BREAST CANCER METASTASIS AND THE IMMUNE RESPONSE: MSP/RON SIGNALING SUPPRESSES CD8 T CELL ACTIVITY AND ENABLES METASTASIS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Many breast cancers have spawned clinically undetectable metastatic colonies even prior to diagnosis. The eventual outgrowth of these microscopic lesions causes metastatic relapse and death of an estimated 40,000 women in the US. However, how disseminated cancer cells convert to overt metastases remains largely unknown. Immunosuppression within the primary tumor microenvironment enables tumor progression; however, how tumors maintain immune suppression once outside the immune-protective environment of the primary tumor is unclear.

Macrophage Stimulating Protein (MSP) is activated via cleavage by a protease, matriptase, and binds to its receptor, Ron, which is found on epithelial cells, osteoclasts, and macrophages. Co-overexpression of *MSP/Matriptase/Ron* is a strong independent prognostic factor for both metastasis and death in breast cancer patients. Additionally, overexpression of MSP in mouse mammary tumor cells resulted in increased tumor growth rate and significantly increased spontaneous metastasis to the lungs, lymphatics and bones.

We utilized a model of breast cancer metastasis, along with tissue complementation strategies, to interrogate the role of MSP/Ron in metastasis. Tumor growth was similar between WT and Ron knockout hosts. However, loss of host Ron abrogated pulmonary metastasis, specifically by preventing the outgrowth of seeded metastatic colonies.

We discovered that tumor-bearing Ron knockout hosts had a significant increase in CD8+ T cell levels in their spleen and increased levels of infiltrating CD8+ T cells in the tumor stroma. $CD8^+$ T cells from Ron knockout hosts had increased cytolytic ability *in vitro* and *in vivo*, while ablation of $CD8^+$ T cells in Ron TK-/- hosts restored levels of metastasis. Therapeutically, BMS-777607, a Ron inhibitor, decreased lung colonization in both a prophalytic and adjuvant setting. This was dependent on $CD8^+$ T cells, as depletion of $CD8^+$ T cells in the context of drug treatment did not decrease colonization.

In summary, my dissertation shows that MSP/Ron pathway is a key mediator of conversion of micrometastases to metastatic lesions in lungs, by suppressing antitumor $CD8^+$ T cell immunity. Clinically, our findings suggest that Ron inhibitors may be immunotherapeutic drugs. This may impact clinical development of Ron inhibitors, including clinical trial design, monitoring of clinical efficacy, patient selection, and combination therapies.

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ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION TO BREAST CANCER AND THE PROBLEM OF METASTASIS

Introduction to mammary biology

The mammary gland is a milk-producing exocrine organ, comprised of the stroma (adipocytes, fibroblasts, blood vessels, inflammatory cells, extracellular matrix) and epithelium (branching ductal-lobular system) (1). Mammary gland development occurs in defined stages, consisting of embryonic, prepubertal, pregnancy, lactation and involution. During embryonic development, crosstalk between the epithelium and mesenchyme specifies the mammary bud (2). After birth, mammary development is arrested until puberty. Subsequently, during puberty, elongation of the ducts and secondary branching occurs. The final developmental fate of the mammary gland occurs with pregnancy and lactation. During this period, reproductive hormones cause the differentiation of the mammary epithelium into secretory, milk-producing lobular alveoli (3). As pregnancy progresses, milk proteins are expressed, and there is formation of lipid droplets. Finally, following lactation, there is extensive remodeling, wherein the alveolar cells and most of the mammary epithelia are removed by apoptosis, a process called involution (4).

Breast cancer is reminiscent of many aspects of mammary gland development (5). For example, during development, epithelial cells invade the stroma, undergoing repeated branching to create the ducts that deliver milk. Additionally, pregnancy results in massive tissue remodeling, accompanied by massive epithelial cell proliferation. A large number of genes influence ductal and alveolar morphogenesis, including estrogen receptor (ER), progesterone receptor (PR), and transforming growth factor-β (TGFβ), all genes that are involved in tumorgenesis (3,6). Each estrous cycle causes a burst of proliferation, and the number of menstrual cycles correlates with breast cancer risk. However, full term pregnancy can be protective in young women (7), perhaps due to the contribution of involution in removing many potentially transformed cells (4). Another proposed

mechanism is that terminal differentiation of mammary stem cells after pregnancy decreases the number of cells that can undergo future oncogenic mutations (8).

Breast cancer epidemiology, diagnosis and treatment

Breast cancer is the most common cancer among women, with approximately 1.4 million women diagnosed per year and an estimated 460,000 dying annually due to the disease (9). The incidence of breast cancer worldwide is increasing due to a number of factors, including the introduction of mammography, which allows better detection, as well as changes in reproductive patterns like delayed childbearing and decreased number of children (10).

Breast cancer is diagnosed in a variety of ways. Classic mammographic findings of breast cancer include the presence of a soft tissue masses with spikes and clustered microcalcifications, or calcium particles of various size and shapes (11). A significant limitation of mammography is the obscuring of the tumor due to the dense overlying tissue, which limits the sensitivity of detection (12). In this setting, magnetic resonance imaging (MRI) may complement mammographic screening. MRI is also typically used to screen women at the highest risk for breast cancer to avoid cumulative radiation from frequent mammographies (13). Although the increased use of screening mammography has substantially increased the detection and incidence of breast cancer (14), the effect of early screening on breast cancer mortality is less significant. Recent studies have shown that early screening is responsible for a small percentage of the reduction of breast cancer mortality, suggesting that the decreasing mortality trends may be largely due to improved treatment rather than early detection (15).

Following diagnosis based on biopsy, the patient undergoes testing to ascertain the subtype, staging and extent of the cancer to guide therapeutic decisions. Newly diagnosed breast cancers are tested for estrogen receptor (ER) and progesterone receptor (PR) expression and for overexpression of human epidermal growth factor 2 (HER2) (16). These clinical markers have both prognostic value and therapeutic value (17). Women with ER/PR+ breast cancer who receive no systemic therapy have a 10% decreased chance of recurrence at five years than patients whose tumors are ER/PR negative (18). Patients whose tumors are ER/PR+, however, are candidates for endocrine therapy as neoadjuvant or adjuvant treatment. Endocrine therapy results in a drop in the risk of recurrence of 47% for ER/PR+ patients (19). Neoadjuvant therapy refers to the systemic treatment of breast cancer before surgery, while adjuvant therapy refers to treatment postsurgery. Patients whose tumors are HER2 positive are also candidates for HER2-directed therapies, both in the adjuvant and neoadjuvant setting. Trastuzumab/Herceptin, an antibody that binds to the extracellular domain of HER2, is used in both the adjuvant and neoadjuvant setting (20). Lapatinib, a small molecule tyrosine kinase inhibitor of HER2, is used to treat women that have been previously treated with trastuzumab, and have progressed (21). In addition to the three clinical markers, molecular profiling based on gene expression is often used to characterize breast cancer proliferation and to predict response to therapy and clinical outcome (17). There are several distinct subtypes of breast cancer, originally identified by gene expression profiling. These are luminal breast cancers, HER2 enriched breast cancers, and triple negative breast cancers (22).

Luminal breast cancer, the most common subtype of breast cancer, is similar in nature to normal luminal epithelial cells of the breast, typically expressing luminal cytokeratin 8, cytokeratin 18, ER and PR (23). Luminal breast cancers are further

subdivided into luminal A and luminal B subtypes, with luminal A comprising 40% of breast cancer. Luminal A tumors are characterized by increased ER related genes, decreased HER2 cluster genes, and decreased proliferation genes (24). Luminal B, on the other hand, comprises 20% of breast cancers and are characterized by lower levels of ER related genes, expression of HER2 and higher expression of proliferation clusters (25) .

HER-2 enriched breast cancers comprise 10-15% of breast cancers and are characterized by increased levels of HER-2, proliferation gene clusters and decreased luminal and basal-like clusters. They are also usually negative for ER and PR (25).

Triple negative breast cancers include the basal-like and claudin-low cancers, and are similar to the basal epithelia of normal breast (22). They lack ER and PR as well as HER2. Basal-like subtypes comprise 15-20% of breast cancer, and are characterized by the decreased expression of luminal and HER2 gene clusters, increased proliferation, and high grade (23). Claudin-low subtype comprises 5-10% of breast cancers. Claudin-low subtypes are enriched for the expression of epithelial-mesenchymal transition genes, low expression of cell-cell adhesion genes and increased expression of immune response genes (26).

In addition to receptor testing and gene expression profiling, breast cancer is further staged using the American Joint Committee on Cancer and the International Union for Cancer Control (AJCC-UICC) classification system for tumor, nodes, and metastases (TNM). These are based on information on the tumor size (T), lymph node involvement (N) and the presence and absence of distant metastasis (M). Once the TNM status is determined, a stage of 0, I, II, III, or IV is assigned, with 0 being in situ, stage I being early stage invasive and stage IV being most advanced with distant metastases. In addition to TNM classification, grade may be taken into account during staging. Grade is based on

the differentiation and proliferation state of breast cancer cells (27). Poorly differentiated tumors are associated with worse prognosis. Accordingly, a Grade of 1, 2, or 3 is assigned, with Grade 1 tumors being well differentiated, Grade 2 tumors moderately differentiated, and Grade 3 tumors looking poorly differentiated (27).

Thus, breast cancer treatment selection depends on the stage and receptor status of the tumor. Women with early stage breast cancer typically undergo primary surgery (lumpectomy or mastectomy) to the breast and regional lymph nodes with or without radiation therapy (28). Subsequently, adjuvant systemic therapy may be offered based on primary tumor characteristics, such as tumor size, grade, number of involved lymph nodes, the status of ER and PR, and expression/amplification of HER2. For women with locally advanced breast cancer, care consists of therapy employing systemic and regional therapy. Patients with locally-advanced breast cancer sometimes receive neoadjuvant systemic therapy, with the goal being to induce a tumor response before surgery and enable breast conservation (29). Subsequent adjuvant therapy results in long-term improved distant disease-free survival and overall survival.

Targeted therapies also depend on the tumor subtype. Patients with ER/PR+ breast cancer receive endocrine therapy to reduce the risk of breast cancer recurrence and breast cancer-related mortality (30). The treatment options for ER/PR+ breast cancers are selective estrogen receptor modulators such as tamoxifen, aromatase inhibitors and fulvestrant. Tamoxifen binds to the ER, preventing estrogen binding, thereby killing estrogen dependent tumor cells (31). Tamoxifen, when compared to a control, results in a 13% absolute reduction in breast cancer recurrence, and 9% reduction in breast cancer mortality over 15-years (32). Aromatase inhibitors inactivate aromatase, the enzyme that makes estrogen from testosterone, thereby reducing blood estrogen levels in post-

menopausal women. Generally, premenopausal women are treated with tamoxifen, while postmenopausal women are treated with aromatase inhibitors (33). Fulvestrant, which inhibits binding of estrogen to ER and kills estrogen dependent cells, is approved for treatment of postmenopausal women who have progressed on prior antiestrogen therapy (34). Patients with HER2-positive breast cancer also receive treatment with Trastuzumab, an antibody that decreases proliferation of HER2 expressing tumor cells (20). Trastuzumab reduces the risk of recurrence by 9.5%, and reduces the risk of mortality by 2% (35). Patients that have progressed while on trastuzumab are subsequently treated with lapatinib, a small molecule HER2 inhibitor (36).

The majority of breast cancer recurrences occur within the first five years of diagnosis, particularly with hormone receptor-negative disease. The five-year survival rate for those who present with localized cancer is 99%, and 84% for regional disease with lymph node involvement. For women presenting with metastatic disease the five-year survival rate is a dismal 18% (Figure1.1) (American Cancer Society).

The problem of metastasis

Metastatic disease is the most dangerous part of breast cancer; >90% of breast cancer deaths are due to metastasis (37). The median survival for metastatic breast cancer is 18 to 24 months, depending on the subtype of tumor, sites of metastatic involvement, and burden of metastatic disease (38). Common sites (and symptoms) of metastatic breast cancer include bone (e.g., back or leg pain and fracture), liver (e.g., abdominal pain, nausea, jaundice), lungs (e.g., shortness of breath or chronic cough) and brain (e.g., headaches, seizures, memory problems or personality changes) (39). Systemic treatment for metastatic breast cancer, although usually toxic and noncurative, is given to prolong

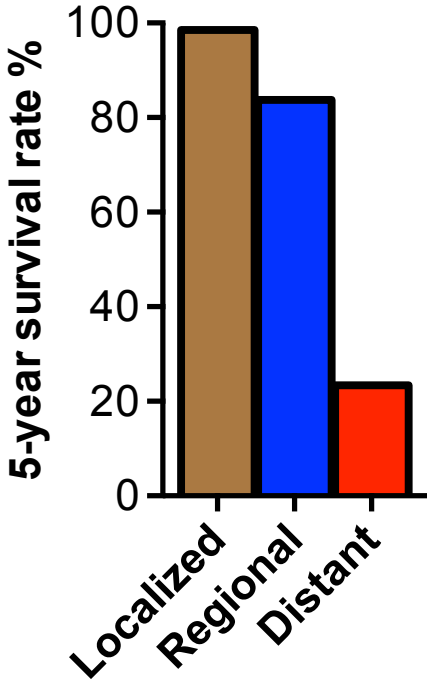

Figure 1.1. Survival is significantly lower for women with metastatic breast cancer. Five year relative survival upon diagnosis is 99% for localized breast cancer, 84% for regional breast cancer, and 23% for metastatic breast disease[1].

