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Role of TLR2-dependent inflammation in metastatic progression

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Abstract

Inflammation is a part of the host defense system, which provides protection against invading pathogens. However, it has become increasingly clear that inflammation can be evoked by endogenous mediators through Toll-like receptors (TLRs) to enhance tumor progression and metastasis. Here, we discuss the roles of TLR-mediated inflammation in tumor progression and the mechanisms through which it accomplishes this pathogenic function.

Keywords

Toll-like receptor; inflammation; TNF- α ; tumor progression; metastasis

Introduction

A link between chronic inflammation and cancer has been suspected since the 19th century, when Rudolf Virchow first noted that malignant tumors arise at regions of chronic inflammation and contain inflammatory infiltrates.^{1–5} Recent studies have clearly shown that chronic inflammation increases the risk of tumor development and progression.⁶ However, the source of inflammation in tumors that are not associated with chronic infection remains poorly understood.

Inflammation is a part of the host defense system originally evolved to protect organisms against invading pathogens. However, it has become apparent that inflammation can also be evoked by intrinsic mediators (endogenous mediators) in the absence of infection (Fig. 1). For instance, it has been established that necrotic cell death, resulting in the release of molecules that are normally stored within cells, such as high mobility group box 1 (HMGB1) and interleukin (IL)-1 α , acts as a potent inflammatory stimulus.^{7,8} Toll-like receptors (TLRs), which recognize both extrinsic and intrinsic mediators, play key roles in the initiation of inflammation and innate immune responses and the eventual activation of adaptive immunity.^{9,10} Interestingly and importantly, many studies have shown a correlation

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between TLR signaling and tumor progression and metastasis, and endogenous mediators, including HMGB1, have been implicated in the triggering of tumor-associated inflammation.⁷

Here, we will discuss and mechanistically explain the roles of endogenous mediators and TLR-mediated inflammation in tumor promotion, progression, and metastasis.

Endogenous and exogenous inflammatory mediators and tumorigenesis

Under normal conditions, programmed cell death is tightly regulated by the adenosine triphosphate (ATP) concentration of the cell and the balanced expression of antiapoptotic and proapoptotic proteins.¹¹ When it does occur, apoptotic cell death results in cell condensation, nuclear fragmentation, and formation of apoptotic bodies that are rapidly cleared by phagocytic cells without release of cellular constituents to the extracellular milieu.¹¹ By contrast, necrotic cell death, which occurs upon injury or upon shortage of oxygen and nutrients in the core of solid tumors, results in the release of normal cellular constituents into the extracellular space.¹² Some molecules normally kept within cells act as potent inflammatory mediators through activation of TLRs or other receptors.¹³ We refer to such molecules as endogenous inflammatory mediators and one notable example is HMGB1.⁷ Several studies showed that HMGB1, normally a component of chromatin, interacts with TLR2, TLR4, and receptor for glycation end-products (RAGE), thereby triggering inflammation through the production of chemokines and cytokines that stimulate tumor growth, progression, and metastasis.^{8,14} Several cancers, including breast cancer, colon cancer, melanoma, pancreatic cancer, and prostate cancer were found to contain higher amounts of HMGB1 than normal tissues, suggesting a close link between the necrotic release of HMGB1, inflammation, and tumor progression and/or metastasis.¹⁴ Other endogenous mediators include S100 proteins in melanoma cells, which also can trigger inflammation and enhance tumor growth.¹⁵ Heat shock proteins (HSPs) are also well known as potent activators of innate immune signaling.^{16,17} HSPs such as Hsp60, Hsp70, and Hsp90 can induce expression of proinflammatory cytokines, including tumor necrosis factor (TNF), IL-1, IL-6, and IL-12, and stimulate the release of nitric oxide (NO) and C–C chemokines by monocytes, macrophages, and dendritic cells (DCs) via TLR-dependent mechanisms.¹⁸ HSPs also induce the maturation of DCs as demonstrated by upregulation of major histocompatibility complex (MHC) classes I and II molecules, and co-stimulatory molecules such as CD80 and CD86.^{16,17} There is clear clinical evidence showing a positive correlation between elevated HSP amounts and metastatic tumor progression.^{19,20} Similar proinflammatory effects have also been reported for other cellular constituents, some of which are secreted by live cells, including fibrinogen,²¹ surfactant protein A,²² domain A of fibronectin,^{23,24} heparan sulfate,²⁵ short hyaluronan (HA) fragments (soluble HA),²⁶ (β-defensin 2-lymphoma antigen idiotype sFv fusion protein,²⁷ tRNA synthase,²⁸ and versican.^{29,30} Nucleic acids released from necrotic cancer cells or by injured normal cells can also serve as inflammatory mediators that activate nucleic acid sensing TLRs, such as TLR3, 7, 8, and 9.³¹ There is also a report showing potent induction of inflammation by a complex of DNA and HMGB1 released by necrotic cells.⁷ Importantly, the absolute amounts of circulating DNA in serum and plasma appear to have diagnostic and prognostic significance for various cancers.³² The integrity of circulating DNA, measured as the ratio

of longer to shorter DNA fragments, is higher in cancer patients than in normal individuals.³³ In general, there is growing evidence that intrinsic TLR activators that are released during tumor progression, by both dying and living cancer cells, may be responsible for the persistent low-grade inflammation found in the tumor microenvironment.³⁴ However, a full understanding of the roles played by most of these intrinsic mediators in tumor progression and metastasis is yet to be achieved.

