMI 1640 medium (Cleveland Clinic, OH) supplemented with 10ng/ml recombinant murine macrophage-colony stimulating factor (M-CSF, Shenandoah Biotechnology, PA), 20% fetal bovine serum (FBS), and 10% L292-conditioned medium (LCM) which contains abundant M-CSF. RPMI medium stands for Roswell Park Memorial Institute medium, and it is traditionally used for the cultivation of many cell types, especially human T/B-lymphocytes, bone marrow cells and hybridoma cells. Macrophage colony-stimulating factor (M-CSF) is a secreted cytokine essential for both the proliferation and differentiation of monocytes/macrophages from hematopoietic stem cells. Macrophages generated from mouse bone marrow cell culture were harvested after 7 days culture and were used for in vitro experiments. To assess macrophage endocytosis, phagocytosis ability and cell motility, macrophages were activated with LPS (0.5μg/ml) for 48 hours.
2.2.2 Endocytosis and phagocytosis assay

As introduced in 2.1.2, macrophages internalize specifically-targeted particular ligands through receptor-mediated endocytosis and phagocytosis, whereas pinocytosis is a less specific mechanism of endocytosis. To assess whether RNase L influences macrophage most important functions, specific ligands conjugated with fluorescence signal were selected to assess macrophage endocytosis and phagocytosis ability. As shown in Table 2.1, FITC-Dextran 40,000 (Sigma, MO) was used to determine macrophage endocytosis and FITC-conjugated E. coli particles (Molecular Probes, Invitrogen, CA) were selected to study macrophage phagocytosis. Cells were washed twice with cold PBS, and incubated in fresh DMEM (10% FBS, 25mM Hepes) with FITC-Dextran 40,000 (1g/ml) or FITC-E.coli (bacterial to macrophage ratio=50:1) at 37°C for 1 hour. Control groups were incubated on ice. After incubation, cells were fixed with 4% paraformaldehyde for 10 min and fluorescence of extracellular particles was quenched with 1-2 ml 0.02% trypan blue for 3 minutes. Prolong cold antifade reagents with 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, CA) were used as mounting solution. DAPI binds strongly to A-T rich regions in DNA and is wildly used in fluorescence microscopy to stain cell nucleus. Prepared slides were allowed to dry and observed under microscope.
<table>
<thead>
<tr>
<th>Probe</th>
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<th>Receptor</th>
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<tr>
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<td>Molecular Probes</td>
<td>Unknown</td>
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<tr>
<td>FITC holo-transferrin</td>
<td>Molecular probes</td>
<td>Transferrin receptor</td>
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<td>Di-I LDL</td>
<td>Per Immune</td>
<td>LDL receptor</td>
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<tr>
<td>Di-I acetylated LDL</td>
<td>Per Immune</td>
<td>SR-A, CD36, MARCO</td>
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<td>HRP</td>
<td>Sigma</td>
<td>Mannose receptor on macrophages</td>
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<tr>
<td>Lucifer yellow</td>
<td>Sigma</td>
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<tr>
<td>Mannosylated BSA</td>
<td>E-Y lab</td>
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<td>Diamedix, Miami, FL</td>
<td>CR3 if coated with complement FcR if coated with IgG</td>
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<td>Molecular Probes</td>
<td>Multiple</td>
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<td>FITC-S. aureus bioparticles</td>
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<tr>
<td>Zymosan</td>
<td>Sigma</td>
<td>MR and b-glucan receptor</td>
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</tbody>
</table>

**Table I.** Commonly used endocytic tracers and phagocytic particles in experiment.

(Book: Macrophages. Donna M. Paulnock. 2000 Oct; page 83)
2.2.3 Macrophage in vitro migration assay

For studying macrophage migration ability, BMMs were used to perform in vitro transwell cell migration assay (Figure 2.3). The transwell migration assay is performed by adding cells to the upper, coated surface of the transwell inserts and medium with chemoattractant to the lower well of the chamber. After incubation, especially under the induction of chemoattractant, cells invade through the matrix barrier and pass through the pores on the membrane, and migrate onto the lower surface of the inserts and attach on it. Migrated cells can be quantified by methods as simple as fix/stain and count.

When BMMs grew to 80% confluency, growth medium was replaced with serum free DMEM to starve cells for 20 hours. Transwell filter inserts (8μm pore size; Greiner bio-one) were coated with 20μg/ml fibronetin (from human plasma; Sigma, MO), which helps the attachment of migrated cells on the membrane, at 4°C overnight. After aspirating solution out of apical and lower chamber, macrophages (2.07x10^5) were seeded in the upper chamber with serum free DMEM. In the lower chamber, media with different macrophage migration stimulator were added respectively, including serum-free DMEM, DMEM, DMEM with M-CSF (100ng/ml), or with IFN-γ (100 μg/ml), or with GM-CSF (100ng/ml). Cells were incubated at 37°C for overnight to allow enough time to migrate. After incubation, chambers were turned upside-down and gently submerged in PBS several times to remove unattached cells. Then cells left on membrane were firstly fixed with 10% formalin, and then stained with eosin for 5 minutes. Under the microscope, cells were counted in x200 fields and averaged.
Figure 2. 3 Illustration for tranwell insert cell migration assay.
2.2.4 Cytokine enzyme-linked immunosorbent assay (ELISA)

ELISA is a widely used method for measuring the concentration of a particular molecule in a fluid such as serum, urine or cell culture medium. It is performed in a 96-well plate with a high throughput result and high sensitivity.

To determine certain cytokine and chemokine secretion level in macrophages, ELISA was performed with macrophage cell culture medium. Primary macrophage and RAW 264.7 macrophage cell culture medium were collected and CCL2, TGF-β, M-CSF and IL-10 secretion was measured with commercially available ELISA kits (eBioscience, CA). The ELISA process is shown as in Figure 2.4. Briefly, according to manufacturer’s directions, flat bottom 96 well ELISA plates were coated with a capture antibody at 4°C. After overnight incubation at 4°C, the plates were washed three times, blocked with blocking buffer provided in the kit and then incubated with standards or cell culture supernatant samples for 2 hours at room temperature. Serial dilutions of standard solutions were run in duplicate on each plate. After washing the plates, biotinylated detection antibody was added to each well and incubated for 1 hour, which followed by washing and 30 minutes incubation of strepavidin peroxidase. Substrate containing 3, 3' 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide was added into plates, and phosphoric acid was used as stop solution after 30 minutes. Plates were read at 450nm within an automated microplate reader and cytokine concentrations were determined against the standard curve.
Figure 2. 4 Main steps of the Sandwich ELISA Kit.

2.2.5 RNase L knocked down in RAW264.7 macrophage cell line

2.2.5.1 RNA interference (RNAi) and short hairpin RNA (ShRNA) lentiviral particles

RNA interference (RNAi) was first identified in 1997 in C. elegans by Fire and Mello as a biological response to exogenous double-stranded RNA (dsRNA), which induces sequence-specific gene silencing (38). It is a natural process that expression of a targeted gene can be knocked down with high specificity and selectivity (39). The discovery introduced an extraordinarily powerful and promising laboratory tool for researchers, and also has exciting clinical potential. The RNAi pathway is initiated by the enzyme Dicer, which cleaves long dsRNA molecules into short fragments of ~20 nucleotides that are called small interfering RNA (siRNA). SiRNA assembles with the RNA-induced silencing complex (RISC) and unwound into two single-stranded (ss) RNAs. One of the two strands will be degraded, and the guide strand remaining on RISC specifically binds with a complementary sequence of mRNA and induces cleavage by the catalytic component of the RISC complex, resulting in post-transcriptional gene silencing (40) (Figure 2.5).

Methods of mediating the RNAi effect involve siRNA and short hairpin RNA (shRNA), which is packed and transfected as shRNA plasmid or shRNA lentiviral particles (Figure 2.6) (39). siRNA provides a fast and efficient, though short-term, decrease in target gene expression, whereas shRNA plasmids or shRNA lentiviral
particles transfection is more stable and last longer. A transfection reagent is required when siRNA and shRNA plasmid are used. With the same encoded sequence against a target gene, shRNA lentiviral particles are ready to transduce by directly adding them to cell culture. Moreover, shRNA lentiviral particles can easily transduce typically hard-to-transfected cell lines, such as primary cells, non-dividing cells, and also macrophages.
Figure 2. 5 RNAi machinery.

(Daniel C, Doris L, Volker WK. Clinical Science. 2006)
Figure 2. 6 siRNA, shRNA Plasmid and shRNA Lentiviral Particles as gene silencer.

(http://www.scbt.com/gene_silencers.html Santa Cruz Biotechnology, CA)
2.2.5.2 RNase L knocked down in RAW264.7 cell line

RAW264.7 cells were cultured in a 12-well plate to 50% confluent on the day of infection. Fresh complete medium with Polybrene (Santa Cruz Biotechnology, CA) at a final concentration of 5μg/ml was added in each well and cells were infected by directly adding mouse RNase L shRNA lentiviral particles or control shRNA lentiviral particles in the control group. After overnight incubation, culture medium was replaced with complete medium without Polybrene or shRNA lentiviral particles. RNase L and Control shRNA lentiviral particles contain a puromycin resistant gene, which enables stably transduced cells to survive in puromycin selective culture medium. Puromycin dihydrochloride (10mg/ml) was added after 2 days to select shRNA stably transduced cell colonies. A puromycin titration over RAW264.7 cells was performed previously to select an amount sufficient to kill the non-transduced cells. Medium with fresh puromycin was replaced every 3-4 days in two weeks, until resistant colonies can be formed. All stably transduced colonies were picked and expanded to check the expression level of RNase L by Western Blot.