[1] Modified from American Cancer Society

survival, alleviate symptoms, and maintain or improve the quality of life (40). Therefore, to impact clinical outcomes, we must understand the process of metastasis in order to develop new drugs for metastatic breast cancer.

Tumors develop when normal cells undergo genetic alterations that impact the regulated systems for cellular control. Hanahan and Weinberg have described "the hallmarks of cancer," an organizational concept to convey the complexity of tumorgenesis (37). These hallmarks include sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, and invasion and metastasis (Figure 1.2) (37). Cancer cells sustain proliferation by activating growth factor signaling pathways through mutations or producing ligands that activate their cognate receptors (41). Alternatively, mutations downstream of signaling pathways, including phosphatidylinositide 3-kinases and mitogen-activated protein kinases pathways also allow sustained tumor cell proliferation (42).

Inactivation of tumor suppressor genes, such as retinoblastoma and tumor protein 53 removes important checkpoints that prevent cell growth and proliferation (43). During tumor progression, tumors release factors such as vascular endothelial growth factor (VEGF) that induce sprouting of new blood vessels, sustaining continued tumor growth (44). As carcinomas progress, they invade local tissues, and eventually can metastasize to distant organs. The mechanisms that drive metastasis are still unclear (37).

The main cause of breast cancer mortality is metastasis. Although early surgery is often the only way to prevent metastasis, this may not be sufficient. Indeed, recent data indicate that metastasis can actually occur years prior to diagnosis (45). Klein et al. have calculated that the growth of a tumor from initiation to a size of 1 cm, when it can be detected using current imaging tools, requires an average of 12 years (46). Because

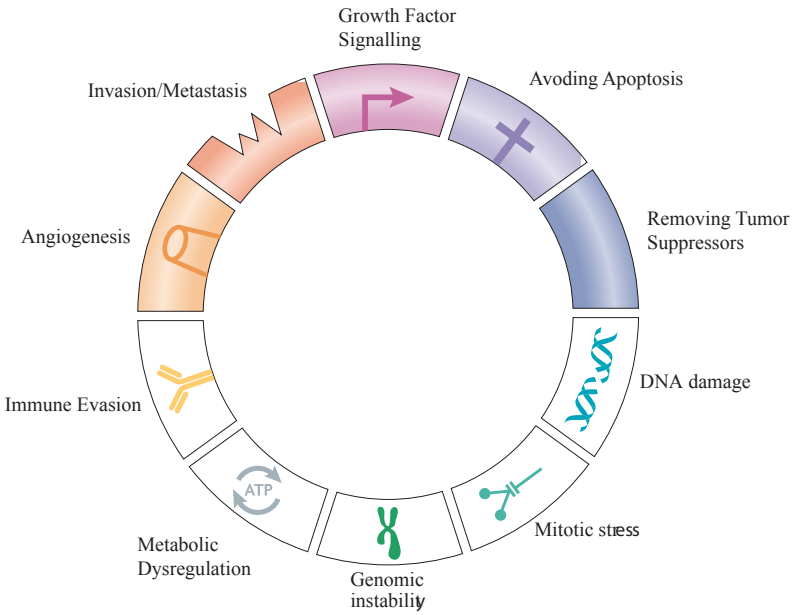

Figure 1.2. The hallmarks of cancer. These are an organizing principle to understand the multiple steps of cancer development. Cancer development is fueled by tumor cell intrinsic alterations accompanied by tumor microenvironmental adaptations. Tumor intrinsic alterations include sustained growth factor signaling, removing tumor suppressors, circumventing apoptosis, genetic abnormalities, and metabolic changes. These are accompanied a diverse entourage of "normal" cells that create the tumor microenvironment. The tumor microenvironment enables angiogenesis, invasion, metastasis and immune evasion[2].

[2] Modified from Hanahan D, and Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011: 144:646-674

tumor volume doubling time is similar between the primary tumor and the metastatic lesion, much of the tumor evolution and metastasis is hypothesized to have occurred prior to detected using current imaging tools, requires an average of 12 years (46). Because tumor much of the tumor evolution and metastasis is hypothesized to have occurred prior to diagnosis (46). Epidemiological data supports this hypothesis, with the median time from tumor resection to a diagnosis of metastasis for patients with a < 2 cm tumor being 35 months versus 20 months for patients with >5 cm tumor (46). Clinically relevant inhibition of metastasis, therefore, may need to focus more on outgrowth rather than initial spreading (47). Thus, to prevent metastatic outgrowth, it is critical to understand how cells metastasize, and elucidate pathways that convert metastatic microcolonies into overt metastases.

The metastatic cascade

In 1887, Paget presciently proposed the "seed and soil" hypothesis (48). He posited that primary neoplasms (and metastases) consist of both tumor cells and host cells, and that metastatic development occurs in specific organs or microenvironments ("soil"), which are biologically unique (49). This conceptual leap has been validated by several subsequent studies. For example, one such study was in ovarian cancer patients with cancer cells growing in the peritoneal cavity as ascites. The ascetic fluid was drained into the venous circulation, resulting in palliation with minimal complications. However, it allowed the entry of cancer cells into the jugular vein. Subsequent autopsy findings from the patients revealed that the shunts did not increase the risk of metastasis to organs outside the peritoneal cavity, despite continuous entry of millions of tumor cells into the circulatory system. Surprisingly, metastases to the lung, the first capillary bed

encountered, were rare (50). Clinically, ovarian cancer rarely metastasizes to the lung (51). This study demonstrates that metastasis is not a passive process of cancer cell dissemination, but is an active process that can be deciphered and potentially stopped.

The past ten years have seen a tremendous effort to understand metastasis (52). Metastases are thought to form following a series of events whereby epithelial cells in primary tumors: *(1)* invade locally through surrounding extracellular matrix and stromal cell layers, *(2)* intravasate into blood vessels, *(3)* survive in the circulation, *(4)* arrest at distant organ sites, *(5)* extravasate into tissue parenchyma, *(6)* survive in these foreign microenvironments and form micrometastases, and *(7)* proliferate at metastatic sites, to generate clinically detectable lesions (Figure 1.3) (53). Each process is summarized briefly in the sections below.

Local invasion consists of cancer cells invading into the surrounding tumor-associated stroma and into the adjacent normal tissue parenchyma (52). Mechanistically, invasion may occur by integrin-and protease-dependent mesenchymal invasion, or by integrin-dependent amoeboid invasion (54). The invading cells encounter stromal components, including fibroblasts, endothelial cells, and immune cells (55). These stromal cells in turn are able to enhance the aggressive behaviors of carcinoma cells via various signaling mechanisms. For example, secretion of interleukin-6 by adipocytes in the microenvironment increases breast cancer invasiveness (56). Tumor cell secretion of interleukin-4 stimulates tumor-associated macrophage cathepsin protease activity, which in turn increases breast cancer invasiveness (57). Many of the pathways that are activated during the invasion process are also part of the normal wound healing process. Because of the histologic similarities between the tumor microenvironment and wound healing, tumors have been called “wounds that never heal” (58).

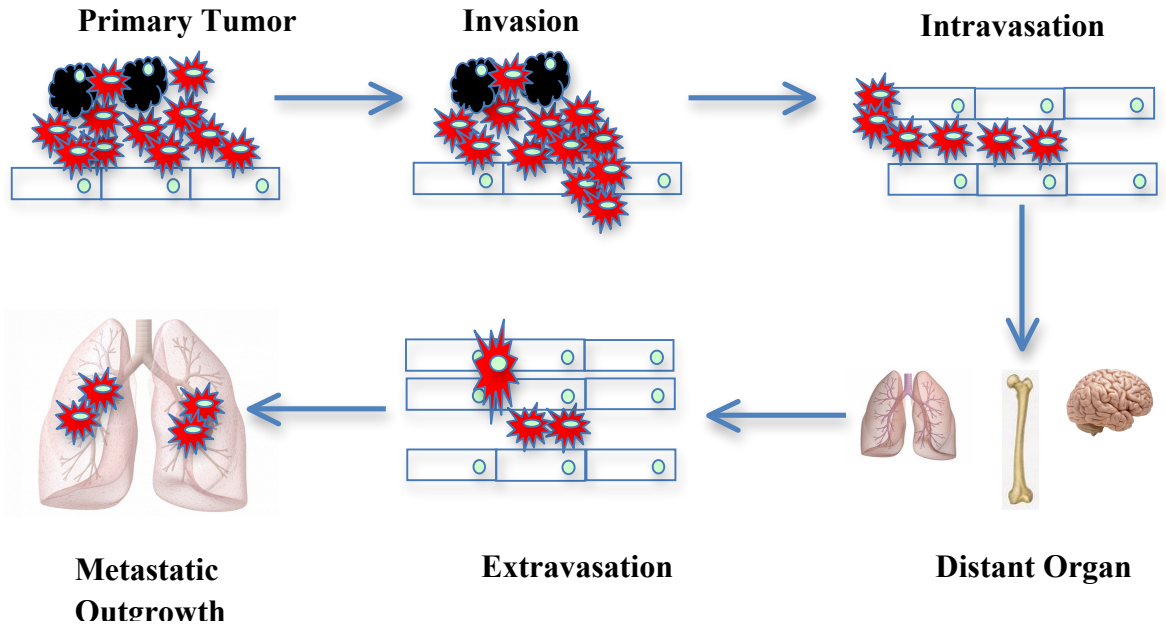

Figure 1.3. The metastatic cascade. During metastatic progression, tumor cells *invade* into the local stroma. They then penetrate blood vessels, and enter into the circulatory system, also known as *intravasation*. They survive in the circulation, and become entrapped in distant organs. They then exit into the distant organ, a process called *extravasation*. Proliferation at the distant organ results in macrometastases. Cancer cells are red[3].

[3] Modified from Gupta GP, and Massague J. Cancer metastasis: building a framework. *Cell*. 2006: 127:679-695

Intravasation occurs when tumor cells enter lymphatic or blood vessels (53). Intravasation can be significantly enhanced by the tumor microenvironment. For example, TGFβ enhances mammary cancer intravasation by increasing the ability of tumor cells to penetrate blood vessels (59). Tumor-associated macrophages also engage in a positive-feedback loop with cancer cells, comprised of the reciprocal secretion of epidermal growth factor (EGF) and colony-stimulating factor-1 (CSF-1) by macrophages and cancer cells, respectively, resulting in intravasation: macrophages are good at getting through blood vessels, with cancer cells following (60).

Survival in the circulation is another critical step for metastasis. Circulating tumor cells (CTCs) have been observed in the blood of cancer patients, and can aid in diagnosis and drug development (61). In the circulation, CTCs need to survive the stress of matrix detachment, the hemodynamic shear forces and escape the immune system. Cancer cells evade these stresses, in part, by binding and activating platelets, enabling successful arrest at the vessel walls and masking them from immune response (62).

Extravasation occurs once cancer cells lodge in the vasculature of distant organs, whereby CTCs may initiate microcolonies that rupture surrounding blood vessels (63). There is evidence suggesting that primary tumors may influence these distant microenvironments, allowing for favorable environments for the CTCs. For example, secretion of angiopoietin-like-4 (64), upregulation of cyclooxygenase-2 (65) and secretion of matrix metalloproteases (66) may disrupt the pulmonary vascular endothelial cell-cell junctions to enhance the pulmonary extravasation of breast cancer cells.

Micrometastasis formation and colonization occurs once cancer cells are in a distant organ. Extravasated carcinoma cells must survive in this foreign microenvironment, which often differs vastly from the primary tumor site (52). This

makes colonization the rate limiting and most difficult step in the metastatic cascade for tumor cells (67). Accordingly, occult micrometastases may successfully grow, remaining in a "dormant" state for years. This process, called tumor dormancy, results in minimal residual disease that can cause eventual relapse (68). The relapse rate for breast cancer after 5 years is about 20% (68).