Molecules derived from invading microorganisms such as bacteria, fungi, and viruses are the major extrinsic inflammation-inducing molecules. Such molecules are recognized by TLRs and other pathogen-recognition receptors. Approximately 15% of all cancers worldwide develop in the context of chronic infections, including gastric cancer (*Helicobacter pylori*), cervical cancer (Human papilloma virus), liver cancer (Hepatitis virus B and C), and hematologic malignancies (Cytomegalovirus and Epstein-Barr virus).³⁵ Mediators released by infectious organisms also cause chronic inflammation that promotes tumor development and progression.³⁶

Link of TLR-mediated inflammation and tumor development

Toll-like receptors (TLRs), the mammalian homologues of the *Drosophila* Toll protein, are evolutionarily conserved pattern recognition receptors.^{9,37} Eleven TLRs (TLR1 to TLR11) have been identified in humans so far. They are expressed in different types of immune cells and are located either at the plasma membrane or within early endosomes. TLRs play key roles in activation of inflammatory responses and host defense against invading pathogens by virtue of their ability to recognize conserved molecular motifs of microbial and viral origins, referred to as pathogen-associated molecular patterns (PAMPs).^{38–40} TLRs may also be activated by a number of intrinsic agonists produced by mammalian cells, such as HMGB1⁸ and HSPs.¹⁸ Any TLR agonist, either microbial or mammalian in origin, can trigger the activation of myeloid and lymphoid cells as well as stimulate DC maturation.^{7,41–43}

The first mammalian TLR to be identified, TLR4, is the receptor for lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria.⁴⁰ Since then different TLRs were found to recognize a wide range of microbial components: TLR1 (in association with TLR2) being activated by tri-acyl lipopeptides;⁴⁴ TLR2 by lipoproteins and peptidoglycans;⁴⁵ TLR3 being a receptor for double-stranded RNA;⁴⁶ TLR5 being a receptor for flagellin;⁴⁷ TLR6 (in association with TLR2) being activated by di-acyl lipopeptides;⁴⁸ TLR7 and TLR8 being receptors for single-stranded RNA;⁴⁹ and TLR9 being a receptor for nonmethylated CpG DNA.⁵⁰ No ligands have been identified as yet for TLR10 and TLR11. Upon binding of their cognate ligands, the TLRs trigger several different intracellular signal transduction pathways that culminate in induction of proinflammatory cytokines, chemokines, as well as interferons (IFNs).^{39,51,52}

Several studies have shown a statistically significant correlation between elevated TLR protein expression in tumor and clinical grade. For example, pancreatic ductal adenocarcinoma and colorectal cancers expressing higher amounts of TLR4 show larger tumor size, lymph node, and liver metastasis, respectively, and reduced overall survival rate.^{53,54} However, it is not clear whether the above-mentioned patients exhibit more

inflammation; but most likely, upregulation of TLR expression should increase responsiveness to endogenous TLR agonists produced by cancer and noncancer cells alike, which should alter the tumor environment and overall disease condition. In colon, liver, and lung tumors, which are exposed to bacteria and bacterial-derived products, elevated TLR expression should also enhance the response to microbial-derived TLR agonists. Such factors should be considered when designing improved personalized treatments for such patients.

Inflammation and cancer

Inflammation and early tumor promotion—Cancer is a chronic disease that is caused by defective genome-surveillance and aberrant signal-transduction mechanisms.⁵⁵ If infection and inflammation enhance tumor development they could do so through effects on cell cycle control, cell survival, or genomic surveillance. Chronic inflammation was proposed to act as an initiating factor in malignancy through the generation of reactive oxygen and nitrogen species (ROS and RNS), which subsequently leads to DNA damage.⁵⁶ Although persistent inflammation certainly results in excessive and prolonged generation of ROS and RNS by resident and infiltrating inflammatory cells, there is little evidence so far that this may directly result in induction of oncogenic mutations. One of the enzymes involved in free radical generation is the inducible form of nitric oxide synthetase, iNOS, which is frequently expressed not only in inflamed tissues, but also in premalignant lesions and tumor tissues.^{57,58} However, as mentioned earlier, there is little genetic evidence that chronic inflammation acts as a direct tumor initiator rather than a tumor promoter.⁵⁹ Furthermore, a mouse mutant defective in the repair of oxidative DNA lesions was found to be highly susceptible to induction of chronic inflammation, while exhibiting only a very small increase in oncogenic mutations and tumor load.⁶⁰ In addition, chronic low-grade inflammation is immunologically distinct from acute inflammation elicited by infections. The latter is characterized by CD8⁺ effector T cells, which have a central role in tumor-associated antigen (TAA)-specific immunity and thus can eliminate microbes as well as tumors.⁶¹ Activated natural killer (NK) cells also stimulate the maturation of DCs and facilitate antitumor immunity. Chronic inflammation lacks these functions, which is why it may result in net stimulation of tumor growth through a variety of mechanisms, including the production of cytokines that act as growth factors.⁶² Furthermore, it is clear that not every form of chronic inflammation can enhance tumor development. Usually, the site in which chronic inflammation occurs is of great importance, and it seems that only in tissues that are also exposed to environmental carcinogens, such as the colon or the liver, can chronic inflammation strongly enhance tumor development.⁶³