2.2.6 Determination of RNase L protein expression level with Western Blot

2.2.6.1 Introduction of western blot

Western blot is a widely used technique for detection of known proteins in the given sample of tissue homogenate or extract with specific antibodies. It comprises four
parts, including protein separation by gel electrophoresis, protein transference, antibody blotting, and probe detection. The most common type of gel electrophoresis is SDS polyacrylamide gel electrophoresis (in x200 fields), which employs polyacrylamide gels and loaded with sodium dodecyl sulfate (SDS) buffer. Polypeptides were denatured by treating with strong reducing agents to remove secondary and tertiary structure and thus allow SDS-PAGE to separate proteins only by their molecular weight. When voltage is applied along the gel, proteins migrate into it at different speeds. Smaller proteins migrate faster through the gel and therefore proteins in samples can be separated by their different molecular weight (Figure 2.7).

After gel electrophoresis, proteins are transferred from the gel to a membrane such as polyvinylidene difluoride (PVDF) or nitrocellulose membrane, by wet (tank) transfer or semi-dry transfer. An antibody is then added onto the membrane which is able to bind to its specific protein. After washing, a secondary antibody conjugated with an enzyme (e.g. alkaline phosphatase or horseradish peroxidase) will be used, which could be detected by incubating it with a substrate, usually chemiluminescent substrate. The attached enzyme could convert colorless substrate to a colored product that can be detected and photographed (Figure 2.8).
Figure 2. 7 Separation of proteins in SDS-PAGE according to molecular weight.

(http://www.imb-jena.de/~rake/Bioinformatics_WEB/proteins_purification.html)
Figure 2. 8 Detection of horseradish peroxides (HRP)-linked secondary antibody.

(http://www.cellsignal.com/products/7072.html, Cell Signaling, CA)
2.2.6.2 Determine RNase L expression level by Western Blot.

All western blot experiments performed here were using SDS-PAGE and wet (tank) transfer. Cells were lysed in RIPA lysis buffer (5ml 1M tris HCl pH 7.4, 3ml 5M NaCl, 1ml 10% NP40, 0.5g sodium deoxycholate, 1ml 0.5M EDTA 0.2ml 0.5M EGTA, 0.1% SDS) with freshly added a protease inhibitor cocktail (Calbiochem, CA), and followed by centrifugation. Protein samples were separated on 10% SDS-PAGE gel and thereafter transferred onto PVDF membrane (Pall Cooperation, FL). After blocking, the membrane was incubated in PBST containing 5% non-fat milk and a polyclone antibody specific to RNase L (1:1000 dilution, generated in our lab) over night at 4°C. HRP-conjugated anti-rabbit IgG (1:2000 dilution, Cell Signaling, MA) was used as a secondary antibody and incubated at room temperature for 1 hour. The membrane was incubated in an ECL plus reagent (GE health, OH) and then exposed to hyper film.
2.3 Results

2.3.1 RNase L enhances the extent of macrophage endocytosis

Endocytosis plays an essential role in cell physiology and pathology. The pathways followed by particles internalized by its three main models (fluid-phase endocytosis, receptor-mediated endocytosis and phagocytosis) have been amply delineated in innate and adaptive immunity. We found from our previous studies that RNase L was highly expressed in macrophages. To determine if RNase L influences macrophage endocytosis activity, endocytosis and phagocytosis assay was performed. In endocytosis assay, bone marrow-derived macrophages (BMMs) from RNase L deficient and wild type mice were incubated with FITC-labeled Dextran 40,000 after activation by lipopolysaccharine (LPS) and fluorescence of engulfed FITC-Dextran 40,000 was observed and analyzed under a microscope. As shown in Figure 2.9A, FITC-Dextran 40,000 were internalized at a higher level in macrophages from RNase L\(^{+/+}\) mice, compared to the cells in the absence of RNase L. Average numbers of total cells and cells ingested FITC-Dextran 40,000 or FITC-\(E.\ coli\) was counted from 8 fields under a microscope. In the endocytosis assay, 61.1 ± 5.0% of RNase L\(^{+/+}\) macrophages was observed with endocytosis, but only 34.0 ± 8.3% RNase L\(^{-/-}\) macrophages accounted for total cells (Figure 2.9B). The lack of RNase L in macrophages resulted in a dramatic reduction of endocytosis ability by 30%. However, when activated macrophages were exposed to FITC-labeled \(E.\ coli\), similar amount of \(E.\ coli\) were efficiently phagocytosed by RNase L\(^{+/+}\) and RNase L\(^{-/-}\) macrophages (Figure 2.10A). As shown in Figure
2.10B, 67.4 ± 5.4% of RNase L+/+ and 64.5 ± 3.0% of RNase L−/− macrophages internalized FITC-E.coli in phagocytosis assay, indicating that phagocytosis activity of E.coli by macrophages was not altered in the deficiency of RNase L. Experiments were repeated 3 times and similar results were obtained.
Figure 2. 9 RNase L enhances endocytosis of FITC-DX40,000 in macrophages.

(A) Deficient endocytic capacity of FITC-Dextran 40000 was observed in BMMs in the absence of RNase L. (B) Average numbers of total cells and cells ingested FITC-Dextran 40,000 was counted from 8 fields under a microscope. In the endocytosis assay, 61.1±5.0% of RL+/+ and 34.0±8.3% of RL−/− macrophages was observed with endocytosis.
Figure 2. 10 RNase L does not affect macrophage phagocytosis of FITC-\textit{E.coli}.

(A) RL\textsuperscript{+/+} and RL\textsuperscript{-/-} macrophages have efficient and similar phagocytic ability to engulf FITC-\textit{E.coli}. (B) Average numbers of total cells and cells ingested FITC-\textit{E.coli} was counted from 5 fields under a microscope. In the phagocytosis assay, 67.4±5.4\% of RL\textsuperscript{+/+} and 64.5±3.0\% RL\textsuperscript{-/-} macrophages internalized FITC-\textit{E.coli}. 
2.3.2 RNase L deficiency represses migration ability of BMMs

We next studied the influence of RNase L on macrophage migration by using in vitro transwell cell migration assay. BMMs were seeded on transwell filter inserts with pore size at 8μm. M-CSF, GM-CSF and CCL2 were added respectively to trigger macrophage migration. Migrated cells were stained (red dots) with eosin.

As shown in Figure 2.11A, fresh serum, M-CSF, GM-CSF, and CCL2 all successfully triggered macrophage migration in vitro. Moreover, not only after treatment of M-CSF, GM-CSF, and CCL2, but also in normal condition (Serum free and Serum group), RNase L deficiency resulted in significantly reduced migration of macrophages when compared with wild type control, and the difference in the migration level between RNase L \(^{+/+}\) and RNase L \(^{-/-}\) macrophages was more clear with statistic analysis (Figure 2.11B). This result indicated the involvement of RNase L in macrophage migration. Furthermore, RNase L was capable to modulate macrophage migration without any inducement, suggesting that the regulation of RNase L on macrophage migration was independent of M-CSF, GM-CSF, and CCL2 inducement, and RNase L plays a central role in macrophage migration.
Figure 2. 11 RNase L deficiency represses migration ability of BMMs.

(A) Eosin staining and (B) migrated cell number counting showed that, not only after treatment of M-CSF, GM-CSF, and CCL2, but also in normal condition (Serum free and Serum group), RNase L deficiency resulted in significantly reduced migration of macrophages when compared with wild type control,
2.3.3 Effect of RNase L on macrophage cytokine production

Macrophages are known to secrete a large number of cytokines and chemokines, which in turn regulate the characteristics of macrophages (22). Since RNase L has strong capability to modulate macrophage migration, we were intrigued to study the effect of RNase L on macrophage migration inducing factors. In our study, the secretion of CCL2, TGF-β, M-CSF and IL-10, which were found to be positively associated with macrophage migration, were determined in RNase L +/+ and −/− macrophage cell culture medium by using ELISA. Our data showed that secretion of CCL2 was greatly reduced without RNase L after induction by LPS or M-CSF, whereas the reduction was not clearly detectable without treatment (Figure 2.12A). Similar results were obtained for TGF-β and IL-10 (Figure 2.12B, D). However, the M-CSF secretion level was not reduced in the deficiency of RNase L, and in contrast, a larger amount of M-CSF was detected in RNase L −/− macrophage cell culture medium after LPS treated (Figure 2.12C).

To extend this finding from primary macrophage cells to mouse macrophage cell line RAW264.7, we constructed a RAW 264.7 cell line in which RNase L was constitutively knocked-down by shRNA lentiviral particles. Lentiviral particles are provided as transduction-ready viruses, delivering a shRNA encoding plasmid to target cells for stable knock-down of a target gene. Cell colonies stably transduced with shRNA Lentiviral particles were selected by puromycin for two weeks. The RNase L protein level was then determined in selected colonies and as shown in Figure 2.13A, RNase L was clearly knocked down in the RAW106 cell line in comparison to RAW Con which
was transfected with shRNA control lentiviral particles. To confirm the effect of RNase L on macrophage migration related factors, we performed an ELISA assay to determine the secretion level of CCL2, TGF-β, M-CSF and IL-10 in RAW Con and RAW106 cells. As shown in Figure 2.13B, similar to what we observed in BMMs, a reduction level of CCL2, TGF-β and IL-10 was found in RAW106 cells compared to RAW Con cells, only treated with LPS or M-CSF. In contrast to other migration inducing factors, the level of M-CSF was elevated in RNase L knocked-down macrophages compared to RAW Con cells. Nevertheless, our results demonstrated that RNase L positively regulates the secretion of CCL2, TGF-β, and IL-10 in macrophages. With the reduction of those migration related factor, deficiency of RNase L may consequently resulted in declined migration ability of macrophages.
Figure 2. 12 Effect of RNase L on BMMs cytokine and chemokine production. Secretion of CCL2 (A), TGF-β (B) and IL-10 (D) was greatly reduced in RNase L deficient BMMs after induction of LPS or M-CSF, whereas the reduction was not clearly detectable without treatment. (C) M-CSF secretion level was not reduced, but elevated in the deficiency of RNase L after LPS treatment.
Figure 2. 13 Effect of RNase L on migration inducing factors in RAW264.7 cells.