Tumor dormancy

Tumor dormancy is the pause in cancer progression and the absence of clinical symptoms following treatment of the primary lesion (68). Dormancy at new sites may occur due to several reasons. First, proliferation may be absent or slowed due to stress signals in the new microenvironment, activating growth arrest programs (69,70). Second, angiogenesis may be lacking at the new site, preventing expansion of the lesions (71). Third, the immune system may keep micrometastases in check (discussed in more detail in later sections). Thus, escape from dormancy may be accomplished by alterations in the cancer cells that allow proliferation or prevent apoptosis in the new environment, and/or activation of signaling pathways that allow escape from the immune system and recruitment of blood vessels (68).

In sum, these observations may explain why CTCs are prevalent in the bloodstream of a majority of cancer patients, but only few of them develop into overt metastases. Indeed, preclinical studies show that survival in the circulation, arrest at distant sites, and extravasation occurs efficiently in different cancer cell types, with more than 80% of intravenously injected tumor cells extravasating (72). In contrast, less than 3% of these cells survive to form micrometastases. Even more striking, the subsequent process of metastatic colonization is even more inefficient; less than 0.02% of

intravenously injected cells generate macroscopic metastases (72). Thus, it is a testament to our current lack of mechanistic knowledge about metastatic outgrowth that, even taking into account the inherent inefficiency and difficulty of metastatic colonization, there are no approved drugs designed to specifically block metastatic outgrowth (73). Instead, systemic cytotoxic agents are standard care in the metastatic setting.

Developing therapeutics for metastasis

Understanding the metastatic process is critical in developing antimetastatic drugs. Truly effective antimetastatic therapeutics must prevent the outgrowth of disseminated tumor cells, rather than just blocking escape of these cells from the primary tumor (73). Unfortunately, existing adjuvant therapies exhibit limited activity against metastatic lesions once they are detected, perhaps due to pharmacological or biological barriers (74). For example, this lack of efficacy may be related to drug delivery, or by the specific features of the target organs (i.e., the blood-brain barrier) (75). Alternatively, this lack of efficacy may reflect biological differences between the primary tumors and the metastatic lesions that make the latter resistant to therapy (76).

There are several criteria that candidate genes must satisfy to be considered as potential targets for antimetastatic therapy (73). First, their expression should correlate with disease-free survival or therapeutic response. Second, the manipulation of these genes must impact metastasis in preclinical animal models without causing systemic toxicity. One promising avenue for targeting metastasis is manipulating the signaling nodes between tumor cells and the new metastatic microenvironment, because adaptation to the new microenvironment is a limiting step in metastasis (as noted above). Tumor

microenvironment can comprise vascular and lymphatic endothelial cells, fibroblasts, leukocytes, and structural tissues like bone, expanding the possible targets for therapeutic intervention (55). Targeting the tumor microenvironment has several advantages (77). First, metastatic tumor cells rely on the tumor microenvironment to provide necessary signals, cytokines and nutrients. Second, cells of the microenvironment are genetically stable, and are thus less likely to become resistant to the drug. Therefore, understanding the interactions between the host and tumor cells, especially during the metastatic cascade, should yield high value targets for therapeutic targeting. There have been recent successes in targeting the tumor microenvironment, specifically in bone metastasis (78). For example, bisphosphonates and the antireceptor activator of nuclear factor kappa-B ligand antibody, denosumab, slow down bone degradation in breast and lung cancer patients with bone metastasis (79). These treatments have significantly improved quality of life for cancer patients: however, the effects are mostly palliative, and do not generally kill tumor cells.

Tumor microenvironment

Although cancer cells initiate tumors and drive tumor progression, tumors contain a diverse entourage of "host" cells, including fibroblasts, innate and adaptive immune cells, endothelial cells, and pericytes (55). The numerous interactions that occur between epithelial cancer cells and the tumor microenvironment influences both cancer progression and metastasis. This makes it clear that the biology of a tumor can best be understood by exploring the role that different cell types, neoplastic or normal, manifest during the tumorigenic process.

Increased vessel density is associated with poor prognosis in a wide range of human cancers (80). Tumor angiogenesis, the growth of new vessels from preexisting vascular beds, is regulated by factors that elicit a proangiogenic effect by stimulating perivascular cell proliferation, migration, and tube formation (37). For example, hypoxic conditions result in increased secretion of VEGF, resulting in increased blood vessel recruitment (81). Important contributors to blood vessel formation are pericytes, which provide physical support, stabilization, and prosurvival factors for their associated endothelium (82).

Fibroblasts synthesize and remodel the extracellular matrix (ECM) by producing ECM proteins such as collagens and structural proteoglycans, as well as various classes of proteolytic enzymes and their inhibitors (83). Moreover, "cancer-associated" fibroblastic cells secrete various growth factors that regulate cell proliferation, morphology, and survival (83).

The ECM is an integral part of the tumor microenvironment (84). The ECM contains a mixture of fibrillar proteins, glycoproteins, proteoglycans, cytokines, and growth factors. It acts as a physical scaffold, facilitating interactions between different cell types, and providing survival and differentiation signals (84). Moreover, ECM proteins facilitate cell migration and invasion. For example, the ECM protein periostin may enhance metastasis by concentrating Wnt ligands in the metastatic niche (85). Tenascin C, another ECM protein, may also enhance metastasis by signaling through the Notch signaling pathway and supporting metastasis-initiating cells (86).

Immune cells, including granulocytes, dendritic cells, macrophages, natural killer cells, mast cells, and lymphocytes, are prominent components of neoplastic tissues (37). They are important mediators of tumorgenesis, paradoxically acting in both an antitumor

and a protumor fashion (87). Immune cells release multiple factors that regulate cell proliferation, migration, angiogenesis, and tissue remodeling (37). The role of the immune system during cancer progression is addressed in more detail in the next chapter.

Dissertation goal

The focus of my dissertation is the Macrophage Stimulating Protein (MSP) signaling pathway (reviewed in Chapter 3). Briefly, MSP is a serum protein that is released into the blood as an inactive proform and is then activated via cleavage by the protease Membrane Serine Protease I (MTSP1 or matriptase) (88). Activated MSP binds to its receptor, Macrophage Stimulating I Receptor (MST1R or RON), which is found on epithelial cells, osteoclasts, and macrophages (88).

Ron signaling pathways have been reported to play a role in a wide range of human cancer, including breast cancer (88). Mouse models where Ron is overexpressed under the mouse mammary tumor virus (MMTV) promoter resulted in metastasis to the liver and/or lungs (89). On the other hand, overexpressing MSP in the same model results in a broader range of metastasis, including to lung, lymphatics, bone and spleen (90). Furthermore, MSP-induced bone metastases in the mice were osteolytic, similar to human breast cancer patients (90). In human breast cancer patients, co-overexpression of MSP, its activating enzyme matriptase, and Ron (collectively referred to as MSP/matriptase/Ron) is a significant independent prognostic factor for metastasis. Importantly, overexpression of MSP or Ron mRNA alone did not significantly correlate with patient outcome (90).

These observations have led to the question that my dissertation has attempted to resolve. Specifically, how can apparent activation of the same pathway result in different ranges of metastatic phenotypes? Our hypothesis was that Ron function in metastasis of

breast cancer could be largely ligand-dependent, even when the receptor is overexpressed. Thus, instead of MSP/Ron signaling in tumor cells causing invasion, metastasis and migration, MSP/Ron signaling may be acting on macrophages, causing changes in the tumor microenvironment that facilitate the increase in metastasis. The tumor microenvironment has been shown to play an instrumental role in tumor progression (Chapter 3).

To resolve the role that MSP/Ron signaling has during metastasis, we utilized a genetic mouse model of metastasis. My dissertation has uncovered that MSP/Ron signaling suppresses $CD8^+$ T cells, enabling metastasis (Chapter 4). This finding has generated new questions about the how MSP/Ron signaling suppressed the immune system, both in the normal setting and pathological settings (Chapter 5).

In summary, the most challenging aspect of breast cancer is metastasis. There is an urgent need for new treatments to prevent metastatic outgrowth. Multiple interactions between tumor cells and stromal cells play an integral role in metastasis. Thus, it is important to understand tumor-stroma interactions during metastasis, in order to develop therapies that target both tumor cells and supporting stroma.

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CHAPTER 2

INTRODUCTION TO IMMUNOLOGY AND ITS ROLE IN TUMOR BIOLOGY

The immune system functions to defend the body against a wide variety of external and internal pathogenic events, including cancer. The immune system is broadly divided into innate and adaptive immunity. I will discuss the general function of each group, followed by a section on tumor immunology.

The innate immune system

The innate immune system defends the host from infection in a nonspecific manner (1). Innate immunity does not confer long-lasting or protective immunity to the host. Accordingly, the innate immune system produces cytokines and chemokines that recruit other immune cells to sites of infection, clears dead cells and pathogens by phagocytosis, and activates adaptive immunity through antigen-presentation (1,2). The innate immune system consists of neutrophils, eosinophils, natural killer (NK) cells, dendritic cells (DC) and macrophages (MΦ).

Neutrophils are polymorphonuclear cells that are among the first responders to sites of infection (3). They have various receptors, like complement receptors and interferon gamma receptors, that enable them to sense chemical gradients as they migrate to sites of infection (4). At the sites of infection, neutrophils release cytokines that recruit other immune cells and also clear pathogens by phagocytosis (5). Neutrophil deficiency leads to increased susceptibility to invasive bacterial infections and fungal infections (6,7). Neutrophils make up a significant portion of the tumor microenvironment (8), and have been reported to contribute to tumor development by producing protumor chemokines such as interleukin 8 (9) and hepatocyte growth factor (10).

Eosinophils are leukocytes that are involved in diverse inflammatory responses (11). For example, they are important mediators of immunity to parasites, viral infections

and allergies (12). They produce reactive oxygen species, cytokines such as interleukin-6 and interleukin-1, and growth factors such as vascular epithelial growth factor (VEGF) (11). Eosinophil-deficient mice are susceptible to helminthic infections (13). Tumor-associated infiltration of eosinophil cells is a favorable prognostic factor (14,15), perhaps due to their toxic granules, which they release following activation (16).

NK cells are cytotoxic lymphocytes that do not express B and T cell antigen-receptors and cannot form memory cells (17). However, like $CD8^+$ T cells (discussed in the adaptive immunity section), their main function is to kill infected cells and tumor cells with cell-mediated cytotoxicity (18). NK cells release cytotoxic proteins, such as granzymes and perforin, when they come in contact with a target cell (19). Additionally, NK cells also cause antibody-dependent cell mediated cytotoxicity, using their CD16 receptors to recognize immunoglobulin G (IgG) antibodies that bind to antigens (20). Loss of NK cell function is associated with recurrent viral and bacterial infections (21). Decreased activity of NK cells in human cancer patients is associated with increased cancer risk (22), demonstrating that NK cells play a role in tumor immune-surveillance by inducing tumor cell death (23).

The main function of DCs is to process and present antigens to T lymphocytes (24). DCs are the most potent antigen presenting cells (APCs) for T cells, expressing more antigen peptide–major histocompatibility complexes (MHC) structures for longer periods of time when compared to the other professional APCs (B cells and macrophages) (25). Immature DCs are attracted to areas of inflammation, where they ingest antigens (26,27). Once captured, the antigens are processed and presented to T cells via MHC molecules (28). In addition to presenting antigens, DCs use their cell surface receptors, CD80 and CD86, to stimulate T cell proliferation and activation (29). Loss of DCs increases

susceptibility to viral infections such as lymphocytic choriomeningitis virus (LCMV), bacterial infections such as *Listeria* and parasitic infections such as *Plasmodium* (30). Within the tumor microenvironment, DCs can be immunosuppressive and/or angiogenic, and express low levels of costimulatory molecules such as CD80 and CD86 (31) or angiogenic factors such as VEGF (32), respectively.

MΦs are functionally diverse cells, and play a role in multiple aspects of biology, including development, homeostasis, and immune response (33). MΦs can phagocytose and destroy particulate material, and subsequently present antigens to T cells (33). MΦs have surface receptors for binding particulate antigens, including receptors for certain sugars (e.g., mannose) and for bacterial lipopolysaccharides (via interaction with LPS-binding protein) (34). MΦs fragment, crystallizable receptors (FcRs) and complement receptors enable them to bind antigens that have been coated (opsonized) with either IgG antibodies (35) or C3b complement protein (36), respectively. MΦs also rapidly clear apoptotic cells, due to their receptors for phosphatidylserine that is expressed on the outer surface of apoptotic cells (33). Loss of MΦ, for example through mutation in macrophage colony stimulating factor (M-CSF), results in osteopetrotic mice that lack osteoclasts (op/op mice) and are more susceptible to bacterial infection (37). MΦs play multiple roles in the tumor microenvironment (38); from stimulating tumor cell migration (39), to inducing angiogenesis (40) to suppressing the antitumor response (41).