Inflammatory infiltrates in tumors and their significance—Although inflammation at certain sites can promote tumor development, it is also clear that primary oncogenic events, such as Ras oncogene activation, can trigger inflammation through effects on expression of various proinflammatory genes, especially those that code for chemokines.^{4,64} Thus, primary oncogenic events in epithelial cells can establish an inflammatory microenvironment even in tumors that do not develop in the context of chronic inflammation, and this microenvironment can have a major impact on the course of tumor progression.^{3,65} The inflammatory microenvironment of neoplastic tissues is characterized

by presence of infiltrating cells of hematopoietic origin, such as leukocytes, macrophages, dendritic cells, mast cells, and T cells.⁶⁶ However, it is still being debated whether activation of the innate immune system, whose major manifestation is inflammation, contributes to tumor promotion and progression or whether it enhances tumor surveillance and elimination.⁶⁷ It was suggested that whereas acute inflammation may inhibit malignancy through activation of T and NK cells, and induction of death cytokines such as tumor necrosis factor (TNF)- α -related apoptosis-inducing ligand (TRAIL), chronic low-grade inflammation promotes carcinogenesis through activation of macrophages and mast cells that produce tumor-promoting cytokines, such as IL-6, which enhance the proliferation and survival of cancer cells^{3,65,68}

Pollard and coworkers have shown that macrophages, major components of the inflammatory microenvironment of most tumors, are important to the growth and progression of mammary carcinomas by using macrophage colony stimulating factor (M-CSF)-1-deficient mice.⁶⁵ Such tumor-associated macrophages (TAMs) can promote tumor development and metastatic progression through multiple mechanisms, including the inhibition of antitumor T cell-dependent immunity through production of immunosuppressive indoleamine dioxygenase metabolites, inhibition of DC maturation via secretion of IL-10, transforming growth factor (TGF)- β and M-CSF, as well as attraction of T regulatory (T_{reg}) cells to the tumor.⁶⁶ In addition, TAMs produce numerous cytokines, for example, TNF- α , IL-1 β , and IL-6, chemokines, for example, IL-8, macrophage inflammatory protein (MIP) 1 and MIP2, and enzymes that catalyze production of inflammatory mediators, such as cyclooxygenase (COX)-2. All of these act to support survival, proliferation, invasiveness, motility, and metastasis of cancer cells. It is now evident that TNF- α , initially heralded for its anticancer activity,⁶⁹ can actually serve as a tumor-promoting factor,^{70,71} and a similar function has been demonstrated for IL-6.⁷² TAMs also secrete matrix metalloproteinases (MMPs) and proangiogenic factors such as vascular endothelial growth factor (VEGF) that stimulate invasion of surrounding tissues and angiogenesis, as well as ROS and RNS that enhance genomic instability, cell proliferation, and tumor progression.^{73,74}

The capability to express distinct functional programs in response to different microenvironmental signals is a key feature of macrophages, which are typically displayed during pathological conditions such as infections and cancer.⁷⁵⁻⁷⁷ In response to cytokines and microbial products, mononuclear phagocytes express specialized programs, manifested by different cytokine production profiles that are referred to as M1 and M2. Classically activated M1 macrophages are induced by IFN- γ , either alone or in concert with microbial stimuli, for example, LPS, or by combinations of cytokines, for example, TNF- α and granulocyte-macrophage colony-stimulating factor (GM-CSF). On the other hand, IL-4 and IL-13 induce alternatively activated M2 macrophages.⁷⁸ M1 and M2 macrophages display a number of distinct features. M1 macrophages are characterized by high capacity to present antigens, high IL-12 and IL-23 production, and consequent activation of type I T cells that may have cytotoxic ability toward tumor cells.⁷⁹ On the other hand, M2 macrophages have poor antigen-presenting capacity; have an IL-12^{low} IL-10^{high} cytokine expression phenotype; suppress inflammatory responses as well as Th1 adaptive immunity; actively scavenge cellular debris; and promote wound healing, angiogenesis, and tissue

remodeling.⁷⁶ Earlier studies with TNF- α -stimulated macrophages or TAMs indicated that under certain conditions these cells display cytotoxic functions against cancer cells.^{80,81} However, it is clear that in the absence of M1-orienting signals, TAMs promote cancer cell growth *in vitro* and *in vivo* partially through the release of angiogenic and lymphoangiogenic factors that promote lymphatic metastasis of cancer cell.^{77,81,82} Importantly, in many human tumors a high TAM content has been associated with poor prognosis.^{1,66,82}