(A) In RAW106 cells, RNase L was successfully knocked down compared to RAW Con cells. (B) Similar results of the secretion of migration inducing factors were obtained in RAW106 and RAW Con as in BMMs.
2.4 Discussion

This study is the first time to demonstrate that RNase L is associated with macrophage functions, including endocytosis, migration, and cytokine production, which all have crucial significances for the innate immune system. Although other groups have reported that RNase L plays an important role in innate immunity through antiviral immune response (41), the involvement of RNase L in macrophage functions has never been determined.

In phagocytosis and receptor-mediated endocytosis, macrophages have evolved a restricted number of receptors, like the mannose receptor, that recognize conserved motifs on pathogens. However, in phagocytosis and receptor-mediated endocytosis, different receptors and underlying molecular mechanisms were utilized for internalizing different agents. FITC-Dextran is mainly taken up through the mannose receptor into macrophages (42, 43), and the entry of FITC-\textit{E.coli} requires Fc receptor and scavenger receptors (44, 45). Our results suggested that RNase L may stimulate mannose receptor-mediated endocytosis. Transient transfection with the mannose receptor cDNA revealed that mannose receptors were sufficient to mediate phagocytosis of Candida albicans, zymosan, Pneumocystis carinii, Klebsiella, and mycobacteria, as well as endocytosis of radiolabeled mannose-BSA glycol conjugate, indicating that the mannose receptor is both a major phagocytic and an endocytic receptor and is able to engage pathogens directly (46-48). Therefore, no alternation on phagocytosis of FITC-\textit{E.coli} does not rule out the possibility that RNase L may also influence on macrophage phagocytosis, especially a
process to enter cells via mannose receptor. Ongoing work is focused on elucidating the molecular mechanism underlying the regulation of RNase L on mannose receptor-mediated endocytosis.

Macrophage migration is regulated by cytokine profile it produced. In a complete cell culture medium, deficiency of RNase L resulted in decreased macrophage migration activity, whereas a strong alternation was only observed in TGF-β expression, but not CCL2, IL-10, or M-CSF. This experiment suggested it is with high possibility that TGF-β, rather than CCL2, IL-10, and M-CSF, is involved in the direct regulation of RNase L on macrophage migration. In addition, RNase L played an important role in the induction of CCL2, TGF-β, and IL-10 by both LPS and M-CSF, and with the positive correlation between those factors and macrophage migration, RNase L may indirectly stimulate macrophage migration. Although those speculations were not confirmed here, further studies will provide insights into this hypothesis.

In summary, we provide the data to indicate that RNase L is not only required for efficient macrophage endocytosis, but also migration ability. We further report that RNase L has the capability to regulate macrophage cytokine profile, which may be involved in regulating macrophage migration ability. Taken together, our findings provide new insight into how RNase L regulates macrophage functions, and suggest a novel role of RNase L in immunity.
2.5 References


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CHAPTER III

RNASE L MANIPULATES TUMOR-ASSOCIATED MACROPHAGES (TAMs) IN TUMOR GROWTH

3.1 Introduction of tumor-associated macrophages (TAMs)

3.1.1 Macrophage polarization

Macrophages display significant heterogeneity in function, with remarkable plasticity and can change their physiology in response to local environmental factors (1, 2). Different environmental stimuli activate macrophages to express distinct chemokines, cytokines and surface markers, and ultimately give rise to various subsets of macrophages with diverse functions in inflammatory and noninflammatory settings (2, 3). According to their ability to regulate inflammatory response, macrophage activation has
been operationally classified into 2 major polarization states. Type 1 macrophages (M1) are generally characterized by elevated expression of inflammatory cytokines, including interleukin (IL-12), tumor necrosis factor α (TNF-α), reactive oxygen species and nitric oxide (NO). M1 type macrophages are induced by endo- and exogenous stimuli, such as IFN-γ and LPS, and are involved in Th1 type response and engaged of Toll-like receptors (TLRs) (4). Type 2 macrophages (M2), also known as alternative activated macrophages, are diverse, but in general, are induced by IL-4, IL-13, IL-10 and TGF-β (3, 5) and involved in Th-2 type response, including humoral immunity and wound healing (4, 5). Also, M2 macrophages have low pro-inflammatory cytokine expression and are believed to participate in the blockade of inflammatory responses and in the promotion of tissue repair and tumor progress (3, 6-8). Systematic analysis of M1 and M2 macrophages revealed that a distinct stimulus is able to induce a specific macrophage phenotype with unique functional properties (Figure 3.1).
Figure 3.1 The orchestration of macrophage activation and polarization. (BioLegend)
3.1.2 Dual role of macrophages in tumor growth

As previously described, monocytes are recruited from the circulation into surrounding tissues, either normal healthy tissues or sites of injury, inflammation, malignancy where they differentiate into tissue macrophages. Macrophages, as well as dendritic and natural killer cells, are attracted into tumor tissues.

The local microenvironment is critical for macrophage differentiation and activation. Investigators have found that macrophages infiltrated into tumor tissues have a dual role on tumor progression through either helping or inhibiting tumor cell growth (4, 9). In tumors, macrophages activated by microbial products or interferon γ (IFN-γ) to express an M1 phenotype and express high levels of proinflammatory cytokines and major histocompatibility complex. The classical phenotype of macrophages (M1) in tumors participate in the immune response against tumor progressions by directly presenting tumor antigens to activate T cells and rapidly secreting cytokines to attract more dendritic and NK cells, which could stimulate the cytotoxic lymphatic system against tumor growth. Additionally, in situ evidence proved that macrophage phagocytosis is also a critical mediator of tumor immune surveillance to phagocytose tumor cells (10).

However, recent work has shown that tumor cells, besides having the capability to acquire stem-cell like machinery to allow self-renewal, are also able to escape the immune system when tumor immunosurveillance is not sufficient (11). Once tumor cells
escaped, some of them are capable to impair antigen processing and presenting pathway, or suppress the induction of pro-inflammatory cytokines, resulting in deficiency of immune detection (11). At this stage, the immune system would help rather than inhibit tumor growth, notably by promoting the tumor cell survival and infiltration. In this regard, the mostly known switch in the immune system is alternative activated macrophages. Macrophages are likely to differentiate or switch from their immunosurveillance phenotype to an immunosuppressive phenotype, which would promote rather than suppress tumor growth. The immunosuppressive macrophages phenotype presented in tumor tissues are referred to as tumor-associated macrophages (TAMs), which produce less inflammatory cytokines, promote Th2 T-cell responses, have poor antigen presenting capability, suppress T-cell proliferation and increase local angiogenesis (2, 11-16). It should be noted that in most clinical cases, patient prognosis in solid tumors is generally correlated inversely with TAM density. In the PyMT mouse model of breast cancer (17, 18), higher populations of macrophages are recruited at the adenoma intraepithelial stage when the tumors progressing to malignancy (4, 18-21). There is also strong evidence shown the association between poor survival and increased macrophage density in thyroid, lung, and hepatocellular cancers (22-24).

M2 phenotype macrophages influence multiple steps in tumor development, including tumor growth, invasion, angiogenesis and suppression of antitumor immune response by releasing proteases, angiogenic factors, and cytokines (4, 12, 25). Studies have shown that TAMs express a number of factors, which stimulate tumor cell proliferation and survival, including epidermal growth factor (EGF) (26, 27), platelet-
derived growth factor, TGF-β1, hepatocyte growth factor, and basic fibroblast growth factor (bFGF; 28). Macrophage synthesized EGF and CSF-1 (also known as M-CSF) are also found to stimulate tumor cell invasion. Results from co-cultured experiments with tumor cells and macrophages indicated that tumor cell invasion was stimulated by EGF and CSF-1(12, 26). TAMs also release a wide range of cytokines to stimulate tumor angiogenesis, including VEGF, TNF-α, IL-8, bFGF and a broad array of angiogenesis-modulating enzymes, as MMP-2, MMP-7, MMP-9, MMP-12, and cyclooxygenase-(COX-2; 29-31), leading to the formation of new, mature blood vessels (11).
Figure 3.2 The dual role of macrophages in tumor tissue.

(Chrystelle Lamagna, Michel Aurrand-Lions and Beat A. Imhof J Leukoc Biol, 2006.)
Figure 3.3 The roles of different subpopulations of TAMs in tumor progression.