The adaptive immune system

Adaptive immunity is an antigen-specific defense mechanism that is designed to remove pathogens or cells expressing a specific antigen. Adaptive immunity is characterized by somatic hypermutations and variable, diverse and joining (VDJ)

recombination that allows a vast number of different antigen receptors (42). During adaptive immunity, DCs and MΦs present antigens to antigen-receptors on lymphocytes (discussed in the following section). This results in activation and proliferation of antigen-specific lymphocytes, culminating in production of pathogen specific antibodies, cytotoxic T lymphocytes, as well as cytokine production (43). The adaptive immune system comprises B cells and T cells.

B cells, also known as humoral immunity, produce antibodies. B cells express a B cell receptor (BCR), and binding of a matching antigen with the BCR results in B cell activation (44). B cells recognize and bind to antigen directly, in contrast to T cells that recognize short peptide fragments of protein antigens (45). An activated B cell differentiates into a plasma cell that secretes specific antibodies. These antibodies bind to antigens, which opsonize them for recognition by innate immune cells (46) . B cells have been reported to infiltrate the tumor microenvironment (47), where they regulate tumor development by secretion of proinflammatory cytokines, such as IL-10 and TGFβ (48), inhibition of $CD8^+$ T cell activity (48), and recruiting and activating innate immune cells (49).

T cells circulate in the bloodstream and lymphatic system, and are responsible for regulating the activity of other immune cells or directly kill infected or malignant cells (50). A key feature of T cells is that they cannot recognize antigens in their native form, but only when they are presented on the surface of APCs. The antigen receptors of T cells interact with peptides derived from the degradation and processing of foreign antigenic proteins (51). These peptides are bound MHC molecules on the surface of APCs. There are two types of MHC molecules, MHC class I and class II, which present antigen peptides to $CD8^+$ and $CD4^+$ T cells, respectively (28).

As alluded to previously, T cell receptors recognize antigenic peptides that are associated with MHC molecules on APCs. APCs internalize antigens, process them into peptides, and load the peptides onto MHC I or MHC II molecules, a process called antigen presentation (28). Exogenous and endogenous antigens are processed and presented to T cells by different mechanisms (Figure 2.1). Exogenous antigens (e.g., bacteria) are ingested by APCs via phagocytosis or pinocytosis. Subsequently, they are degraded into peptide fragments in the lysosome (52). The peptide fragments are loaded onto MHC II molecules, where they are recognized by $CD4^+$ T cells (53). On the other hand, endogenous antigens are generated within a cell (e.g., tumor antigens) by proteasomal degradation (54). Subsequently, the peptides are transported to the endoplasmic reticulum, where they are loaded onto MHC I molecules, where they are recognized by $CD8^+$ T cells (28,54). However, APCs can also present exogenous antigens via MHC I molecules to stimulate $CD8^+$ T cells, a process called cross-presentation (55). Cross-presentation may occur when an intracellular pathogen or tumor does not affect APCs, or the endogenous MHC I pathway is compromised. Thus, APCs take up the exogenous antigen, and load it unto MHC I molecules. Although the exact mechanism is unclear (56) , $CD8^+$ T cells activated in this manner are called cross-primed, and play important roles for immune defense against viruses and tumors (57).

$CD4^+$ T, or helper T (T_H) cells, facilitate the immune response mainly by stimulating other cells of the immune system. Accordingly, they stimulate B cells to become activated and to secrete antibodies, and cause macrophages to become more effective at killing pathogens (58). Depending on the cytokine expression profile of T_H cells, they are further subdivided into two groups (59). T_H1 cells secrete cytokines (e.g. interleukin 2 and interferon gamma) that mainly promote cell-mediated immunity by

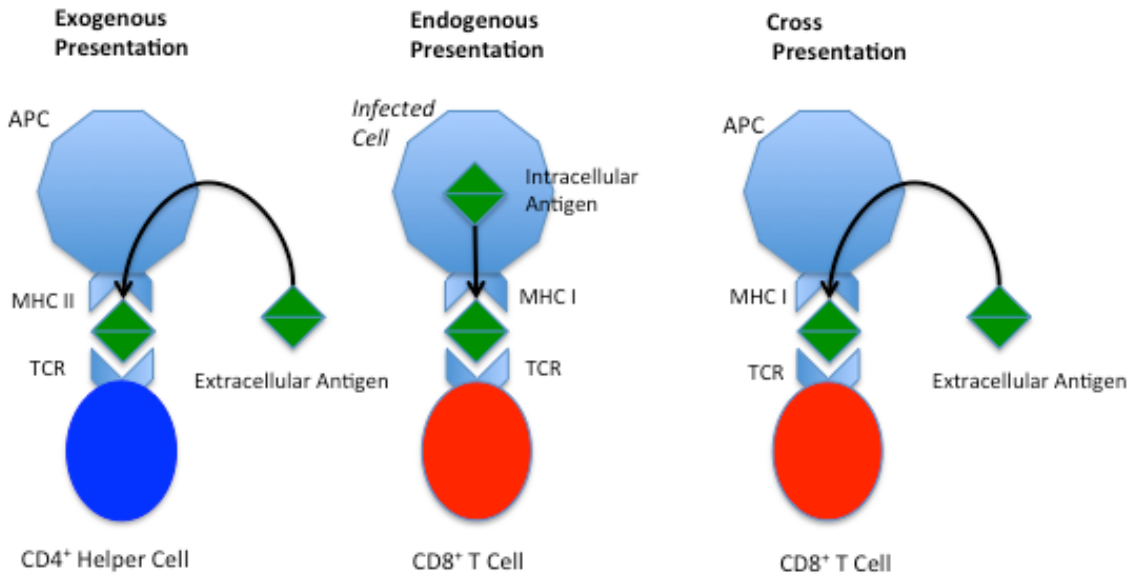

Figure 2.1. Antigen presentation. Antigens are processed and presented to T cells by several mechanisms. *Exogenous presentation* occurs when an antigen-presenting cell processes an extracellular antigen and presents it to $CD4^+$T cells via the MHC II molecule. *Endogenous presentation* occurs when antigens that are generated within a cell (e.g., tumor antigens in any infected cell) are processed and presented to $CD8^+$ T cell via MHC I molecules. *Cross-presentation* occurs when extracellular antigen is processed by an antigen-presenting cell and presented to a $CD8^+$ T cell via the MHC I molecules[4].

[1]Modified from Germonprez P, Valladeau J, Zitvogel L, Thery C, and Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol*. 2002; 20;621-67

activating $CD8^+$ T cells (see below) and MΦs, whereas the cytokines produced by T_H2 cells (e.g., interleukin 4 and interleukin 10) primarily stimulate antibody production by B cells (60).

$CD8^+$ T cells provide defense against viral, bacterial, protozoal infections and tumorgenesis (43). Naïve $CD8^+$ T cells reside secondary lymphoid tissues, such as lymph nodes and spleen (61), where they are activated by APCs. $CD8^+$ T cells recognize processed antigen presented on MHC molecules via the T cell receptor (TCR) (51). A naïve T lymphocyte requires a series of signals to become an effector cell (Figure 2.2) (62). The first signal occurs when the TCR of the naïve T lymphocyte directly interacts with the antigen peptide bound to the MHC molecule on an APC (Signal 1) (45) . The second signal for activation comes from interactions between the APC and T cell via co-stimulatory molecules such as CD80 and CD86 (on APCs), which bind to CD28 on the T cell surface (Signal 2) (63–65). If this costimulatory signaling fails to occur, the T lymphocyte will not become activated and becomes anergic, a condition where the T cell is functionally inactive but still alive (66), a control mechanism that prevents inappropriate T cell activation. The third signal involves inflammatory cytokine signaling by APCs, leading to robust T-cell activation and proliferation (Signal 3) (67,68).

Antigen uptake in the absence of inflammatory signals renders phenotypically immature APCs that express low levels of MHC molecules and costimulatory molecules, leading to T-cell tolerance (69). Thus, to achieve maximal expansion, $CD8^+$ T cells integrate multiple signals. These include signaling from the TCR, costimulatory signals, and inflammatory cytokines such as IL-12. Moreover, many members of the tumor necrosis factor receptor (TNFR) family on APCs also deliver important costimulatory signals to $CD8^+$ T cells (70). Following recognition of antigen, activated T cells expand by

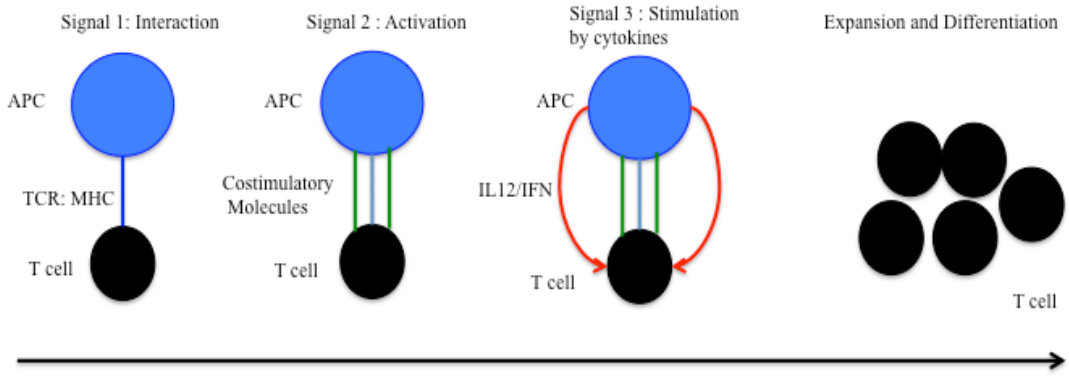

Figure 2.2. T cell activation. Activation of naïve $CD8^+$ T cells requires three signals. *Signal one* occurs when the T cell receptor recognized a peptide in the MHC molecules. *Signal two* involves the binding of costimulatory molecules CD80 and CD86 on APCs by CD28 on $CD8^+$ T cell. This results in extensive proliferation. *Signal three* occurs when IL-12 or IFNβ/α are present, resulting in increased survival, and robust effector functions[5]

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[5] Modified from Curtsinger, JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. *Curr. Opin. Immunol*. 2010; 22:333-40

proliferating and enter into peripheral tissues.

As mentioned above, $CD8^+$ T cells are specifically activated following binding to MHC I molecules, either on APCs or other "host" cells, such as infected or transformed epithelial cells. Peptides that are derived from intracellular abnormalities, such as viral proteins from infected cells are processed and presented on the surface of MHC I molecules, making the presenting cell a target for $CD8^+$ T cell killing (43). Alternatively, $CD4^+$ T cells can activate $CD8^+$ T cells (71). In this process, APCs present peptides in the context of MHC class II molecules to $CD4^+$ T cells. The interaction between the $CD4^+$ T cell and the APC induces transient expression of CD40 ligand by the $CD4^+$ T cell. Binding of CD40 ligand to CD40 on the same APC enables the APC to present peptides in MHC class I in order to specifically activate $CD8^+$ T cells (72,73).

To activate $CD8^+$ T cells, antigen-presenting cells undergo a series of steps. Danger signals from pathogens transform DCs and MΦs into efficient APCs and robust T cell activators (74). Particulate and soluble antigens are efficiently internalized by phagocytosis and macropinocytosis, respectively. Phagocytosis is generally receptor mediated, whereas macropinocytosis is a cytoskeleton-dependent type of fluid-phase endocytosis (75). Phagocytosis is a major route for antigen uptake and presentation, and is mediated by complement receptors, CD14, integrins, and serine/arginine rich family members (56). Experimentally, forcing internalization of antigen by phagocytosis strongly increases the efficiency of cross presentation, or the presentation of extracellular antigens via MHC-class I molecules (76). Physiologically, phagocytosis of apoptotic cells also results in efficient cross presentation of antigens (28). In addition FcR-mediated uptake of immune complexes, opsonized liposomes, or opsonized dead cells promote efficient cross

presentation. The cross-presented antigen can be acquired in several different forms including deoxyribonucleic acid, ribonucleic acid, or peptides (77–79).

Once activated, there are several mechanisms of $CD8^+$ T cell mediated cytotoxicity (80). These include granule-mediated apoptosis, Fas-mediated apoptosis and TNF-α-mediated apoptosis. Granule-dependent apoptosis occurs when preformed granules or lysosomes of the cytotoxic cell are released upon contact with the target cell (81). Lytic molecules such as perforin, granzymes (Grz), and granulysin then come into contact with the target cell. Perforin polymerizes on the target cell membrane, forming a pore through which Grz-A, Grz-B, and granulysin enter (82). These proteins form a complex with mannose-6-phosphate receptor, and are then internalized and released into the cytoplasm (83). Granzymes activate caspase 3, resulting in fragmentation of the DNA, nuclear membrane and cytoskeleton. Granzymes also cleave Bid, which induces cytochrome C release from mitochondria, causing activation of caspase 9 and mitochondrial-induced apoptosis (19).