Myeloid-derived suppressor cells (MDSCs), which can be recruited by IL-6 and IL-1 β , are also an important component of the tumor microenvironment.⁵⁴ MDSCs have been recently recognized as critical mediators of cancer progression. They inhibit the antitumor immune responses by release of arginase, NO₂, and TGF- β .^{83,84} Plasmacytoid DCs that express CXCR4 are also recruited into the tumor microenvironment and potentiate IL-10 production by T cells and therefore act as immunosuppressants.⁸⁵ Vascular DCs in the tumor microenvironment increase tumor vascularization and subsequent metastasis.⁸⁶ Cancer-associated fibroblasts (CAFs) are another important component of the tumor stroma and the inflammatory microenvironment. Unlike normal fibroblasts, CAFs are perpetually activated and can produce a variety of chemokines.⁸⁷ The origin of CAF is not well understood, but they appear to be as important as immune cells in the tumor microenvironment.⁸⁸ A recent study proposed that TGF- β has a crucial role in CAF activation.⁸⁹ Activated CAFs promote the proliferation and progression of cancer cells through the production of growth factors and metalloproteinases,⁹⁰ and can attract immune and inflammatory cells through chemokine production.⁹¹ Therefore, a TLR-related increase in TGF- β production might lead to recruitment and activation of CAFs in the tumor microenvironment.

The mechanisms that lead to inflammatory cell recruitment and activation within tumors to promote growth, angiogenesis, and metastatic progression are not fully understood, and need to be further investigated. However, in various models of cancer metastatic progression, the appearance of distant site metastasis was found to correlate with infiltration of the primary tumors with various immune and inflammatory cells. For instance, immune cells that express high levels of the TNF family members RANK ligand (RANKL) and lymphotoxin (LT) α are likely to promote metastatic progression in the TRAMP model of prostate cancer.⁹² RANKL was also found to stimulate the metastatic growth of mouse and human mammary carcinomas (W. Tan *et al.*, in preparation), and clinical trials suggest that it is also of importance for bone metastasis.⁹³ Such findings suggest that activation of genetically unaltered myeloid and lymphoid cells in the tumor microenvironment can provide a major impetus to tumor growth, survival, angiogenesis, and metastasis.^{3,65,68} Our current mechanistic understanding of such processes will be discussed later.

Mechanisms

The inflammatory microenvironment

Cancer and inflammation can be linked by two pathways; one is dependent on an underlying inflammatory activation and the other one is not (Fig. 2). The latter pathway is activated by the genetic events that cause neoplasia, which include activation of proto-oncogenes by mutations, chromosomal rearrangements, gene amplification, and genetic and epigenetic inactivation of tumor-suppressor genes. Genetically transformed cancer cells produce

different mediators, which generate an inflammatory microenvironment even in tumors without an underlying inflammatory condition or infection, which include breast cancer. For instance, it was shown that h-Ras activation can result in increased production of the chemokine CXCL-8/IL-8 that recruits inflammatory cells that produce factors that stimulate the growth of malignant, h-Ras-transformed cells.⁶⁴ The inflammation-dependent pathway, on the other hand, is based on underlying infections or chronic inflammatory and autoimmune diseases that generate an inflammatory microenvironment rich in cytokines and chemokines that provide a “fertile soil” within which genetically transformed cancer cells can survive and rapidly proliferate (e.g., colorectal and gastric cancers). The two pathways converge through activation of transcription factors, such as NF- κ B, signal transducer and activator of transcription (STAT) 3 and hypoxia-inducible factor (HIF) 1 α .^{5,94} Within the malignant cell these transcription factors control expression of prosurvival genes, proangiogenic factors, and MMPs. Within inflammatory cells, NF- κ B controls the production of cytokines and chemokines that act on the malignant cells, as well as directing the production of proangiogenic factors, such as VEGF.⁹⁵ Interestingly, NF- κ B is also required for full activation of HIF1 α through its effect on transcription of the *Hif1 α* gene.⁹⁶ In addition to its critical role in activation of the proangiogenic program, HIF1 α is important for the survival and activation of macrophages and other myeloid cells in the oxygen-poor environment of primary tumors.⁹⁷ The concerted action of these transcription factors and the reciprocal interactions between malignant cells and inflammatory cells are likely to play a key role in formation of the inflammatory microenvironment typical of advanced tumors.

Chemokines, initially defined as soluble factors that control the directional migration of leukocytes during states of inflammation and immune responses, can be produced by multiple cell types including most human neoplastic cells⁹⁸ and play an important role in formation of the inflammatory microenvironment.² The importance of chemokines in malignant progression was first reported using mice lacking T or NK cell functions, which still exhibited typical inflammatory infiltrates when challenged with tumors, suggesting that neoplastic cells either produce chemotactic factors that recruit inflammatory cells or induce the expression of such factors in nearby host cells.⁹⁹ Importantly, certain tumor cells not only use chemokines to recruit inflammatory cells but also directly respond to these factors to further enhance their own growth and survival.^{100–102}

Inflammatory mediators and their effects on malignant cells

The microenvironment of human and murine cancers is rich in cytokines, chemokines, and enzymes that produce inflammatory mediators, which collectively modulate cancer cell migration, invasion, and metastasis (Fig. 2).^{1,2}