(Claire E. Lewis and Jeffrey W. Pollard. Cancer Res, 2006)
3.1.3 The recruitment of macrophages into the tumor mass

Both M1 and M2 phenotype macrophages and their monocyte progenitors are recruited to the tumors from the blood circulation after released from bone marrows. Macrophages may also migrate to tumors from the surrounding tissue (32). Most of monocytes, which become TAMs in the tumor, are attracted into the tumor mass by the chemokines and cytokines in tumor microenvironment. CCL2 is firstly identified as a tumor-derived chemotactic factor (33). Early work shows that CCL2 induces recruitment of monocytes into fibrosarcomas to enhance tumor growth (34). The low-level expression of CCL2 by tumor cells correlates with a reduced monocyte infiltration and its absence is associated with increased survival in cervical cancer patients. In addition, in the model of choroidal neovascularization, reduction of the number of infiltrating macrophages is observed with inactivation of CCR2, the receptor of CCL2 (35). With its essential role in macrophage recruitment, CCL-2 is found to be overexpressed in a wide range of cancers (36) and is associated with poor prognosis in breast, colorectal, and thyroid cancers (37-40).

Moreover, it is well established that M-CSF is also a critical factor implicated in the recruitment of macrophages into neoplastic tissues (41). It has been shown that infiltration of monocytes into tumors is reduced markedly in the absence of M-CSF, resulting in delayed angiogenesis and tumor progression. Overexpression of M-CSF is associated with poor prognosis in breast, ovarian, endometrial, prostate, hepatocellular, and colorectal cancer (36, 42-47). Tumor growth factor (TGF-β) has also been reported
to be associated with the presence of macrophages within tumors. Studies revealed that in patients with gallbladder cancer, expression of TGF-β increases along with cancer progression and strongly influence angiogenesis and macrophage infiltration, which contributes to tumor proliferation (48, 49).
Figure 3. 4 Macrophage recruitment in tumor tissues by CSF-1 and CCL2.

(Binzhi Qian and Jeffrey W. Pollard. Cell, 2010)
3.2 Materials and Methods

3.2.1 Allograft tumor models

Allograft mouse tumor systems, also known as syngeneic models, consist of tumor tissues derived from the same genetic background as a given mouse strain. Since the cancer tissues and the host mouse share genetic background, the transplant is not rejected by the host’s immune system. In this experiment, $\text{P53}^{-/-}$, $\text{RL}^{-/-}$ fibrosarcoma cells were injected subcutaneously into RNase L wild type and knocked-out C57BL/6 mice, respectively with $4.3\times10^6$ cells for each mouse. Mice with the same gender and age were used in this experiment. Tumors were formed and continued to grow for 3 weeks and then excised.

3.2.2 Immunofluorescence staining

Immunofluorescence staining is one specific example of immunohistochemical staining, which utilize the enzyme, such as peroxides, conjugated on antibodies to catalyze a color-producing reaction, and therefore allows visualization of the location of biomarkers or proteins in tissue. In immunofluorescence staining, a fluorescent dye, instead of peroxides, is conjugated with an antibody to visualize specific antigens. In this experiment, tumor tissues isolated from mice were immediately fixed in 10% formalin, embedded with paraffin and sectioned at $5 \, \mu\text{m}$ (Department of Oral Pathology, Case Western Reserve University School of Dental Medicine). Tumor tissue section
were de-paraffinized in xylene for 3 times, hydrated with serially diluted ethanol (90%, 70%, 50%, 5 min each), and then washed with distilled water for 20 min. Antigens were retrieved by incubating in a sodium citrate buffer at 95°C for 20 min. This process is necessary because, fixatives used to preserve cellular morphology, also causes protein cross-linking, resulting in the inability of some protein epitopes to bind complementary antibodies. Antigens retrieve could improve staining by modifying the molecular conformation of ‘target’ proteins. Tissue sections were first blocked with 10% donkey serum for 1 hr, then incubated with goat anti-mouse F4/80 polyclonal antibody overnight at 4°C, which followed by incubation with AlexFluor488 (Cell signaling, MA) conjugated donkey anti-goat IgG antibody for 1 h. Nuclei were visualized with DAPI (Vector Laboratories Inc., Burlingame, CA). Isotype controls were simultaneously performed using isotype-matched IgG, corresponding to primary antibody. Immunofluorescent images were generated using a Leica DMI 6000B automated inverted fluorescence microscope.

3.2.3 Determine cytokine expression in tumor tissues with ELISA

As described in 2.2.4, ELISA was used to assess the production level of CCL2, IL-10, and TGF-β in tumor tissues from both RNase L wild type and knocked-out mice. ELISA kits (eBioscience, CA) were used for this assay.
3.2.4 Macrophage depletion

Clodronate liposome was used to experimentally deplete macrophages. Clodronate is captured in liposome, which will be ingested and digested by macrophages. Clodronate is then released out and accumulated in macrophages till a certain concentration to induce macrophage apoptosis. Using clodronate liposome to deplete macrophages has been applied in various models of autoimmune disease, transplantation and tumor research.

In this experiment, clodronate liposome was used to deplete macrophage in both wild type and RNase L knocked out mice. Clodronate was purchased from Roche Pharmaceuticals (Germany) and was encapsulated in liposomes by Encapsula Nanosciences (Nashville, TN). Control liposomes containing phosphatidylcholine and cholesterol without clodronate was injected into the control groups. Four mice were used in each group, with P53⁻/⁻ RL⁻/⁻ cell injection. Mice were injected with clodronate liposome 0.2mg Intraperitoneally (i.p.) 3 days before carcinoma cell injection. Carcinoma cells were cultured and suspended in PBS with concentration at 2x10^6/100μl. Mice with the same gender and age were injected with 100μl cell solution subcutaneously, which followed by another 3 times clodronate liposome (0.1mg) injection on day1, 2, and 6 to continuously deplete macrophages (Figure 3.4). In control group, mice were received with the same volume of control liposomes and carcinoma cells. To evaluate the effect of clodronate liposome, tumors from mice were stained with an anti F4/80 polyclonal
antibody and analyzed under a microscope as described in immunofluorescence assay (3.2.2).

Figure 3.5 Time scale for macrophage depletion experiment.
3.3 Results

3.3.1 RNase L promotes tumor growth

Based on previous results that RNase L impacts on macrophage phagocytosis and migration ability, we were prompted to hypothesize whether RNase L would suppress tumor growth via its modulation on macrophage innate immunity. To test our hypothesis, we used an allograft tumor model. A fibrosarcoma cell line with mutation of P53 gene were injected subcutaneously into gender and age matched RNase L\(^{+/+}\) and RNase L\(^{-/-}\) mice. P53 is a tumor suppressor gene, which mutation results in severely reduced tumor suppression. After implantation of tumor cells, tumor growth was monitored and tumors were excised from mice after 3 weeks.

Surprisingly, tumors grew in RNase L\(^{+/+}\) mice were significantly larger than that in RNase L\(^{-/-}\) mice (Figure 3.5A). RNase L-wild type mice with carcinoma cells injection produced, on average, 3-fold greater weight of tumors than that in RNase L\(^{-/-}\) mice (Figure 3.5B). This observation was consistent in both female and male mice. The results suggest that RNase L promotes rather than suppresses tumor growth, which was opposite as we expected. It has been reported that although classically activated macrophages are important effectors in anti-tumor immunity, macrophages in tumors are more likely to enhanced tumor progression in the majority of experimental and clinical cases (4, 11, 12). Thus, our results implicated that RNase L may promote tumor growth through impacting
M2 macrophage function.

Figure 3. 6 RNase L promotes P53⁺⁺ RL⁻⁻ tumor growth in mice.
3.3.2 RNase L affects macrophage recruitment in tumor growth

It should be recognized, however, that although classically activated macrophages are effectors in anti-tumor immunity, alternatively activated TAMs enhanced tumor progression to malignancy. Macrophage polarization and recruitment in a tumor are important for tumor cell survival, polymerization, and invasion (4, 12). Based on the current results, we next determined macrophage distribution in tumor tissues. We performed immunofluorescence staining to detect macrophage presence in tumor tissues from RNase L \(^{+/+}\) and RNase L \(^{-/-}\) mice with P53 \(^{-/-}\) RL \(^{-/-}\) cell injection. Tumor tissue sections were stained with an antibody that specifically recognizes the macrophage marker F4/80 and nuclei were stained with DAPI. Interestingly, we observed clear difference of macrophage presence in the tumors from RNase L \(^{+/+}\) and \(^{-/-}\) mice. As shown in figure 3.6A, macrophages were observed only to gather in tumors from RNase L \(^{+/+}\) mice. By contrast, this accumulation of macrophages was not detected in tumors from RNase L \(^{-/-}\) mice. This result indicated that RNase L contributes to macrophage accumulation in tumor tissues. Because in most clinical and experimental models, macrophages in tumor tissue promote tumor cell growth and tumor progression, the effect of RNase L on macrophage recruitment and accumulation may explain the outcome.

To confirm that less extent of macrophage recruitment in tumors is resulted from deficiency of RNase L, we investigated the RNase L protein level in tumor tissues. As shown in Figure 3.6B, with the same P53 \(^{-/-}\) RL \(^{-/-}\) carcinoma cell injection, tumors from
RNase L $^{+/+}$ mice still expressed a high level of RNase L, whereas expression of RNase L in tumors from RNase L $^{-/-}$ mice was significantly reduced.