$CD8^+$ T cells can also mediate cytotoxicity by Fas-mediated apoptosis. Fas is a member of the Tumor Necrosis Family Receptor (TNFR-1) family (84). $CD8^+$ T cells express Fas ligand (FasL), while target cells express Fas receptor (85). Binding of Fas with FasL causes trimerization and subsequent recruitment of Fas-associated death domain (FADD) proteins (86). Subsequently, FADD recruits caspase 8 or 10, which assemble to form the death-inducing complex (86). This activates effector caspases 3,6 or 7, which cleave DNA or hydrolyze Bid, triggering mitochondrial-mediated apoptosis (87).

$CD8^+$ T cells can also kill target cells by secreting tumor necrosis factor-α (TNF-α), a cytokine that induces apoptosis (88). Following binding by TNF-α, the TNFR undergoes multimerization to form the death-inducing signaling complex (DISC),

signaling downstream through the caspase activation cascade and mitochondria (89). The TNF-TNFR complex has also been shown to enhance nicotiniamide adenine dinucleotide phosphate (NADPH) oxidase activity, promoting oxidative stress that results in necrotic cell death (90).

The immune system and tumor development

The various mechanisms of CD8+ T cell mediated cytotoxicity that I described are also utilized by the immune system to prevent tumor development, using a process called immunosurveillance (91). Thus, the immune system can be an extrinsic tumor suppressor. Paradoxically, however, the immune system can also act as an extrinsic tumor promoter (92). This contradictory role of the immune system has been recently encapsulated under the cancer immunoediting hypothesis (Figure 2.3) (93). This hypothesis posits that cancer development must take place in the context of dynamic immune processes that control and shape the cancer. When normal cells are transformed, they frequently express ligands and antigens which innate and adaptive immune cells recognize, resulting in an antitumor response (94,95). However, if antitumor immunity is unable to completely eliminate transformed cells, the surviving tumor cells enter into the equilibrium phase, where they are somewhat, but not completely, resistant to immune- mediated killing (96). The adaptive immune system keeps the tumor cells in check, preventing tumor outgrowth (97,98). Eventually, however, tumor cells acquire further alterations that result in the evasion of the immune system, and progress to clinically detectable lesions (99,100). For example, tumor cells downregulate human leukocyte antigen class I antigens (101) and express ligands for immune inhibitory receptors such as programmed cell death 1 ligand 1

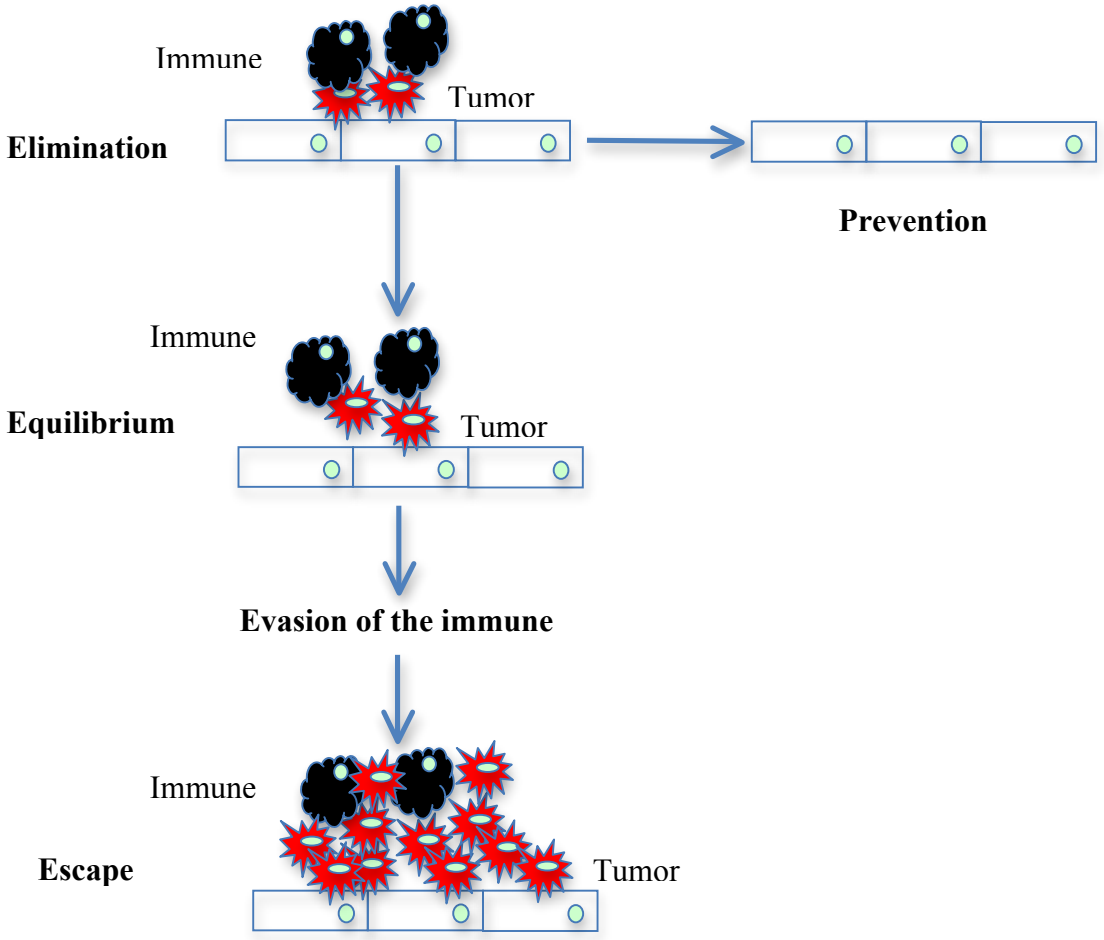

Figure 2.3. Cancer immunoediting. Cancer immunoediting consists of three stages: elimination, equilibrium, and escape. *Elimination* occurs when immune cells destroy developing tumors. Effective elimination results in a tumor-free host. *Equilibrium* occurs when a cancer cell is not destroyed in the elimination phase. The immune system constantly selects for tumor cell variants with increasing capabilities to survive immune attack. *Escape* is the process wherein tumor cells that are resistant to immune attack expand in an uncontrolled manner and emerge to cause clinically apparent disease[6].

[6] Modified from Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*; 2006; 6;24-37

(PDL1) (102). The immune system, at this stage may paradoxically promote tumorgenesis (103).

Although the cancer immunoediting hypothesis is difficult to prove in human patients, tumor-infiltrating lymphocytes are reported to be significantly correlated with patient survival (104,105). Tumor infiltration by T cells or NK cells has been associated with an improved prognosis for a number of different tumor types. Indeed, the type and density of lymphocytes infiltrating epithelial tumors such as colorectal (105), ovarian (106) and breast cancers (107) are powerful prognostic indicators. For example, a tumor immune signature consisting of $CD68^{high}/CD4^{high}/CD8^{low}$ significantly correlates with reduced overall survival of breast cancer patients (107).

Additional support for the cancer immunoediting hypothesis comes from immune-manipulated animal models (99). Cells of both the innate and adaptive immune system have been shown to be critical for the elimination of cancers. Lymphocyte-deficient recombination activating gene 1 and 2 ($Rag1^{-/-}$, $Rag2^{-/-}$), severe combined immunodeficient (SCID), and nude mice all display an increased susceptibility to tumor induction after exposure to carcinogens (108–110), and in their normal lifespan (111). Moreover, depleting T cells in previously carcinogen-treated mice results in sarcomas that rapidly grow at the original site of carcinogen injection, as well as metastatic lesions, suggesting that T cells were keeping transformed cells in check (97).

Immunity is a significant barrier that metastatic tumor cells must overcome to establish metastatic disease. This was dissected in a model using transgenic mice (RET-AAD) that express the human *RET* oncogene and a chimeric mouse/human MHC antigen (AAD) specifically in melanocytes (112). These mice develop extensive disseminated metastases, and depletion of $CD8^+$ T cells in RET-AAD mice significantly accelerated the

outgrowth of metastatic lesions to visceral organs (112). In addition, in the mammary mouse tumor virus Polyoma Middle T antigen model of breast cancer, the absence of $CD8^+$ T cells significantly potentiated metastasis (113).

Thus, over time, tumors must evolve mechanisms to elude or inhibit the immune system (114). These mechanisms can be cell-autonomous adaptations that enable direct evasion of the immune system or noncell autonomous manipulations of the microenvironment to create an immunosuppressive network (114). Tumor-intrinsic changes that allow immune evasion include downregulation of antigen presentation (MHC) molecules (115), upregulation of inhibitors of apoptosis (116), or expressing inhibitory cell surface molecules (PDL1) that directly kill cytotoxic T cells (117). Additionally, tumor cells secrete various factors, such as TGF-β (118) and arginase (119), which can remodel the tumor environment, inhibiting effector immune cell functions and generating a generally immunosuppressive microenvironment.

The immunosuppressive tumor microenvironment is comprised of various immune cells that not only contribute to suppressing the immune system, but also can directly facilitate tumor progression (120,121). Macrophages can promote tumor angiogenesis, invasion, intravasation and metastasis in animal models (122). Macrophages can be subdivided into two broad categories, depending on their function (123). 'Classically activated' (M1) macrophages contribute to tumor rejection through T_H1-type cytokine production and antigen presentation, whereas 'alternatively activated' (M2) macrophages enhance angiogenesis and remodeling through T_H2-type cytokine production (41). Tumor associated macrophages (TAMs) have been shown to have M2 characteristics, and are associated with poor prognosis (124,125). TAMs secrete cytokines like TGF-β (126) , IL-10 (127), and platelet derived growth factor (PDGF), which inhibit T cells (128) .

Myeloid-derived suppressor cells (MDSCs), which resemble poorly differentiated granulocytes (129), are also often increased in tumors. They are immunosuppressive, in part through inhibition of T-cell activation (130,131). Type 1 $CD4^+$ T cells (T_H1) aid $CD8^+$ T cells in tumor rejection (132,133), whereas type 2 $CD4^+$ T cells (T_H2) and $CD4^+$ T regulatory cells inhibit $CD8^+$ T cells through multiple mechanisms (134,135), including expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4), a cell surface molecule that is expressed on $CD8^+$ T cells and is a critical inhibitory regulator of T cell expansion (136).

As mentioned above, $CD8^+$ T cells are the effector cells of the adaptive immune system that specifically recognize and destroy cancer cells through perforin- and granzyme-mediated apoptosis. However, $CD8^+$ T cells in the tumor microenvironment are ineffective in killing tumor cells for reasons that remain largely unknown. This ineffectiveness is hypothesized to be due to the heavily immunosuppressive tumor microenvironment (137). First, $CD8^+$ T cells stimulation is hindered by the lack of positive costimulatory signals and expression of negative costimulatory signals on APCs (138,139). Second, there is ample expression of immunosuppressive cytokines like IL-10 and TGF-β (140–142). Third, tumor cell expression of proapoptotic molecules, such as Fas ligand, induces T cell apoptosis (143,144). Fourth, tumors express the tryptophan-depleting enzyme, indoleamine 2, 3-dioxygenase (IDO), which causes nutritional deficiency for T cells, rendering them unable to survive in the tumor microenvironment (145,146). Fifth, there is significant accumulation of T regulatory cells, MDSCs, and TAMs that all inhibit T cell effector functions (147,148).

How tumor-immune cells interact at metastatic sites, and how tumor cells escape immune surveillance during metastatic growth, remains largely unknown (149,150). Thus, identifying and targeting the key mechanisms by which tumor cells mediate suppression

of $CD8^+$ T cells during metastatic outgrowth holds potential as a strategy to reduce or prevent escape from dormancy, and thereby block progression of metastasis. Disseminated cancer cells are hard to treat, due to biological barriers (e.g., blood-brain barrier) and potential genetic drift from the original cancer leading to treatment resistance (151). However, the immune system can efficiently search and specifically prevent tumor cells outgrowth. For example T_H1 immunity has been reported to prevent cancer proliferation by inducing cancer cell senescence through IFN-γ- and TNF-α (152).

In summary, the immune system plays a multifaceted role during cancer development and metastasis. The dual role of the immune system, both antagonizing and supporting tumor development, suggests that there are multiple pathways that are potential therapeutic targets. The recent approvals of ipilimumab, a CTLA-4 inhibitor for metastatic melanoma, and sipuleucel-T, a vaccine for prostate cancer, demonstrate the promise of harnessing the immune system for cancer therapy. Thus, deciphering the tumor-immune interactions will allow us to develop truly targeted anticancer therapy.