Of particular interest among these factors is TNF- α , a pivotal cytokine in inflammatory reactions. Induced by a wide range of pathogenic stimuli, TNF- α induces expression of other inflammatory mediators and proteases that orchestrate inflammatory responses.¹⁰³ High doses of extrinsic TNF- α cause hemorrhagic necrosis and can stimulate antitumor immunity.¹⁰⁴ However, there is increasing evidence that low amounts of TNF- α are produced by malignant and stromal cells within tumors and act as an endogenous tumor promoter.¹⁰³ TNF- α is frequently detected in human cancers, being produced by malignant

epithelial cells, as in ovarian and renal cancers, or by stromal cells, as in breast cancer.¹⁰³ A tumor-promoting function for TNF- α was first demonstrated in two-stage chemical skin carcinogenesis and later in other cancer models.^{70,103} The absence of TNF- α , or its type I receptor TNFR1, confer resistance to skin carcinogenesis.¹⁰⁵ TNF- α does not influence the initiation phase of carcinogenesis; DNA adducts and initiating h-Ras mutations occur in its absence. However, TNF- α production by keratinocytes is a critical component of tumor promotion by phorbol esters, which act via PKC α and an AP-1–dependent intracellular signal transduction pathway to induce TNF- α gene expression in keratinocytes.⁷⁰ In the absence of TNF- α , the epithelial production of other cytokines and MMPs that are thought to be important in skin carcinogenesis, and tumor–stroma communication is either delayed and/or completely absent. Similarly, in a model of chemically-induced liver cancer, TNF- α production by hepatocytes has been implicated in tumor development.¹⁰⁶ However, in a different model of liver cancer induced by chronic inflammation rather than a chemical carcinogen, TNF- α was found to be produced by the tumor stroma.¹⁰⁷ Nonetheless, stromal TNF- α serves as an important tumor promoter. In this system, as well as in inflammation-induced colon cancer,¹⁰⁸ tumor promotion depends on activation of NF- κ B transcription factor. Selective inhibition of NF- κ B in hepatocytes, or inhibition of TNF- α production by neighboring parenchymal cells, induced programmed cell death of transformed hepatocytes and subsequently reduced the incidence of hepatocellular carcinomas.¹⁰⁷ Tumor TNF- α production is associated with a poor prognosis, loss of hormone responsiveness, and cachexia. An interesting genetic link between TNF- α and malignancy was identified in renal cell cancer, where the pVHL tumor suppressor gene is a translational repressor of TNF- α .¹⁰⁹ Despite its ability to induce necrosis at high concentrations in certain cell types, TNF- α frequently acts as a survival factor due to its ability to promote NF- κ B activation.^{71,107} TNF- α also increases vascular permeability and can stimulate the migration and extravasation or intravasation of cancer cells.¹¹⁰ In certain cases, TNF- α can also act as a growth factor.⁷¹ Likewise, ablation of IKK β , a protein kinase critical for NF- κ B activation, in intestinal epithelial cells was found to prevent the development of colitis-associated cancer (CAC) induced by the procarcinogen azoxymethane (AOM) and repeated cycles of dextran sulfate sodium (DSS), which induced colonic inflammation.¹⁰⁸ Deletion of myeloid cell IKK β also interfered with CAC development, but in this case it mostly reduced tumor size rather than tumor multiplicity. More recent work has shown that part of the tumor-promoting function of myeloid cell IKK β is mediated through induction of the proinflammatory cytokine IL-6.⁷² TNF- β also promotes the development of CAC,¹¹¹ and during colitis macrophage-enriched lamina propria mononuclear cells produce high amounts of TNF- α .³¹

Another key inflammatory cytokine, IL-1 β also increases tumor invasiveness and metastasis, primarily by promoting production of angiogenic factors by stromal cells in the tumor microenvironment.^{112–114} IL-1 β is mainly produced by myeloid cells where its synthesis is subject to intricate transcriptional and posttranscriptional control.¹¹⁵ Curiously, although NF- κ B stimulates IL-1 β gene transcription it inhibits the processing of pro-IL-1 β to IL-1 β .¹¹⁶ A related cytokine is IL-1 α , which, unlike IL-1 β , is mainly secreted by epithelial cells undergoing necrosis.^{8,117} IL-1 receptor activation by either form of IL-1 can lead to induction of IL-6. Curiously, blood levels of IL-6 are elevated with age,^{118,119} due to loss of

inhibitory sex steroids.¹²⁰ Loss of hormonal suppression of IL-6 production has been implicated in the pathogenesis of several chronic diseases,¹²¹ including B cell malignancies, renal cell carcinoma, and prostate, breast, lung, colon, and ovarian cancers.¹²² Many of these cancers appear with old age, when circulating IL-6 is high. In multiple myeloma, for example, IL-6 promotes the survival and proliferation of cancer cells via activation of STAT3 and extracellular signal-regulated kinase (ERK) signaling.¹²³ Using a combination of *in vitro* experiments and mouse models, we showed that IL-1 α released by necrotic hepatocytes can induce the secretion of IL-6 by resident liver macrophages (Kupffer cells).⁸ In turn, IL-6 acts to promote chemically-induced liver carcinogenesis most likely through activation of the prooncogenic transcription factor STAT3.¹²⁴