Figure 3. 7 RNase L facilitates macrophage recruitment in tumors.
3.3.3 RNase L modulates tumor microenvironment

The immunofluorescence staining results demonstrated that macrophage recruitment was remarkably reduced in the mice with deficiency of RNase L. It is well established that macrophage infiltration, differentiation and activation in tumor tissues are mainly regulated by tumor microenvironment, including various cytokines and chemokines. As described in 3.1.2 and 3.1.3, M-CSF, IL-4 and IL-13 are known to induce M2 type macrophage polarization, and CCL2, M-CSF, TGF-β and VEGF secreted in tumors are important to attract macrophage into tumor tissues. To determine the involvement of RNase L in tumor microenvironment, we evaluated the expression level of some factors important for macrophage recruitment in tumors. Our results showed that there was a twofold decrease of CCL-2 expression in tumors from RNase L-deficient mice, compared to that in the tumors from RNase L wild type mice (Figure 3.7A). Similarly, the expression of TGF-β and M-CSF was positively associated with the level of RNase L in the tumors. The higher RNase L was expressed, the more TGF-β and M-CSF were produced (Figure 3.7B, C). The results implicated that RNase L was involved in regulating the production of CCL2, M-CSF, and TGF-β in tumors, as a result, macrophage recruitment in tumors from RNase L deficient mice was reduced.
Figure 3. 8 RNase L regulates CCL2, M-CSF, and TGF-β expression.
3.3.4 Macrophage depletion

Previous data suggested that RNase L is required for macrophage recruitment in tumor tissues, and indicated that RNase L may contribute to tumor growth by increasing macrophage presence. To provide further evidence about the role of macrophages population in P53\(^{+/−}\) RL\(^{−/−}\) tumors, macrophages were experimentally depleted in vivo by injecting mice with liposome encapsulated clodronate. Cladronate liposome causes transient depletion of macrophages in the liver and spleen within 24 hrs and the population is not restored in 2 weeks thereafter (50). In this study, RNase L \(^{+/+}\) and \(^{−/−}\) mice received cladrosome or control liposome 3 days prior to tumor cell inoculation, and 1, 2, 6 days post injection (51-53). Mice were sacrificed 3weeks later, and the tumors from cladronate liposome treated mice were significantly smaller than that from the control group (Figure 3.8). Depletion of macrophages effectively reduced tumor growth in both RNase L- wild type and -deficient mice, while there was no effect from control liposome treatment. In addition, the difference of size and weight of tumors between RNase L\(^{+/+}\) and RNase L\(^{−/−}\) mice after macrophage depletion treatment was similar, suggesting that the impact of RNase L on tumor growth might be mainly from the effect of RNase L on macrophages.

To determine whether cladronate liposome effectively eliminated macrophages, tumor sections were incubated with antibodies specific for the macrophage antigen F4/80.
As shown in Figure 3.9, the infiltration of macrophages in tumors from RNase L $^{+/+}$ mice was significantly decreased, confirming that macrophages were efficiently depleted.

Figure 3.9 Tumor growth after macrophage depletion.
Figure 3. 10 Clodronate liposome depleted macrophages in tumors.
3.4 Discussion

Based on previous findings that the role of RNase L plays in macrophages, we were prompted to hypothesize whether RNase L suppresses tumor growth via its modulation of macrophages endocytic and migration ability. Carcinoma cell lines with mutation of P53 gene were used to test our hypothesis. However, we found that with injection of P53<sup>-/-</sup> RL<sup>-/-</sup> carcinoma cells, tumors grew significantly bigger in RNase L<sup>+/+</sup> mice compared to that in RNase L<sup>-/-</sup> mice. The results suggested that RNase L promotes rather than suppresses tumor progression. In recent years persuasive evidence has emerged that, in majority of clinical cases and experimental models, macrophages could enhance tumor cells invasion, proliferation and survival (4, 11, 12). Bingle et al. reported that, in over 80% of studies, macrophage density was positively correlated with poor patient prognosis (54). It has been firmly established that monocytes extravasate into tumor tissues and differentiate into a distinct type of macrophages termed tumor-associated macrophages (TAMs) (4). TAMs show greatly reduced antitumor functions, and are possibly driven by tumor microenvironment with tumor-derived molecules as IL-6, IL-8, CCL2 and PGE2. Furthermore, the tumor promotion activity of TAMs depends on the expression of a number of growth factors and cytokines, including VEGF, TNF-α, IL-8, and basic fibroblast growth factor (bFGF), which could stimulate tumor growth and angiogenesis (11, 12, 55). To definitely establish that macrophages promote P53<sup>-/-</sup> RL<sup>-/-</sup> carcinoma cell proliferation, we adapted a classical protocol in which clodronate liposome was used to deplete macrophages in a mouse model. Consistent with other
results for TAMs, we demonstrate that P53<sup>-/-</sup> RL<sup>-/-</sup> tumor growth in our mouse model was largely reduced in the absence of macrophages.

Collectively, these data indicate that RNase L does not contribute to tumor growth through its modulation of macrophage innate immunity, but may rather through its effect on macrophage density. Using immunofluorescence staining, we observed sparse macrophages presenting in tumors from RNase L<sup>-/-</sup> mice compared to that in RNase L<sup>+/+</sup> mice, suggesting that RNase L is indeed required for macrophage recruitment. Consistent with tumor size and macrophage recruitment results, we observed a reduction expression level of M-CSF, TGF-β, and CCL2 in tumors from mice that lack RNase L compared to the wild type mice, which indicates that suppressed tumor growth in RNase L<sup>-/-</sup> mice at least in part is attributed to impaired production of cytokines and chemokines. Our studies revealed for the first time the promotion role of RNase L in tumor growth, which maybe owing to its regulation on macrophage presence and efficient cytokines release. Nevertheless, it remains to be determined whether our findings extend to other carcinoma cell lines.
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CHAPTER IV
RNASE L REGULATES CYCLOOXYGENASE (COX)-2
PROMOTER ACTIVITIES

4.1 Introduction of cyclooxygenase (COX)-2

COX-2 is a unique enzyme, catalyzing the conversion of an arachidonic acid to prostaglandin G2 (PGE2), which play an essential role in inflammation pathway. There are two known forms of cyclooxygenase, COX-1 and COX-2, which are encoded by two genes (1, 2). It is known that COX-1 is wildly distributed in all cells, constitutively expressed in the majority of tissues and displays characteristics as a housekeeping gene (3). However, another isoform, COX-2 is highly expressed under stress, especially in response to growth factors, cytokines and pro-inflammatory molecules (4). COX-2 has been widely studied since it is a target of non-steroidal anti-inflammatory drugs (NSAIDs), which exert their anti-inflammatory function through inhibiting COX-2
activity to release pain (4). Studies found that COX-2 expression can be stimulated in vivo by wounding and inflammation (5, 6) and in vitro COX-2 could be induced by lipopolysaccharide (LPS) through a Tpl2 dependent pathway (7-9).

The functional relationship between the inflammation response and cancer is widely recognized. Inflammatory events can create a local microenvironment capable of promoting tumor growth progression. As an essential mediator of inflammation and pain, COX-2 also participates in the processes of carcinogenesis. COX-2 is overexpressed at sites of inflammation and tumor tissues, where COX-2 was found to enhance cell motility and invasiveness (10). Moreover, COX-2 is strongly associated with macrophages in inflammation and tumorigenesis and highly expressed in macrophages, normally with a strong correlation to enhanced macrophage migration and infiltration. In mouse cornea tissues, infiltrating macrophages near the IL-1β induced neovasculature were also COX-2 positive, and a COX-2 inhibitor was found to reduce macrophage infiltration in Lewis lung carcinoma cells (11). In Alzheimer’s disease brain, COX-2 positive macrophages were found to infiltrate perivascular spaces and neuropil (12). Studies by Masatoshi Hori et.al showed that in RAW264.7 macrophages, COX-2 inhibitors completely inhibited macrophage migration at 4 hrs (13).

Based on our previous results that RNase L is necessary for the induction of COX-2 by LPS in fibroblasts and adipocytes, together with the strong association of COX-2 with macrophage infiltration and tumorigenesis, the effect of RNase L on COX-2 in macrophages was assessed.
Figure 4.1 The current COX concept.

(David Gotlieb, 1999)
4.2 Materials and Methods

4.2.1 Determine COX-2 protein level in macrophages by Western Blot.

All western blot experiments performed here were using SDS-PAGE and wet (tank) transfer.

To determine the effect of RNase L on COX-2 expression in macrophages, BMMs from both RNase L wild type and knocked-out mice were treated with LPS (1μg/ml) or M-CSF (10ng/ml) for 14hrs, respectively. Western Blot was performed as previous described with a primary antibody specific to COX-2 (1:1000 dilution, Cayman Chemical, MI) and HRP-conjugated anti-rabbit IgG (Cell signaling, MA) as a secondary antibody.

4.2.2 RNA extraction and real-time polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction (qRT-PCR), also known as quantitative PCR is developed based on PCR, which is the most powerful technology in the laboratory to measure target DNA. Various fluorescent reporters were used in qRT-PCR, and a qRT-PCR machine could record the amount of PCR products by detecting fluorescence throughout a reaction. The fluorescent signal increases in proportion to the amount of PCR product. In this experiment, a non-sequence specific fluorescent intercalating agent, SYBR-green, was used as the fluorescent reporter. SYBR green is a fluorogenic minor
groove binding dye that exhibits little fluorescence when in solution but emits a detectable fluorescent signal upon binding to dsDNA (14; Figure 4.2).

In experiment, total cellular RNA was extracted from macrophages with TRIzol Reagent. COX-2 mRNA levels were detected using qRT-PCR with a one-step SYBR green qRT-PCR kit (Quanta Biosciences, MD) according to the manufacturer’s instruction. COX-2 primers were synthesized by Integrated DNA Technologies, Inc. Primers were also synthesized to amplify the cDNA encoding GAPDH as a control. RNA concentration was determined and 100ng total RNA was firstly reverse transcript to cDNA at 50°C for 10 min, which followed by 5 min Taq activation at 90°C, and 30 PCR cycles including 10 sec at 94°C and 30 sec at 60°C within the StepOne Plus Real-Time PCR system (Applied Biosystems, CA). SYBR signals and mRNA quantities were analyzed by using StepOne software.
Figure 4. 2 SYBR Green during PCR amplification.