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CHAPTER 3

THE MACROPHAGE STIMULATING PROTEIN/RON PATHWAY AS A POTENTIAL THERAPEUTIC TARGET TO IMPEDE MULTIPLE MECHANISMS INVOLVED IN BREAST CANCER PROGRESSION

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The Macrophage Stimulating Protein/Ron Pathway as a Potential Therapeutic Target to Impede Multiple Mechanisms Involved in Breast Cancer Progression

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Abstract: Macrophage Stimulating Protein (MSP) is the only known ligand for the receptor tyrosine kinase Ron. The MSP/Ron pathway is involved in several important biological processes, including macrophage activity, wound healing, and epithelial cell behavior. A role for MSP/Ron in breast cancer has recently been elucidated, wherein this pathway regulates tumor growth, angiogenesis, and metastasis. Here, we review the recent literature surrounding MSP/Ron function in tumor cells, inflammatory cells, and osteoclasts – cell types that often coexist in breast tumor microenvironments. We discuss the potential implications of MSP/Ron activity occurring concurrently in these cell types on tumor progression and metastasis. Lastly, we outline the potential for targeting MSP/Ron as a novel therapy for breast cancer, and for other cancer types.

Keywords: Breast cancer, macrophage stimulating protein, metastasis, MSP, MST1R, osteolysis, Ron, therapeutic target.

INTRODUCTION

Breast cancer has a relatively low case-fatality rate, but approximately 20% of women diagnosed with breast cancer eventually develop metastatic disease. Because breast cancer is highly prevalent and metastatic breast cancer is rarely curable, a significant number of women, about 40,600 in the U.S. per year, will die of the disease [1]. Although the incidence of breast cancer is much higher in women over 50, breast cancer is the major cause of death from all reasons in women age 35-50 and represents a major health care and societal problem.

The goal of initial treatment is to reduce the risk of both local and systemic recurrence. Initial treatment for localized breast cancer is designed to reduce the risk of in-breast and regional disease; local recurrence in breast, skin, subcutaneous tissues and axilla rarely causes death but often results in significant morbidity and can give rise to systemic metastases [2]. Local therapy begins with surgery: either lumpectomy to remove the tumor from the intact breast, or mastectomy. Radiation therapy reduces the risk of local recurrence and is almost always recommended after lumpectomy. Radiation is also generally recommended after mastectomy if the tumor is large or involves regional lymph nodes. Initial treatment of breast cancer often also includes systemic therapy designed to eradicate occult metastatic disease that is not clinically evident, but which may eventually cause relapse and death. Treatment given in this setting is termed "adjuvant therapy" and, because of the near-universal fatality of metastatic breast cancer, is recommended for the majority of women newly diagnosed with breast cancer in order to improve disease-free survival [3].

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Systemic therapy for breast cancer may be given either as adjuvant therapy or as treatment of metastatic disease, and includes hormonal therapy, chemotherapy, and/or biological agents. Hormonal therapy is used if the malignant cells express estrogen and/or progesterone receptors. Chemotherapy of several different classes can be effective in all types of breast cancer, independent of hormone receptor expression. Targeted therapies used in breast cancer include trastuzumab, a monoclonal antibody approved for use in combination with chemotherapy for HER2 positive breast cancer, and lapatinib, a small molecule used for HER2 positive metastatic breast cancer. Bevacizumab, a VEGF inhibitor, is also approved for use in metastatic breast cancer [3]. Numerous other agents are being evaluated as potentially effective adjuvant therapies, notably bisphosphonates, which are best known for blockade of osteoclast function. Various bisphosphonates are in late-phase clinical trials and may reduce the risk not only of skeletal metastases, but visceral metastases as well [4].

Patients with metastatic breast cancer have a median survival of approximately two years [5]. Women with hormone-sensitive metastases limited to bone and soft tissue have on average a longer survival, while those with extensive parenchymal organ involvement usually have a shorter survival, particularly if the tumor does not express hormone receptors or HER2. These latter tumors are aggressive, and there are no targeted therapies available for this subtype of breast cancer. Clearly, there is a need for new therapies with greater efficacy against this disease, and a particular need for therapies that might reduce or prevent the growth of metastatic lesions. Targeted therapies are generally less toxic than other anti-cancer agents and, when effective, are invaluable in achieving the goal of treatment of metastatic breast cancer: to achieve disease control while avoiding toxicity due to therapy. Here, we discuss the exciting potential for a new therapeutic target in breast cancer: the receptor tyrosine kinase Ron.

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THE MET/RON FAMILY OF RECEPTOR TYROSINE KINASES

The receptor tyrosine kinase Ron (also known as human MST1R, for macrophage stimulating 1 receptor, and as murine Stk1, for stem cell kinase 1) is the cell surface receptor for macrophage stimulating protein (MSP; also known as MST1, for macrophage stimulating 1, and as HGFL, for hepatocyte growth factor-like). In humans, Ron is one of only two members of a distinct receptor tyrosine kinase (RTK) family that also includes Met. The highest amino acid identity between Ron and Met is located within the kinase domain (63% identity); the other regions are not highly conserved (34% overall). The respective ligands for Ron and Met are also similar; MSP is 45% identical to hepatocyte growth factor (HGF), the Met ligand. Both ligands are glycoproteins that are secreted as inactive single-chain peptides and are proteolytically processed into active, disulfide-linked α/β heterodimers [6]. HGF binds and activates Met and MSP binds and activates Ron [7-9]; although there is crosstalk between Ron and Met intracellular signaling [10, 11] the ligands and receptors are not interchangeable [12].

Like their ligands, Met and Ron are cleaved disulfide-linked heterodimers. The mature receptors consist of extracellular α and β chains, involved in ligand binding, and the intracellular portion of the β chain, which is responsible for signaling. Binding of ligand causes receptor homodimerization and phosphorylation of two tyrosine residues within the catalytic site, which regulates kinase activity [8, 13]. Activation of kinase activity results in phosphorylation of the carboxy-terminal docking site of the receptor. The docking site is essential for downstream signaling through direct and indirect binding of SH2 domain-containing adaptor proteins such as Grb2, PI3K, and Src [14]. Ron and Met are both expressed in a variety of tissues during development and, in adults, are expressed mainly on epithelial cells and in the nervous system. However, Ron is also highly expressed on adult macrophages and osteoclasts.

Although the signaling pathways that are activated by Ron and Met are similar, they culminate in related, yet distinct, cellular functions. Both are known to induce "scattering," a phenomenon in which cells detach from one another and migrate away from the central colony [10, 15, 16]; both promote proliferation through the MAPK pathway and survival through both MAPK and PI3K/AKT pathways [17-19]; and both have the ability to promote an epithelial-mesenchymal transition, albeit in distinct situations [20, 21]. However, a major effect of MSP/Ron signaling is on the motility and activation of macrophages – a function clearly divergent from that of Met.

Terminally differentiated macrophages express Ron and were first noted to respond to MSP by rapidly altering their shape and increasing chemotactic and phagocytic ability [22, 23]. More recently, it has been realized that MSP/Ron also plays a critical role in attenuation of the inflammatory response. Mice lacking Ron activity display defects in the inflammatory process, most notably the inability to downregulate TNFα and nitric oxide production in response to infection or injury [24-27]. Thus, MSP/Ron signaling plays a dual role in regulating inflammation: initial stimulation of chemotaxis and phagocytosis – important features of "classical" macrophage activation - and, more critically, resolution of the inflammatory response by promotion of the "alternatively activated" macrophage state (discussed in more detail below).

MSP belongs to a group of kringle domain-containing proteins that diverged from an ancient family of serine proteases involved in blood coagulation and fibrinolysis [28]. Amino acid substitutions in the catalytic domain during evolution rendered MSP inactive as a protease, although it retained the feature of being cleaved and activated by other serine proteases. Such cleavage is, in fact, required for the conversion of pro-MSP to the mature, active form of MSP that can bind and activate Ron.

Activation of pro-MSP was originally discovered in wound exudates, where it resulted in stimulation of macrophage activity [23]. A serine protease responsible for activating pro-MSP was localized to macrophage membranes [29] and later identified as matriptase [30]. However, other proteases, such as hepatocyte growth factor activator, also appear to cleave and activate pro-MSP *in vivo* [31]. Pro-MSP is predominantly secreted from the liver, and exists in the blood plasma in its biologically inactive form at a concentration of about 5nM, and is thus poised to initiate Ron signaling upon cleavage [23].

Functional consequences of MSP/Ron signaling are not limited to macrophages or the inflammatory process. Ron, like Met, is upregulated in many types of epithelial cancer, and they are occasionally co-upregulated [32, 33]. Although the role of Met in cancer has been investigated for more than 20 years, culminating in development of multiple targeted therapies now in clinical trials (for a recent review, see [34]), Ron has more recently been recognized as a major player in progression of human epithelial cancers. This report focuses on the significance of the MSP/Ron pathway in breast cancer, and the ensuing opportunities for therapeutic intervention.

RON EXPRESSION AND FUNCTION IN BREAST CANCER

Ron is expressed at very low levels in normal human breast epithelium, but becomes overexpressed in a large proportion of breast tumors (Table **1**). Interestingly, Ron mutation is not associated with breast cancer, suggesting that overexpression of the wild type protein is sufficient to contribute to tumor development or progression. The reason for overexpression has not yet been established. Both MSP and Ron are located on chromosome 3p21.31, and the 3p21 region is often altered in cancer. Specifically, 3p21 undergoes both loss of heterozygosity [35] and amplification in various tumors and cancer cell lines [36, 37]. This region was amplified in 15-42% of lung, renal, and breast cancers examined [36], which suggests that amplification could contribute to MSP and/or Ron overexpression in breast cancer. Consistent with this, MSP is also overexpressed by up to ~20% of early stage human breast tumors [38].

Mouse models have been instrumental for elucidating the contribution of Ron signaling to breast cancer. When overexpressed under the mouse mammary tumor virus (MMTV) promoter, Ron caused mammary hyperplasia by 12 weeks of age, followed by development of adenocarcinoma

in 100% of female mice [39]. Tumors initiated by Ron exhibited spontaneous metastasis to liver and/or lung in ~90% of animals, which is remarkable given the relatively limited metastatic potential of other transgenic mouse models of breast cancer [40]. Confirmation that the Ron pathway is a significant contributor to breast tumor metastasis was obtained by examining 457 breast cancers from two independent patient cohorts [38]. In these studies, co-overexpression of MSP, its activating enzyme matriptase, and Ron (collectively referred to as MSP/matriptase/Ron) was used as a surrogate indicator of Ron signaling activity, and was a significant independent prognostic factor for metastasis and reduced survival. Importantly, overexpression of MSP or Ron mRNA alone did not significantly correlate with patient outcome, suggesting that Ron function in metastasis of breast cancer could be largely ligand-dependent, even when the receptor is overexpressed. Indeed, activation of Ron by overexpression of MSP in a mouse model of mammary cancer (transgenic mice expressing the polyomavirus middle T antigen under the mouse mammary tumor virus promoter; MMTV-PyMT [41]) was sufficient to cause spontaneous metastasis to lung, lymphatics, and bone. Patients whose tumors expressed MSP/matriptase/Ron also exhibited significantly more metastasis to lung, liver, brain, and bone (bone was the most frequent site of metastasis). Furthermore, MSP-induced bone metastases in the mice were osteolytic, as they are in human breast cancer patients, and appear to be the first example of spontaneous metastasis of a primary (non cell line-derived) tumor from the mammary gland to the bone in mice [38]. Thus, MSP/Ron activity exerts a gain-of-function effect in breast cancer, promoting tumor metastasis to clinically relevant sites.

OPPORTUNITIES FOR RON INHIBITOR THERAPY IN BREAST CANCER

Consistent with the knowledge that breast cancer is a complex and remarkably heterogenous disease, single agent targeted therapy has generally not been effective long term, even in combination with standard chemotherapy. This is particularly true for metastatic breast cancer, which is still considered incurable [42]. Our ever-increasing understanding of mechanisms involved in tumor progression suggests that the ability to simultaneously abrogate several independent processes that are critical for cancer progression would hold great promise for new therapeutic approaches.

It is now realized that many of the processes that contribute to tumor progression and metastasis are not actually intrinsic to the tumor cells. Rather, the tumor microenvironment plays a key role in critical processes such as angiogenesis [43], growth factor production [44], tumor inflammation and immunoediting [45], invasion and intravasation [46], as well as modifying the metastatic site to create a hospitable environment [47]. In fact, it has become clear that tumor progression and metastasis in mouse models can be severely restricted or even eliminated by limiting tumor inflammation [48, 49].

Data obtained *in vivo* using sophisticated mouse models and primary human breast cancer specimens strongly suggest that the Ron pathway is an exciting new target for therapy against solid tumors. MSP/Ron not only plays a causal role in tumor development and progression, but also plays a critical role in the type of inflammation that is known to occur in tumor microenvironments [50, 51]; this is discussed in detail below. Based on the known function of Ron in tumor cells, macrophages and osteoclasts, we suggest that Ron inhibition would simultaneously block essential processes both *intrinsic* and *extrinsic* to the tumor cells: tumor growth and angiogenesis, promotion of metastasis by 'alternatively activated' macrophages, promotion of the wound healing process, and osteolysis due to breast cancer bone metastasis. The specific functions of Ron in each of these processes in the 'normal' state are discussed in detail below, followed by a discussion of the implications for Ron activity in the setting of cancer.