A range of inflammatory enzymes, including COX-2, that catalyzes the conversion of arachidonic acid to prostaglandins (PG) are also induced by cytokines. COX-2 is highly expressed in colorectal, gastric, esophageal, breast, and prostate cancers and in non-small-cell squamous lung carcinoma.¹²⁵ COX-2-produced PGE₂ increases tumor invasion and metastasis and enhances production of IL-6, IL-8, VEGF, iNOS, MMP-2, and MMP-9.¹²⁶ Selective and nonselective COX-2 inhibition exerts chemo-preventive and antimetastatic activity in a variety of human cancers.¹²⁷ Most likely, this activity is due to disruption of the inflammatory microenvironment.

Inflammation and lung cancer metastasis

Lewis lung carcinoma (LLC), a commonly used mouse lung cancer cell line, has strong metastatic activity, and upon tail vein injection or subcutaneous implantation LLC metastasizes to lungs and to a lesser extent to liver, lymph nodes, adrenal glands, and bone.^{128,129} LLC cells grown as a primary subcutaneous tumor induce the expression of MMP-9 by lung endothelial cells, and this was proposed to somehow “precondition” the lung to become a preferential site for LLC cells to migrate into and establish metastatic growths.¹²⁸ Induction of MMP-9 expression in the lung by subcutaneous LLC was shown to be partially dependent on type 1 VEGF receptor (VEGFR1).¹²⁸ These results were confirmed and extended by Lyden and colleagues who found that LLC cells secrete factors that stimulate the migration of bone marrow-derived VEGFR1-positive hematopoietic cells into the lung.¹²⁹ The nature of these factors and their mode of action remained unknown until recently.

Using a biochemical approach, we have identified one of the most critical factors secreted by LLC cells as versican, an extracellular matrix (ECM) prometastatic protein that can induce the activation of macrophages and stimulate the secretion of TNF- α and other cytokines.²⁹ We found that conditioned medium collected from LLC cells activated TLR2 on macrophages to induce NF- κ B and MAP kinase (MAPK) signaling and thereby stimulate the expression of proinflammatory cytokines such as IL-6 and TNF- α . Importantly, subcutaneous LLC tumors led to TLR2 activation *in vivo*, which was important for induction of various inflammatory cytokines and chemokines in the lung, including TNF- α , IL-6, IL-1, CCL3/MIP1 α , CCL4/MIP1 β , CXCL1/MIP2, and CXCL2/KC. Both TLR2 on host bone marrow-derived cells and TNF- α have turned out to be critical for optimal metastatic growth of either tail-vein injected or subcutaneously implanted LLC.²⁹

We used column chromatography and mass spectrometry to identify that the LLC-secreted factor responsible for TLR2 activation and stimulation of metastatic growth is versican,²⁹ an aggregating chondroitin sulfate proteoglycan that was previously found to accumulate both in the tumor stroma and in cancer cells, including lung cancer.¹³⁰ Using *in vivo* experiments in TLR2 knock-out mice, we found that versican signals via TLR2 and its coreceptors CD14 and TLR6.²⁹ Versican expression, which is very high in several cancers, including lung cancer,^{130–132} is stimulated by signaling pathways that are known to be activated in cancer cells.¹³³ Furthermore, versican or fragments thereof can enhance tumor cell migration, growth, and angiogenesis, processes that are of direct relevance to metastasis.¹³⁴ Versican can bind HA, and both versican and HA are highly expressed in non-small-cell lung cancer (NSCLC), especially in advanced disease with high recurrence rates, whereas versican expression in normal lung is low.¹³⁰ In addition to HA, versican can also interact with several adhesion molecules expressed by inflammatory cells and has proinflammatory activity.³⁰ A related ECM proteoglycan, biglycan, was reported to activate both TLR2 and TLR4 on macrophages;¹³⁵ but our results indicate that the proinflammatory activities of versican rely on TLR2 and TLR6, but not on TLR4, activation. Silencing of versican expression in LLC cells eliminates their metastatic behavior.²⁹ In summary, our results suggest that LLC cells secrete versican to activate hematopoietic-derived cells and recruit them to generate an inflammatory microenvironment and to produce TNF- α that stimulates their metastatic behavior. Although the role of versican in the metastatic progression of human NSCLC remains to be confirmed, we have observed that other metastatic cells can also lead to TLR2-dependent macrophage activation, but through other secreted factors. The molecular nature of these factors remains unknown, but the principals that guide their proinflammatory and prometastatic activity are likely to be similar to those established for versican.