4.2.3 Transfection and reporter assays

The reporter gene assay is used in the laboratory to investigate the genetic regulatory elements such as promoters, or transcription factors which influence the activity of regulatory elements. A luciferase reporter assay is one reporter gene assay, which is widely used because of its high sensitivity. In a reporter construct, the promoter of gene of interest was constructed to control one luciferase gene. By adding luciferase substrate, luciferase reporter activity could be measured by produced light signals (Figure 4.3).

The COX-2 promoter (-860/+127)-pGL3 luciferase reporter construct provided by Dr. Narkunaraja Shanmugam was transfected into RNase L^{+/+} and ^/- MEFs by using lipofectamine 2000 (Invitrogen, CA). After transfection, LPS (1μg/ml) was added to induce COX-2 promoter activity for 14hrs. Transfection efficiency was monitored by simultaneous transfection of galactosidase constructs and the relative luciferase value (RLV) was calculated by the ratio of luciferase activity divided by the activity of galactosidase. Groups only with COX-2 promoter transfection or LPS treatment and control group without any treatment were simultaneously performed. After adding the luciferase assay reagent, light was produced and measured in a Wallac 1420 Multilablel Counter (Perkin Elmer, MA). Experiments were repeated twice.
Figure 4.3 Schematic of the luciferase reporter assay.

(Thermo Fisher Scientific, Inc)
4.3 Results

4.3.1 RNase L regulates the induction of COX-2 by LPS and M-CSF in BMMs

Based on our previous results that RNase L is necessary for the induction of COX-2 by LPS in fibroblasts and adipocytes, we were prompted to determine the role of RNase L in COX-2 in macrophages. As shown in Figure 4.4, RNase L deficiency significantly attenuated the induction of COX-2 expression in response to M-CSF and LPS. Consistent with our earlier data, the results indicated RNase L is essential in the induction pathway of LPS and M-CSF on COX-2 expression.

4.3.2 RNase L regulates induction of COX-2 promoter activity by LPS

Furthermore, COX-2 mRNA amount in mouse embryonic fibroblasts (MEFs) was determined by real time-PCR, and similar to the protein expression, RNase L−/− MEFs failed to induce COX-2 transcription level in response to LPS, which suggests that the effect of RNase L on COX-2 stimulation by LPS is mainly at the level of transcription (Figure 4.5). Luciferase reporter assay was subsequently designed to address the role of RNase L in regulating the activity of the COX-2 promoters. RNase L−/− and RNase L+/+ MEFs were transfected with a reporter construct containing a luciferase gene under the control of COX-2 (-860/+127) promoter. β–galactosidase vectors were simultaneously transfected to equalize different transfection efficiency. After 14 hrs treatment of LPS (1μg/ml), the results showed that LPS induced COX-2 promoter
activity, whereas RNase L-deficient cells were greatly resistant to the induction of COX-2 promoter activity by LPS (Figure 4.6).

Figure 4. RNase L regulates the induction of COX-2 by LPS and M-CSF.
Figure 4. 5 RNase L regulates COX-2 mRNA transcription.
Figure 4. 6 RNase L regulates induction of COX-2 promoter activity by LPS.
4.4 Conclusion

As an essential mediator in inflammation, COX-2 was previously found to require RNase L for its expression in response to LPS in fibroblast cells. Here it is further confirmed that in RNase L deficient BMMs, the induction of COX-2 expression in response to LPS has been greatly inhibited. Moreover, our studies demonstrated that the sites where RNase L modulates the induction of COX-2 by LPS are located in COX-2 promoter ranging from -860 to +127. Studies from other groups indicated that COX-2 expression level in macrophage cells may be correlated with their migration ability and their infiltration in inflammation sites and tumor tissues. Moreover, studies implicated that overexpression of COX-2 was strongly associated with tumor progression. Consistent with those findings, RNase L also showed efficient activity to promote macrophage infiltration and tumor growth, which suggest that the promotion function of RNase L on macrophage migration and tumor proliferation may in part due to its regulation on COX-2 expression. Further studies need to investigate the detailed molecular mechanism by which RNase L regulates the expression of COX-2.
4.5 References


5.1. Introduction

5.1.1 COX-2 inhibitor Nimensulides analog Compound 2

Compound 2 is a potent anti-cancer agent structurally derived from the COX-2 inhibitor nimesulide (1). Numerous studies have demonstrated the anti-cancer activity of nimesulide. However, the nimesulide concentrations used in these studies ranged from 200 to 500µM, which greatly exceeded the concentration necessary to inhibit COX-2 activity (2-7). This line of reasoning suggests that nimesulide targeted other pathways in order to achieve anti-cancer activity, and blockage of these pathways required much
higher concentrations. This supported the hypothesis that nimesulide inhibited cancer cell
growth and induced apoptosis independent of its effects on COX-2(3, 4, 8). Structural
modifications were made to eliminate the COX-2 inhibitory activity and the
hepatotoxicity, but it was not clear if the anti-cancer activity would be improved. After
extensive investigations, the first generation non-COX-2 active nimesulide anti-cancer
derivative JCC76 was synthesized (9, 10, 11). It inhibited SKBR-3 breast cancer cell
growth with an IC\textsubscript{50} of 1.38\mu M, which was about 100 fold more active than nimesulide.
Furthermore, compound 2 was recently developed based on compound 3 (JCC76) and
has demonstrated greater anti-cancer potency (1), with IC\textsubscript{50}s of 0.1 \mu M to 0.5 \mu M to
inhibit cancer cell growth. However, the specific molecular targets of compound 3 and 2
were still unclear. Therefore, the major scheme in this experiment was to identify the
molecular targets and to investigate the biologic function of Compound 2 on it molecular
targets.

5.1.2 Tubulin as a target for anti-cancer drugs

Tubulin-containing structures are important for many important cellular functions,
including chromosome segregation during cell division, intracellular transport,
development and maintenance of cell shape, cell motility, and distribution of molecules
on cell membranes (12). The rapid growth of cancer cells leads to their high dependence
on tubulin polymerization/depolymerization, which makes tubulin a good target for anti-
cancer drug development. Paclitaxel (Taxol), the representative tubulin inhibitor
approved by the FDA in 1992 for cancer treatment, is one of the most powerful
chemotherapeutic agents currently in use. Taxol binds to tubulin and results in its precipitation and sequestration, which interrupt many important biological functions of cancer cells that depend on a dynamic tubulin polymerization and depolymerization process (13). This explains the high potency and efficacy of Taxol in fighting cancer.

Besides Taxol, FDA also approved other tubulin inhibitors with complex structures for cancer treatment including epothilone analogs, vinca alkaloid analogs, and halichondrin analogs. Treatment with tubulin inhibitors has led to improvement in the duration and quality of life for many cancer patients (14). However, the majority of them eventually develop progressive disease after initially responding to the treatment (15-17). Drug resistance of most tubulin inhibitors represents a major obstacle to overcome in order to improve the long-term response and survival of cancer patients. In addition to the resistance issue, neurotoxicity is one of the major side effects of the tubulin inhibitors derived from complex natural products, which affects the quality of life of cancer patients (18-20). Furthermore, low oral-bioavailability limits the convenient oral drug administration (21). There is an urgent need to develop new tubulin inhibitors with fewer side effects, good oral bioavailability, and less prone to the development of resistance.

5.2 Materials and Methods

5.2.1. Biotin- neutravidin Pull-Down Assay
SKBR-3 cells \((1.0 \times 10^7)\) were disrupted in a RIPA lysis buffer and sonicated, with freshly added protease inhibitor cocktail (Roche). The cell lysate was incubated with biotin-conjugated compound 2 probe, at room temperature for 1 h. The mixture was further incubated with equilibrated and packed neutravidin resin in columns at room temperature for 30 min, which followed by centrifugation and five times repeated washing with binding buffer to wash out non-binding proteins (Figure 5.1 and 5.2). The pull down assay was performed according to the protocol of neutravidin (Thermofisher). The protein interacted with the biotinylated compound 2 was cleaved from the beads by eluted with binding buffer containing compound 2. The resin was also collected and boiled with SDS buffer to determine the leftover proteins. The elution solution was boiled with 1x loading buffer (100 mmol/L DTT plus bromophenol blue) for 5 minutes and then electrophoresed on a 10% SDS-polyacrylamide gel. The resulting gel was visualized with silver stain kit (For Mass Spectrometry-Compatible Silver Staining Kit, Invitrogen, CA).
Figure 5. 1 Compound 2 probe immobilized with Biotin-neutravidin.
Figure 5. 2 Illustration of Biotin- neutravidin Pull-Down Assay.
5.2.2. Peptide Analysis of the compound 2-binding Protein via Mass Spectrometry