TUMOR-INTRINSIC ACTIVITIES: MSP/RON IN TUMOR GROWTH AND ANGIOGENESIS

Investigation of Ron activity in epithelial cancer cell lines has revealed roles in cell proliferation, survival, migration, and epithelial-mesenchymal transition [15, 52-54]. Selective Ron inhibitors have been generated and were reported to affect these processes, indicating that blockade of Ron function is achievable at least in certain settings [18, 55].

As described above, gain of function studies in mouse models have shown that activation of Ron through either overexpression of MSP [38] or overexpression of Ron [39] was sufficient to increase tumor growth as well as both the frequency and tissue tropism of metastasis in mice, and overexpression of MSP/matriptase/Ron significantly correlated with increased metastasis and death in breast cancer patients [38].

Conversely, loss of Ron function has been demonstrated to affect tumor growth, angiogenesis, and metastasis in a mouse model of breast cancer. Mice lacking the tyrosine kinase domain of Ron (Ron TK-/- [56]) were crossed to MMTV-PyMT mice, which resulted in decreased mammary tumor growth and reduced metastasis to lung – the only site of metastasis in the MMTV-PyMT model. This effect occurred in parallel with decreased vasculature and increased apoptosis in the tumors [57]. Selective Ron inhibitors have also shown some efficacy in xenograft models for other types of cancer (see below). Together, these data suggest that abrogation of Ron activity can impair tumor growth and reduce the likelihood of metastasis, and that pre-clinical studies using Ron inhibitors have shown promising results.

TUMOR-EXTRINSIC ACTIVITIES: MSP/RON IN MACROPHAGE ACTIVITY

Ron is expressed on terminally differentiated resident macrophages, but not on mononuclear phagocytes or circulating monocytes; Ron is upregulated during macrophage differentiation [58]. Ron is expressed on many different types of resident macrophages including alveolar macrophages, microglia, peritoneal macrophages, and dermal macrophages from either normal or wounded skin [23, 59, 60].

As suggested by its name, MSP does function to stimulate macrophages. Activated MSP increases the ability of

macrophages to undergo chemotaxis; stimulation of Ron by MSP leads to rapid changes in cell shape and motility [30, 61]. MSP/Ron activity also promotes rapid phagocytosis of C3bi coated erythrocytes *via* complement receptor 3 [62].

Consistent with its function in macrophage stimulation, *in vivo* experiments demonstrate that the Ron pathway is important in protection against Gram-positive bacteria. When Ron -/- mice were challenged with *Listeria monocytogenes* they showed increased bacterial burden and increased susceptibility to the infection - a phenotype similar to that of interferon-gamma (IFNγ) knockout mice and tumor necrosis factor-alpha (TNF-α) knockout mice. Lack of Ron function may manifest itself in the inability of macrophages to efficiently eliminate the bacteria, as rapid clearance by macrophages *via* the complement receptor is known to be essential in preventing *Listeria* infections [62].

Studies of MSP/Ron signaling in macrophages indicate that, although the Ron pathway is involved in macrophage activation and protection from particular microorganisms, it is *critical* for resolution of inflammation in many models. Mice lacking Ron activity are viable and fertile but have noteworthy defects in macrophage function; Ron is necessary to limit inflammatory responses [56, 63]. Peritoneal macrophages isolated from Ron deficient mice produce increased levels of nitric oxide in response to lipopolysaccharide (LPS) stimulation, and when Ron TK-/- mice are challenged with sub-lethal doses of LPS, they are more susceptible to LPS-induced endotoxic shock [56].

The mechanisms by which MSP/Ron signaling functions to resolve inflammation are elegantly studied. One important function of Ron is to downregulate interleukin 12 (IL-12) production in macrophages. The inability of Ron TK-/- mice to downregulate IL-12 leads to increased IFN-γ production by natural killer cells, and a prolonged inflammatory reaction [50]. *In vitro*, MSP is sufficient to polarize macrophages from the "classically activated" to the "alternatively activated" state [64] (also known as the M1 and M2 states, respectively, and further described below). MSP/RON signaling also suppresses inflammation through several other routes: activation of suppressors of cytokine signaling, down-regulation of IFN-γ, reduction of major histocompatability complex class II cell surface expression, and reduction of IFN-γ-induced STAT1 phosphorylation [50]; downregulation of inducible nitric oxide synthase (iNOS) [64]; increased production of the anti-inflammatory cytokine IL-10 [65]; and downregulation of cyclooxygenase-2 (COX-2) expression through inactivation of Nuclear Factor kappa B (NFkB) [66].

Taken together, the published data indicate that the MSP/Ron pathway plays a dual role in inflammation: a role in initial macrophage activation, as well as an important role in downregulating the inflammatory response. Ron activity results in increased migration of macrophages to sites of infection, and stimulates phagocytosis early in the infection process. Later, Ron is required to resolve inflammation by downregulating iNOS and IL-12 and by upregulating IL-10. Still, much is to be learned: the different roles for Ron signaling in infections elicited by gram-positive versus gram-negative bacteria indicate that MSP/Ron function is context dependent, and the interaction of MSP-stimulated macrophages with cells of the acquired immune system is yet to be discovered.

AN INTERSECTION OF INSTRINSIC AND EXTRINSIC FACTORS: MSP/RON IN WOUND HEALING

Skin wound repair is essential for tissue homeostasis and involves three phases: inflammation, proliferation, and remodeling (for review see [67]). Inflammatory cells play a crucial role in the wound healing process. Macrophages remove dead tissue, stimulate the growth of new blood vessels, regulate fibroblast recruitment, re-growth of the epithelium, and remodeling of connective tissue (for review see [68]). Classically activated macrophages (M1 macrophages) are present early during the wound healing process and function to remove pathogens and stimulate the immune response, whereas alternatively activated (M2) macrophages predominate later in the repair process. M2 macrophages fail to present antigen to T cells, produce minimal amounts of pro-inflammatory cytokines and nitric oxide, and are less efficient than M1 macrophages at killing microbes. Instead, M2 macrophages secrete extracellular matrix (ECM) proteins and polyamines, which influence production of cytokines, inhibit clonal expansion of lymphocytes, and stimulate proliferation of epithelial cells [69]. M2 macrophages are characterized by downregulation of iNOS and upregulation of arginase 1, which metabolizes arginine to urea and ornithine. Consequently, there is increased arginase activity in experimental rat wounds, along with increased ornithine levels [70]. Importantly, MSP/Ron signaling is instrumental in the switch from expression of iNOS to expression of arginase [64].

Successful wound repair entails resolution of the inflammatory response and, as discussed above, MSP is both necessary and sufficient to induce M2 macrophage polarization, which assists in attenuation of inflammation. MSP/RON signaling is involved at various steps of the wound healing process. In experimental excisional wounds in rats, immunostaining revealed both MSP and Ron within the wound, where maximum staining occurred between 7 and 21 days post-wounding [71]. There are also increased levels of activated MSP in fluids collected from burn wounds in humans, and RON-expressing macrophages are scattered throughout the dermis.

In addition to resolving inflammation in wounds, Ron plays a role in repairing wounded skin. This process, referred to as re-epithilialization, involves migration and proliferation of epidermal keratinocytes. Cells at the wound margin loosen their extracellular matrix (ECM)-cell and cell-cell interactions in order to migrate across the wound (for review, see [72]. Ron is upregulated by proliferating and differentiated populations of keratinocytes [23], and MSP promotes keratinocyte migration in mouse wounds and in wound healing assays *in vitro*. In primary keratinocytes, the 14-3-3 protein associates with Ron in response to MSP signaling, which induces spreading and improved migration on laminin 5 ECM [73]. It is notable, however, that MSP deficient mice do not show any defects in specific skin wound healing models [74], suggesting that functional redundancies exist for this important biological process.

The role of MSP/Ron in other models of injury has also been investigated. In two different models of lung injury, Ron proved to be essential for protection from unregulated inflammation. When injected with intrapulmonary LPS, mice lacking Ron function display increased lung injury and damage due to overproduction of nitric oxide and TNFα through the NFkB pathway. Again, MSP/Ron function was deemed necessary to suppress NFkB activation *in vivo* [75]. In a nickel-induced acute lung injury model, in which mice are exposed to aerosolized nickel particles, mice lacking RON function exhibited significantly decreased survival times compared to control mice. The mice showed increased levels of IL-6 and the chemokines Chemokine (C-C motif) ligand 2 (CCL2) and Chemokine (C-X-C motif) ligand 12 (CXCL12), as well as increased serum nitrite levels. These effects were commensurate with earlier onset of pulmonary inflammation, edema and lethality [76]. Gene expression analysis indicated that genes responsible for inflammation, edema and lymphocyte function were significantly altered in mice lacking Ron activity [77].

Paradoxically, in a model of LPS-induced acute liver failure in galactosamine-sensitized mice, RON deficient mice are actually protected from liver injury. This finding was based on histological analysis as well as serum alanine amino transferase levels, and was associated with decreased number of liver cells undergoing apoptosis [78].

The last three examples of Ron involvement in injury indicate that the cytokine milieu and the type of injury likely influence the outcome of MSP/Ron signaling. Data from mice lacking Ron activity indicate that blocking MSP/Ron signaling therapeutically may not adversely affect healing of common skin wounds, but could be a concern for life-threatening infections. An important consideration, however, is whether acute loss of Ron function (as would occur with therapeutic blockade of Ron signaling) would have different effects than the chronic lack of function that develops in genetically engineered mice.

INFLAMMATION AND CANCER: DUAL FUNCTION OF MSP/RON?

A growing body of evidence supports the idea that inflammation contributes to cancer development and progression (for review, see [45]). The risk of developing cancers of the esophagus, colon, pancreas, lung, and gallbladder is heightened by the presence of chronic inflammatory diseases. Chronic inflammation, like chronically unhealed wounds, is characterized by a prolonged cycle of tissue damage, cellular proliferation and tissue repair [79]. The inflammatory environment is enriched with macrophages that generate high levels of reactive oxygen and nitrogen species to fight infection. However, when unregulated, these agents can react with DNA and cause mutations in proliferating epithelial and stromal cells (for review, see [80]).

Increased tumor associated macrophage (TAM) density is associated with tumor progression and metastasis (for review, see [81]). TAMs have many characteristics of an M2 activation phenotype, and are thought to contribute to tumor development by releasing IL-10 and PGE2, which suppress the inflammatory reaction to the tumor [82]. TAMs also release pro-angiogenic factors such as vascular-endothelial growth factor (VEGF), endothelin 2 and plasminogen activator, and pro-proliferative factors such as epidermal growth factor (EGF), fibroblast growth factor, HGF, platelet-derived growth factor, transforming growth factor β (TGFβ), and IL-6 [83, 84]. TAMs are also thought to facilitate tumor cell invasion and metastasis by releasing MMP2 and MMP9, which modify the ECM and basement membrane, and by facilitating a paracrine loop of EGF and colony stimulating factor-1 signaling to promote metastasis [85]. Thus, TAMs endow tumors with an environment that enhances the survival, migration and proliferation of epithelial cells and are a large contributor to the observation that tumors are much like chronically unhealed wounds [86].

Although there is insufficient evidence at this time to suggest that MSP/Ron-induced inflammation *directly* participates in cancer progression, studies indicate that MSP is able to evoke dose-dependent superoxide anion production in human alveolar macrophages *via* src, MAPK, and p38 signaling pathways [59]. In human alveolar macrophages from either smokers or non-smokers, MSP efficiently activates NF-kB. However, MSP evokes superoxide production, cytokine release and NFkB activation to significantly higher levels in cells from smokers versus those from non-smokers, indicating that MSP may enhance inflammation due to cigarette smoke [65]. Although this may contribute to tumorigenesis, there is another likely, and potentially more impactful, role for MSP/Ron in tumor progression and metastasis: polarization of TAMs to an M2 phenotype.

CONSEQUENCES OF MSP/RON ACTIVATION IN TUMORS

As described above, pro-MSP is present in high concentrations in serum, and conversion of pro-MSP into MSP occurs locally at sites of inflammation [23]. A serine protease that was shown to cleave and activate MSP, matriptase, is normally present on macrophages, but is also upregulated in a large percentage of breast cancers [30]. In addition, RON is overexpressed to high levels (and Ron is phosphorylated) in ~50% of breast cancers [87]. It is reasonable to presume that activation of MSP locally, at sites of inflammation in tumors, would not only lead to activation of Ron on TAMs, but also on the tumor epithelium, where Ron has been shown to induce proliferation, survival, cell migration, EMT, invasion, and metastasis (see above).