Similarities between metastasis wound healing and vascular remodeling

Other than the studies described earlier, it has been noted that tumorigenic and metastatic progression shares many features with the process of wound healing.^{136,137} For instance, solid tumors must induce new blood vessels if they are to grow beyond a certain minimal size. As established for the neoangiogenic process that accompanies wound healing, tumors secrete vascular permeability factors, for example VEGF, that render the local microvasculature permeable to fibrinogen and other plasma proteins.¹³⁶ Extravasated fibrinogen is rapidly clotted and invaded by macrophages, fibroblasts, and endothelial cells, and undergoes “organization” and eventually is replaced by vascularized granulation tissue, which finally becomes mature connective tissue.¹³⁶ The same sequence of events (angiogenesis) found in tumors also occurs during wound healing and in a range of chronic inflammatory diseases. Yet, the molecular alterations that allow tumors to behave like wounds that do not heal¹³⁷ (invading tumor cells continually render new vessels hyperpermeable to plasma, which does not occur in the normal wound healing process) are just being elucidated.

TGF- β , a key cytokine during embryonic development and tissue homeostasis,¹³⁸ exerts potent inhibitory effects on epithelial cell proliferation and also can deter tumor growth.^{138–140} TGF- β can be produced by myeloid cells, mesenchymal cells, and cancer

cells subjected to hypoxic and inflammatory conditions during tumor progression, and is one of the major cytokines in the tumor microenvironment.¹⁴¹ Interestingly, TGF- β in breast tumors was found to prime cancer cells for metastasis to the lungs.¹⁴² Central to this process is TGF- β -dependent induction of angiopoietin-like 4 (ANGPTL4) in cancer cells that are about to enter the circulation, which enhances their subsequent retention in the lungs.¹⁴² Tumor cell-derived ANGPTL4 disrupts vascular endothelial cell–cell junctions, increases the permeability of lung capillaries, and facilitates the *trans*-endothelial passage of tumor cells.¹⁴² Although this work describes the molecular basis for the vasculature disruptive activity of TGF- β , it is likely that the prometastatic activity of this cytokine depends on several other processes as well.

Inflammation and transcriptional control of metastatic genes

Inflammatory cytokines, chemokines, and the proinflammatory microenvironment also have a role in shaping the gene expression profile that is required for metastatic behavior of cancer cells, including genes that encode mediators of vascular remodeling, such as integrins, VCAM, and MMPs.^{66,143}

A key transcription factor in metastasis is the helix-loop-helix protein Twist, which regulates cell movement and tissue reorganization during early embryogenesis.¹⁴⁴ Suppression of Twist expression in metastatic 4T1 mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung.¹⁴⁴ Importantly, the ability of these cells to form primary mammary tumors was not affected. Loss of Twist expression hindered the entry of metastatic cells into the circulation.¹⁴⁴ Like several other controllers of tumorigenesis, Twist is likely to exert similar biological activities during metastatic progression as it does during normal development. In *Drosophila*, the *twist* gene is required for mesoderm induction;^{145,146} in vertebrates Twist is predominantly expressed in neural crest cells; and ablation of Twist in mice causes failure in cranial neural tube closure, all of these data indicate a role for Twist in migration and differentiation of neural crest and head mesenchym.^{147,148} Both mesoderm formation and neural crest development depend on a key cellular event termed the *epithelial–mesenchymal* transition (EMT), which involves the conversion of a sheet of tightly attached epithelial cells into highly mobile mesenchymal or neural crest cells.¹⁴⁹ Indeed, ectopic expression of Twist resulted in loss of E-cadherin-mediated cell–cell adhesion, activation of mesenchymal markers, and gain of motility by malignant cells.¹⁴⁴ These results suggest that Twist can contribute to invasion and metastasis by promoting the EMT developmental program. Interestingly, under certain circumstances, Twist expression can be induced in response to NF- κ B activation¹⁵⁰ and, therefore, can be upregulated in response to inflammation. This provides a mechanism through which tumor-associated inflammation may stimulate metastatic progression through induction of Twist-dependent EMT.

We have identified another mechanism by which tumor-associated inflammation can affect expression of key metastasis control genes. By studying the TRAMP model of metastatic prostate cancer we found that appearance of distant site metastasis as well as the metastatic behavior of isolated carcinoma cells are dependent on the activation and nuclear accumulation of IKK α .⁹² By examining expression of 40 known genes that either enhance

or suppress metastasis,¹⁵¹ we found that IKK α exerted its prometastatic effect by repressing transcription of the gene *maspin*.⁹² Maspin is a member of the serpin family with well-established antimetastatic activity in breast and prostate cancers.^{152,153} Repression of *maspin* transcription required nuclear translocation of catalytically active IKK α , and the two processes only occur in advanced prostate tumors that contain inflammatory infiltrates and cells that express RANKL and LT α : β .⁹² Early tumors devoid of inflammatory infiltrates do not show activated IKK α and, in contrast to late tumors, express high amounts of maspin and therefore lack metastatic activity. *In vitro*, RANKL can lead to repression of maspin-expression in an IKK α -dependent manner. Once in the nucleus, IKK α interacts with the *maspin* control region, but the mechanism through which it induces its repressive activity is not clear.