This process is accomplished by Dr. Kerri M. Smith. Bands visualized by silver staining were cut and transferred to 0.65 mL siliconized tubes (National Scientific Supply, Claremont, CA). The silver reagent was removed with the denature reagents (Invitrogen, CA). Proteins were in-gel digested by trypsin (Sequencing Grade, Modified; Promega, Madison, WI). The protein digest was reconstituted in 20 μL of 0.1% (v/v) trifluoroacetic acid prior to LC-QTOF/MS analysis. Peptide separation was carried out using 10-μL sample injection at 50 μL/min flow rate on a Vydac® Protein & Peptide C18 (5 μm, 300 Å, 1 mm × 150 mm) column (Grace Discovery Sciences, Deerfield, IL) proceeded by an inline filter (0.5 μm pore) (Upchurch Scientific, Oak Harbor, WA). The gradient elution profile consisted of 1% of mobile phase A for 5 min, then brought to 60% of mobile phase B over 90 min, and followed by 90 % of mobile phase B for 8 min. The total run time was 105 min. Mobile phase A was 0.1 % (v/v) formic acid in HPLC-grade ddH₂O, and the mobile phase B was 0.1 % (v/v) formic acid in HPLC-grade acetonitrile. Peptide detection was done using the positive information-dependent-acquisition (IDA) mode of AB Sciex QStar™ Elite Q-TOF mass spectrometer (AB Sciex, Foster City, CA). Data acquisition was performed using AB Sciex Analyst QS (v. 2.0). Protein identification through peptides matching was accomplished using Mascot MS/MS Ions Search (http://www.matrixscience.com).
5.2.3. Western blot

To confirm the identity of protein targets which have been discovered in neutravidin resin pull-down assay, Western Blot was conducted. Primary antibody specific to tubulin or Hsp27 (Cell signaling, MA) were incubated over night at 4°C, which followed by HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Cell signaling, MA) incubation at room temperature for 1h. Membrane was incubated in ECL plus reagent (GE health, OH) and then exposed to hyper film.

To determine the effect of compound 2 on Hsp27 phosphorylation, SKBR-3 cells were treated with 0.2μM, 0.5μM and 1μM of compound 2, 50nM, 100nM Nocodazole (Sigma, MO), and 0.5μM, 1μM KRIBB3 (Sigma, MO), respectively for 24 h. Protein lysis buffer (1% Triton X-100, 10 mM Tris·HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM Na₃VO₄) optimal to phosphorylated protein were prepared. Extracted proteins from SKBR-3 cells were loaded on 12% SDS-polyacrylamide gel, which allows a better protein separation. Antibody specific to phosphorylated Hsp27 (Ser 78, Cell signaling, MA) was used to blot membrane, and secondary antibody anti-rabbit IgG was blotted thereafter. Hsp27 and Actin antibodies were also used to confirm equal loading amount.
5.2.4. Compound 2 binding to tubulin and Hsp27

10μM bovine brain tubulin (Cytoskeleton, Denver, CO) or Recombinant Hsp27 (ProSpec, East Brunswick, NJ) were incubated with 2μM biotinylated compound 2 in binding buffer in a total volume of 500 μL for 1h at room temperature before loading onto pre-packed neutravidin resin column and further incubated for 30min. The resin was washed with 500μL of binding buffer 5 times and 2 times with buffer containing compound 2. For the colchicine, Taxol and compound 2 probe competition experiments, tubulin was pre-incubated with 10 μM colchicine or Taxol at room temperature for 1h before the incubation with the probe. The samples were fractionated by SDS-PAGE and examined with western blot assay.

5.2.5. Tubulin Polymerization Assay

Microtubule-associated protein-rich tubulin (2mg/ml, bovine brain, Cytoskeleton, CO) in buffer containing 80mM PIPES (pH 6.9), 2mM MgCl₂, 0.5mM EGTA, and 5% glycerol, were placed in cuvettes 200ul/assay, and incubated respectively with DMSO, 0.5μM and 1μM of compound 2, 5μM of compound 3, and 3μM of nacodazole. Polymerization was started by adding 1mM GTP and incubation at 37°C, and followed by absorption readings at 340nm with Varian Cary 50 Series Spectrophotometer (every 5 sec/min 0- min 3, every 10 sec/ min 3- min 5, every 30 sec/ min 5- min 10, and every 60 sec/ min 10- min 17).
5.2.6. Indirect Immunofluorescence staining

SKBR-3 cells were transferred in chamber slides and cultured to 70% confluence, and then incubated with 0.5μM or 1μM of compound 2 respectively for 12 h and 24h. In parallel, the cells without treatment were used as negative control, and cells treated with 0.05μM nacodazole for 12 and 24 hrs were used as positive control. Cells receiving different treatments were fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized with 0.2% Triton X-100 for another 10 min. After blocking with 2% goat serum for 45 min at room temperature, cells were incubated with biotinylated anti-tubulin antibody (1:200, Molecular Probes, OR) overnight at 4 °C. After washing with PBS, cells were then stained with Alexa Fluor 488 streptavidin (1:1000, Invitrogen, NY) for 45 min at room temperature, which followed by mounting with antifade reagent (ProLong Gold antifade reagent, invitrogen, NY). Fluorescently stained cells were analyzed with Leica TCS SP2 fluorescence microscope.
5.3 Results

5.3.1. Affinity purification of Compound 2-bound proteins

We hypothesized that compound 2 bound to certain proteins to achieve its anti-cancer activity. We mixed compound 2 probe with SKBR-3 breast cancer cell lysate, in order to allow specific binding proteins to attach to the compound 2 moiety of the probe. For the protein isolation, the biotin moiety of the probe was bound to the neutravidin resin to immobilize the probe. After extensive washing with binding buffer, the non-binding proteins were eluted. Then compound 2 was used as a competing agent to wash the immobilized probe, and the proteins specifically binding to the probe at the compound 2 domain were pulled out. The main procedure is described in Figure 5.1. We next examined the molecule weight of the binding proteins using SDS/PAGE and stained the gel with silver staining reagent (Figure 5.3). Lane 2 is the cell lysate, lane 3 is the final elution solution. Lane 5 is the binding buffer with compound 2 (25µM) as elution solution. Lane 6 is binding buffer with compound 2 (50µM) as elution solution, and lane 4 is the resin boiled with SDS buffer. The results suggest that compound 2 was not competitive enough to pull all the proteins out of the probe, and the resin with the probe still held a good amount of proteins. It is also possible that the competition experiment was finished on the resin packed column and only lasted 30 mins, which led to less effective competition. There are seven visible protein bands that can be identified on the gel. Protein a and b have molecular weight above 110 KD, protein c has a molecular weight about 80 KD, protein d, e and f have molecular weight about 55 to 60 KD, and
protein \( g \) has a molecular weight about 28 KD. Apparently, \( d \) and \( e \) are the major proteins bound to compound 2, the other proteins are relatively minor. However, it is too early to speculate which protein(s) is (are) the role player(s) at this stage. Minor attached proteins are not less important than the highly binding proteins. We will identify all the visible proteins on the gel in next step.
Figure 5. 3 Affinity isolation of compound 2-binding proteins.

The last buffer without drug wash solution (lane 3), two times wash solution with compound 2 (lane 5 and 6), and the resin (lane 4) were denatured in SDS sample buffer, and separated by SDS-PAGE. Lane 1 represents the molecular weight markers and lane 2 is the whole cell lysate. Visualization of the separated proteins with silver stain showed the specific binding proteins with compound 2.
5.3.3. Identification of the binding proteins by LC-MS/MS

Compound 2 binding proteins were separated by SDS/PAGE and collected by cutting the gel with the visible protein bands. After silver-staining agent was removed, the protein bands were subjected to tryptic digestion \textit{in situ}. The resulting peptide mixture was identified with mass spectrometry. The molecular weight of the peptide mass fingerprint was used to identify the protein identity via Mascot database (Matrix Science Mascot, Boston, MA). The proteins with the best score (highest possibility) are listed in Table 5.1.

Band a was identified to be the trypsin residue, which possible is the signal of the leftover trypsin in the digestion step, and band a itself did not give any countable signals. Bands b, c and f were keratin proteins with peptide mass fingerprint matching scores below 10%. These low binding, and without clear functions related to cell growth proteins are not listed in table 1. The major bands d and e were identified to be tubulin α and β, respectively. Band g was identified to be Hsp27. Both proteins are very critical for cancer cell proliferation (22).
<table>
<thead>
<tr>
<th>Protein band</th>
<th>Molecular weight (estimated based on the marker)</th>
<th>Peptides (bold and underlined) matching with potential protein</th>
<th>Percentage of the matching</th>
<th>Protein identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>d</td>
<td>58KD</td>
<td>MRECISIHVG QAGVQIGNAC WELYCLEHGI QPDQGQPSDK TIGGGDDSFN TFFSETGAGK HVRPAVFVDL EPTVIDEVRT GTRYQLFHPE QLITGKEDAA NYARGHYTI KGEIDLVLID RIRKLADQCT GLQFLVFHSS FGGGTGSGFT SLLMLRSLVD YGKSKELEFS IYFAPQVSTA VVEPYNSILT THTTLEHSDC AFMVNEAIAY DICRRNLIDIE RPTYNLNLRL ISQIVSSITA SLRFQDGALNV DLTEFQTNLV PYPRIFPLA TYAPVISAEK AYHEQLSVAE ITNACFEPAN QMVKCDPRHG KYNACCLLLRY GDVVPKDVNA AIATIKTKRS IQFVDWCPPTG FKVGINYQPP TVGGPGQDAK LQVAVCMLSN TTAIAEAWAR LDHFKDLMYA KRAFVHYWVG EGMEEGSEF AEDEMAALEK DYEEXGVDSD EGEGEEE EY</td>
<td>21%</td>
<td>Bovin tubulin alpha chain</td>
</tr>
<tr>
<td>e</td>
<td>55KD</td>
<td>MREIVHIQAG QGNCQGIAKAF WEVISDEHGI DPTGTYHGDS DLQLDR YSVY YNEATGKKV YPAILVLDEP GTMDSVGSGF FGQIPRPDNF VFQSQGAGNN WAGHYTEGA ELVDSVLDVV RKEAESCDCL QGFQLTHSLG GGTGSGMTGL LISKIREEYP DRIMNTFSVY PSSKVSSTTV EPYNATLSV QLVENTDETY CIDNEALYDI CFRKLIKTTP TYGDLLHLS LSMSGVSTCL RPPGQLNADL RKLAVNMVF PRLLHEFMGFP APILTSRSQQ YRALTVHELT OQVFDAKNMM AAQDPRHGR Y LTVAAVFR Y MSKKEVDEQM LNQVKNSSY FYEWIPNYKR TAVCDIFPRG LKMNAVTFGIN STAIQELFKR ISEQPTAMFR RKAFLHWYTG EGMDEMEFTE AESNMLDLYS EYQQYQDATA EEEEDFGEAA EEEA</td>
<td>23%</td>
<td>Bovin Tubulin beta chain</td>
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<tr>
<td>g</td>
<td>28KD</td>
<td>MERRVPOSTL LGPGSAPDGPR DYPHSRFLFD QAGGLPRLPE EWSQWLGGG HPPWYRVLPP AAAIESPAAVA PAYSRALRGQ LSGVSEIRH RTRWVRVSLDL VNHFAPDELT VKTKDGVEVI TGKHEERQDE HGYISRCFT RKYTLPPGVD TQPQSSLSPG GTLTVEAPMP LATQSNELT IPVTFEQR AQ LGGPEAAKSD ETAAK</td>
<td>25%</td>
<td>Human heat shock protein 27</td>
</tr>
</tbody>
</table>

Table II. Identify pull-down proteins by LC-MS/MS
5.3.4 Confirmation of protein identity with Western Blot

To confirm the protein identity, we repeated the SDS-PAGE assay combined with western blot. As shown in Figure 5.4, the binding proteins were confirmed by using the corresponding antibodies. Tubulin and Hsp27 as molecular targets are new observation for the non-COX-2 active nimesulide anti-cancer derivatives. It was reported that tubulin has relatively strong interaction with Hsp27, and the two proteins could be co-precipitated in the immunoprecipitation experiment (23). In our study, both proteins were pulled out by the probe, which could be due to the interaction between the two proteins. The probe might only bind either to tubulin or Hsp27, but the interaction between the two proteins could have caused them to both be retained by the probe.
Figure 5. 4 Protein identity confirmation with Western Blot.
5.3.5 Compound 2 binds directly with tubulin and Hsp27

To rule out the possibility of co-precipitation, we used tubulin and Hsp27 pure proteins and our probe to repeat the binding experiments individually. The protein binding ability was determined with western analysis (Figure 5.5). After 5 times extensive washing with binding buffer, the extra proteins were all eluted and there were no proteins detected in the fifth elution solution. However, tubulin and Hsp27 were further eluted after 2 was added in the elution solution. The results indicated that both proteins could specifically bind to the probe and could be eluted with buffer containing compound 2, suggesting that compound 2 was the ligand for both proteins.

Due to the structure similarity between compound 2 and Tubulin inhibitor 1, a colchicine domain binder, we speculated that compound 2 might also be a colchicine domain binder. To prove our hypothesis, we pre-incubated tubulin protein with an equivalent amount of colchicine for 1h, and then repeated the experiment. We found that the probe did not hold any tubulin protein (Figure 5.6), suggesting compound 2 and colchicine share same binding site and colchicine has higher binding affinity in the domain than compound 2. However, pre-incubation tubulin with Taxol did not affect the binding ability of the probe with the tubulin. These data are consistent with our hypothesis that compound 2 binds to the colchicine binding domain on tubulin. Pre-incubating tubulin with colchicine saturated the binding domain, and blocked the probe to attach to the protein, but Taxol with different binding domain did not affect the binding.
Figure 5.5 Binding of biotinylated compound 2 with pure Hsp27, tubulin.
Figure 5. 6 Compound 2 binds to the colchicine binding domain on tubulin.
5.3.6. Biological activity of compound 2 on tubulin

5.3.6.1 In vivo tubulin polymerization assay

We showed here that compound 2 binds to tubulin, however it is still not clear if the compound can interfere with tubulin function. Tubulin can be affected in two manners. Taxanes and epothilones stabilize tubulin polymerization, whereas vinca alkaloids, halichondrins and colchicine inhibit tubulin polymerization (24-27). Although binding differently, colchicine and vinca alkaloids show same mechanism of inhibition of tubulin. Compound 2, a relatively smaller and nonchiral molecule compared to the bulky and bearing multiple chiral centers natural product tubulin interfering agents, inhibited tubulin polymerization dose-dependently (Figure 5.7). Nocodazole, a well-known tubulin inhibitor, was used as a positive control for the assay. Compound 3, the first generation nimesulide anti-cancer derivative, showed moderate tubulin polymerization inhibitory activity.
Figure 5. 7 Effect of compound 2 on the organization of tubulin.
5.3.6.2 Compound 2 inhibits tubulin polymerization in cancer cells

In addition, the tubulin polymerization inhibitory effect was observed in cancer cells (Figure 5.8). After 12h treatment with compound 2 at 0.5 µM and 1µM, the microtubules were disorganized and their density was significantly reduced in SKBR-3 breast cancer cells. After 24h treatment, the effect was more pronounced. The results indicate that compound 2 could inhibit tubulin polymerization in both an assay with purified protein and in SKBR-3 cells. The results also are in agreement with the cell cycle arrest studies of compound 2 in previous studies (1).
Figure 5. Effect of compound 2 on the organization of the microtubule cytoskeleton in cancer cell.
5.3.7 Biological activity of compound 2 on Hsp27

Hsp27 is a chaperone of the sHsp (small heat shock protein) family. The common functions of sHsps are chaperone activity, thermostolerance, inhibition of apoptosis, regulation of cell development and cell differentiation. They are also partially involved in cell signal transduction (22, 28-31). Compound 2 showed significant anti-cancer activity in our previous studies (1). Tubulin inhibitory activity was responsible for the cancer cell toxic effect of the compound. Did Hsp27 play any role during the cell death since compound 2 bound to this protein? The phosphorylation of Hsp27 is the key step for Hsp27 to participate in the cell signal transduction process (23, 29). We checked the levels of phosphorylated Hsp27 and total Hsp27 in SKBR-3 breast cancer cells after compound 2 treatment (Figure 5.9). The compound significantly increased pHsp27 levels, which was similar to the response after tubulin inhibitor nocodazole treatment. Cells use pHsp27 as a protective molecule when any damage happened to the cells (22, 31). It is very common that cells express higher level of pHsp27 after being treated with cytotoxic agents (32). Compound 2 inhibited tubulin polymerization, and induced cell death, which very likely to promote the activation of pHsp27 in the cells. KRIIBB3, an Hsp27 binder, also retained Hsp27 protein via its biotinylated probe in a protein pull down assay (33). It was used as a control and it significantly inhibited Hsp27 phosphorylation (Figure 5.9). It seemed that compound 2 showed a different manner to KRIIBB3 regarding Hsp27 inhibition, suggesting that compound 2 might bind to a different domain of Hsp27 and this domain did not involve in Hsp27 phosphorylation. It was also possible that the tubulin inhibitory activity of compound 2 dramatically induced Hsp27 phosphorylation
and overridden the direct Hsp27 inhibitory effect of the compound. Tubulin function was relatively easier to check with polymerization assay. However, it was difficult to elucidate whether Hsp27 was an anti-cancer target for compound 2. Besides Hsp27 phosphorylation, the other downstream molecular and cellular consequences of Hsp27 inhibition were not well defined. The tubulin inhibition could lead to Hsp27 phosphorylation as indicated by pure tubulin inhibitor nocodazole in Figure 5.9, suggesting that tubulin function had a close correlation to Hsp27 function. It also made it difficult to solely investigate the Hsp27 effects of the dual targets compound 2. More medicinal chemistry effort is needed to develop more specific ligands and dissociate the two targets of compound 2.
Figure 5. 9 Effect of compound 2 on Hsp27.
5.4 Conclusion

A class of new anti-cancer agents were developed based on the COX-2 inhibitor nimesulide as a lead compound (1). To identify the molecular targets of the agents and elucidate the anti-cancer mechanism, we designed and synthesized the biotinylated nimesulide analog 2 as a probe. The proteins binding with the compound were isolated and subjected to mass spectrometric identification, and the most prevalent binding proteins were determined to be tubulin and Hsp27. Both proteins are very important for cancer cell proliferation. Further investigation revealed that the compound bound at colchicine binding domain on tubulin, and interrupted the polymerization of tubulin. However, the biological activity of compound 2 on Hsp27 was difficult to determine, because the tubulin inhibitory activity of compound 2 stimulated the activation of Hsp27 signals. The direct Hsp27 inhibitory effects of compound 2 could be overridden by the tubulin inhibition consequences. Studies are now underway to further optimize compound 2 in order to generate more potent derivatives. In addition, more specific ligands, which have dissociated targets, i.e., pure tubulin inhibitor or pure Hsp27 inhibitor, might be identified from the new compounds pool. The new sole ligands will allow us to further investigate the consequences of the Hsp27 inhibition.
5.5 References


6. Pan, Y.; Zhang, J. S.; Gazi, M. H.; Young, C. Y. The cyclooxygenase 2-specific