Effect of TLR agonists and cytokines on metastatic growth

Several clinical trials are on-going using TLR ligands in different types of cancer. In these trials, TLR agonists are expected to activate the host immune system including CD8+ effector T cells and NK cells, which should kill tumor cells and subsequently reduce tumor progression and metastasis. One potentially successful example is imiquimod, a TLR7 agonist.^{31,154} This drug is extensively used to treat actinic keratosis and basal cell carcinoma, and it is being studied as an adjuvant therapy for melanoma.³¹ A study of imiquimod 5% cream in 90 patients with basal cell carcinoma reported a 96% clearance rate and only two recurrences during a mean follow-up period of 36 months.¹⁵⁵ Cutaneous side effects were minimal and no systemic side effects were reported. Imiquimod induces IFN- α , IFN- γ , and IL-12, which activate APC function and TAA-specific immunity.¹⁵⁶

On the other hand, several publications have demonstrated that strong proinflammatory stimuli can stimulate tumor growth and have suggested that bacterial contamination during surgery or postoperative inflammation triggered by tissue damage can increase metastatic tumor growth in mice^{71,157,158} and human patients.¹⁵⁹ As described earlier, very high doses of TNF- α administered in close proximity to solid tumors can kill cancer cells by destroying the tumor neovasculature.¹⁰⁴ However, endogenous TNF- α at moderate amounts produced by activated inflammatory cells promotes tumor development and growth.¹⁶⁰ We found that administration of a sublethal dose of LPS, a TLR4 agonist, can stimulate the metastatic growth of colon carcinoma cells in the lung by inducing TNF- α expression.⁷¹ However, LPS administration also leads to induction of the death cytokine TRAIL,⁷¹ a type II transmembrane protein of the TNF family also known as Apo2 ligand, which in contrast to TNF- α is a weak inducer of inflammation.¹⁶¹ Administration of recombinant TRAIL suppresses the growth of tumor xenografts with no apparent systemic toxicity.¹⁶² Endogenously expressed TRAIL on the surface of NK cells can suppress the growth of liver and lung metastases.^{71,163} Inhibition of NF- κ B activation in metastatic colon cancer cells prevents and strongly enhances susceptibility to TRAIL-induced killing.⁷¹ Thus, inhibition of NF- κ B in malignant cells may be used to convert the prometastatic activity of LPS and similar proinflammatory stimuli and TLR agonists to a potent tumoricidal effect. Only future clinical studies will tell whether TLR agonists will be found to have a broad antitumorigenic activity or whether TLR stimulation should be avoided at all costs due to its tumor-promoting and prometastatic activity.

In addition to TNF- α and TRAIL, other cytokines may also affect tumor progression or regression. A considerable effort has been invested in the use of cytokines and anticytokine drugs in cancer therapy.¹⁶⁴ One of the most effective agents identified so far is IFN- α , which evokes antitumor effects in several hematological malignancies and solid tumors.¹⁶⁵ The infusion of high doses of IL-2 was also found to induce regression of renal cell carcinoma and melanoma in a minority of patients.^{166,167} Recently, numerous preclinical studies have established the ability of tumors that were engineered to produce cytokines to serve as cellular vaccines that augment systemic immunity against wild-type tumors (cytokine-based vaccines).¹⁶⁸ On the flip side of the coin, both anti-TNF- α and anti-IL-6 drugs are currently being evaluated for their antitumor activities and encouraging results have been reported.³¹

Can we use anti-inflammatory drugs to battle metastatic cancer?

As discussed earlier, there is ample evidence from experimental cancer models in rodents and investigations of human cancer that continuous/chronic inflammation stimulates tumorigenesis and metastatic progression. If so, therapies that are directed at reducing inflammation, inhibiting the function of inflammatory cytokines, or preventing the recruitment of inflammatory cells onto the tumor microenvironment could reduce cancer risk, slow tumor progression, and may even decrease the burden of metastasis. Indeed, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin was found to reduce colon cancer risk by 40–50%, and may also have a significant preventative effect in lung, esophageal, and stomach cancers.^{169,170} The ability of NSAIDs to inhibit COX-1 and COX-2 underlies their mechanisms of chemo-prevention. Other NSAIDs, for example, flubiprofen, were found to have strong antimetastatic effects that were attributed to their inhibition of platelet aggregation.¹³⁰ However, NSAIDs may act through additional mechanisms, as some NSAIDs lacking COX-inhibitory function are also effective in inhibiting colon carcinogenesis.¹⁷¹

Some of the current NSAIDs—especially those that are COX-2 selective—exert side effects, such as life-threatening stomach ulcers, heart attacks, and strokes, in a considerable number of patients,¹⁷² and this has limited their utility. The continuing study of the molecular mechanisms that lead to inflammatory cell activation within tumors and consequently stimulate tumor growth, angiogenesis, and progression should help in the identification of new therapeutic targets and aid in the design of new drugs free of such side effects. In addition, this understanding may also aid the development of vaccines and other strategies that enhance antitumor immunity.

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Figure 1.

Inflammatory mediators in tumor progression and metastasis. Endogenous molecules released by injured and necrotic cells might activate TLRs expressed on hematopoietic cells, including macrophages, and these activated cells release inflammatory cytokines and chemokines, which lead tumor progression and metastasis.

Figure 2. Cancer-related inflammation (genetic alteration versus inflammation). Genetically altered cancer cells may produce different inflammatory mediators that establish an inflammatory microenvironment within tumors to promote tumor progression and metastasis.