Although the result of Ron signaling in TAMs is still unclear, MSP/Ron activates signaling pathways in macrophages that are known to be involved in tumor progression. Ron activation causes phosphorylation of the signal transducer and activator of transcription 3 (STAT3) protein [88], which is required for the immunosuppressive and tumor promoting effects of TAMs. In fact, STAT3 knockout mice [89] show similar inflammatory phenotypes as RON deficient mice, and several infectious agents are known to cause inflammation-induced cancer *via* STAT3 activation [90]. Furthermore, the MSP/Ron-induced cytokine IL-6 activates STAT3 in both inflammatory cells and epithelial cells [90].

In addition to the immunosuppressive effects of MSP/Ron *via* STAT3 activation, the MSP/Ron pathway also

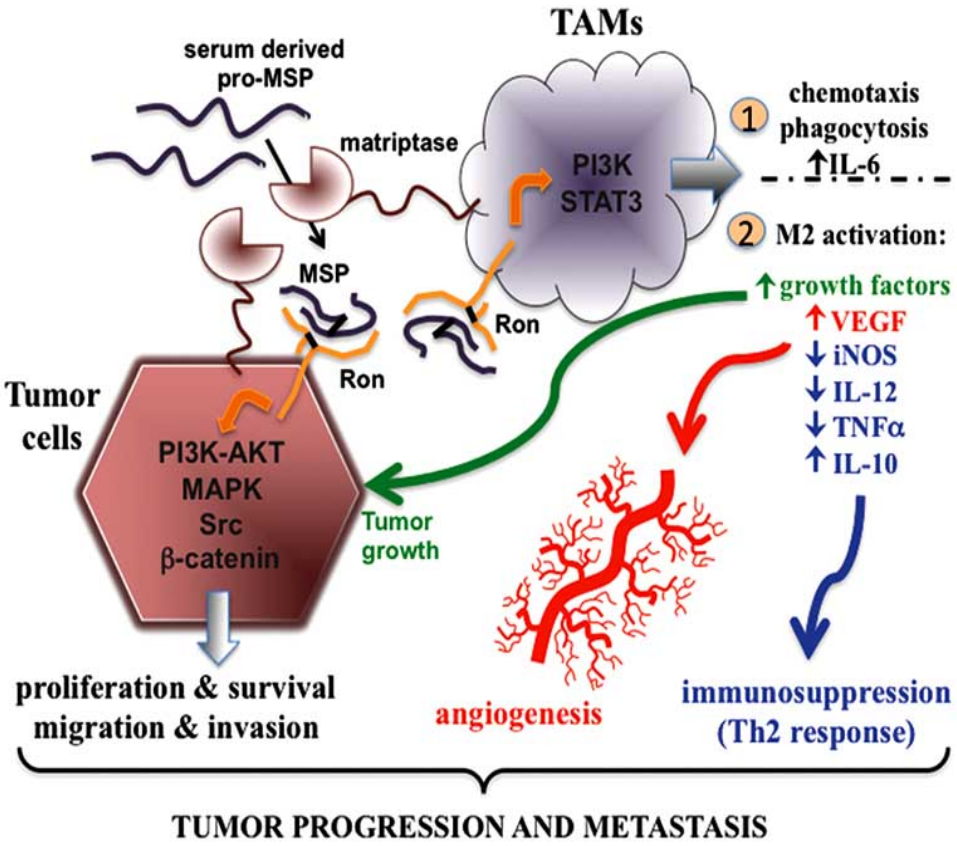

Fig. (1). Model for the contribution of MSP/Ron function in tumor progression and metastasis through both cell autonomous (tumor cell proliferation, survival, migration and invasion) and non-cell autonomous (macrophage activation and polarization) functions.

down-regulates STAT1 activity [27], which is involved in anti-tumor immune responses through upregulation of IL-12 [90]. STAT1 and STAT3 clearly act in opposing roles with regard to immune responses against tumors; genetic deletion of STAT3 in immune cells leads to upregulated STAT1 activity and increased anti-tumor properties [91]. Thus, it is likely that both STAT3 activation and STAT1 inhibition by MSP/Ron may manifest in immune tolerance to the tumor, in addition to the potential function of MSP/Ron in promoting secretion of pro-growth and pro-angiogenic factors by M2-polarized TAMs. The potential consequences for MSP/Ron activation in tumors are summarized in Fig. (**1**).

MSP/RON IN OSTEOLYTIC BONE METASTASIS

In addition to its expression and activity in macrophages, Ron is also expressed on osteoclasts, the specialized macrophages of bone. Ron becomes expressed on the surface of multinucleated osteoclast-like cells when human bone marrow cells are differentiated *in vitro*, and MSP activates osteoclasts, causing bone resorption [92]. *In vivo*, Ron is highly expressed on osteoclasts but does not appear to play a critical role in bone development, since mice lacking Ron function have no overt bone defects [56].

The role of MSP in osteoclast activation is highly relevant to breast cancer, since bone metastasis occurs in 70-80% of patients and is therefore the most common site for relapse [93, 94]. Osteoclasts are activated *in vitro* by breast cancer cells that express MSP, and to a much greater extent than that induced by control tumors [38]. Furthermore, mice with mammary tumors expressing MSP spontaneously developed osteolysic bone metastasis, and breast cancer patients with high MSP/matriptase/Ron experienced significantly more metastasis to bone than those without high MSP/matriptase/Ron [38]. A model for the role of MSP/Ron activity in osteolytic bone metastasis is shown in Fig. (**2**). A specific understanding of whether the MSP/Ron pathway contributes to the "vicious cycle" of breast tumor growth in bone that was previously proposed by Guise and Mundy (for recent reviews on breast cancer bone metastasis, see [93, 95]), or whether MSP/Ron activation defines a new mechanism for osteolysis remains to be determined. Understanding the role of the MSP/Ron pathway in breast cancer bone metastasis would have important clinical implications.

MSP/RON AS A THERAPEUTIC TARGET

The ability to simultaneously block several key pathways that contribute to tumor progression might lead to more efficacious therapy. We suggest that the MSP/Ron pathway holds promising potential in this regard, since it is upregulated in a large proportion of cancers and contributes

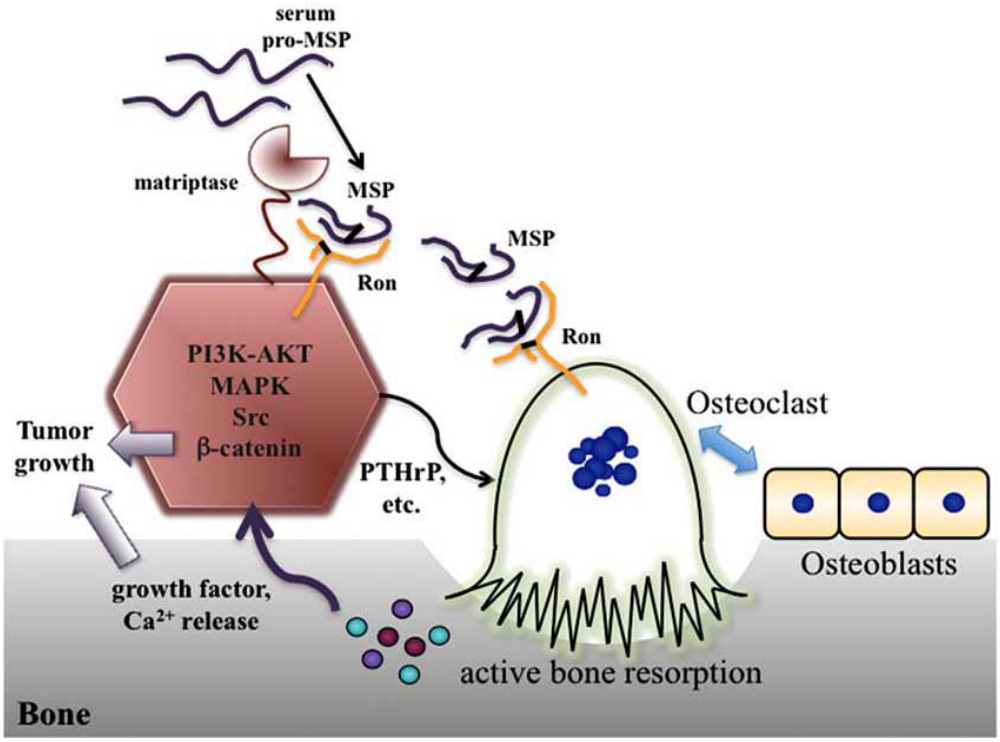

Fig. (2). Model for the role of MSP/Ron activity in osteolytic bone metastasis as a complication of breast cancer. MSP can directly activation osteoclast activity through Ron stimulation. The ensuing bone resorption can release calcium and growth factors that stimulate tumor growth and perpetuate a "vicious cycle" [93].

to proliferation, survival, migration, and invasion of tumor cells. In addition, though, MSP/Ron activity also promotes M2 macrophage polarization, potentially leading to secretion of immunosuppressive cytokines as well as growth and angiogeneic factors that support the tumor. Blockade of MSP/Ron function might therefore interfere with critical tumor-promoting pathways in the tumor itself *and* in the tumor microenvironment.

Strategies for Ron Inhibition

One can imagine several potential strategies to interfere with MSP/Ron function, including prevention of pro-MSP activation, blockade of MSP-Ron interaction and/or receptor dimerization, and inhibition of Ron kinase activity. Inhibition of MSP activating enzymes such as matriptase is unlikely to be effective due to redundancy between several serine proteases capable of activating MSP *in vitro* and *in vivo* [30, 31, 96]. Strategies to prevent ligand-receptor interactions and/or receptor downregulation could be achieved through generation of monoclonal antibodies (mABs). mABs can also have the added benefit of inducing antibody-mediated cellular cytotoxicity, analogous to that achieved by the HER2 antibody trastuzumab (Herceptin) in breast cancer. One Ron inhibitory antibody has been described, and was shown to be effective in slowing growth of colon, lung, and pancreatic cancer xenografts [18].

Inhibition of kinase activity may be less specific, due to high conservation of kinase domains within receptor tyrosine kinases, but would have the added benefit of oral availability and potentially lower cost. One advantage of targeting Ron with a small molecule kinase inhibitor is that Met kinase inhibitors are already available, some of which are being tested in clinical trials [34]. Since the kinase domains of Ron and Met are 68% identical, it is very likely that a Met inhibitor will also block Ron, at least to some extent. A dual Ron/Met inhibitor recently showed promising results in xenograft studies using Met-dependent cell lines or colon cancer cells expressing an endogenous, hyperactive form of Ron (see below) [55].

Challenges for Drug Development Against MSP/Ron

As with all potential new therapies, there are great challenges. The MSP/Ron pathway, in particular, may be even more confounding due to dual effects on the tumor and on the host immune system. One hurdle in drug development for oncology is pre-clinical testing in animal models, and immunodeficient mice are routinely used for initial studies. However, if MSP/Ron functions to promote tumor progression and/or metastasis through alteration of immune function, the results would be very difficult or impossible to discern in such a model. Use of syngenic, immunocompetent mouse models such as the one we developed [38] can overcome the problem of immune involvement, but pre-

cludes testing species-specific drugs such as anti-human Ron antibodies. Likewise, if activation of host macrophages is a key component of MSP/Ron function in tumors, drugs that recognize and inhibit both the human and murine Ron proteins would be required for validation in xenograft models.

The existence of multiple isoforms of Ron poses another challenge for drug development. There are a number of alternative Ron isoforms described. These include hyperactive splice variants [97, 98], and an N-terminally truncated form of Ron, termed short form Ron (sfRon). sfRon is generated through utilization of a second, internal promoter within intron 10 of *RON,* creating a constitutively active form of the receptor that does not require ligand binding for activity [99]. sfRon is expressed in cell lines originating from multiple cancer types, and has been detected in primary breast cancers [99]. In mice, sfRon is required for transformation of erythroblasts by the Friend virus, and mice with a naturally occurring polymorphism in the sfRon promoter are resistant to this form of erythroleukemia [100]. Since the human sfRon promoter is relatively uncharacterized, it is unknown whether polymorphisms exist and are relevant to tumorigenesis. However, methylation of the main RON promoter may contribute to expression of sfRon in cancer cell lines [101]. Upregulation of active forms of Ron in cancer could serve as an important contributor toward resistance to therapies designed to interfere with ligand binding, for example. Thus, in our view, Ron kinase inhibitors may hold the greatest promise for targeted therapy against this pathway in breast cancer.

RELEVANCE OF THE MSP/RON PATHWAY IN OTHER CANCERS

The Ron pathway may also be an excellent therapeutic target in cancers other than breast. Ron is overexpressed in a wide variety of human cancer tissues (Table **1**) and, although its function in the epithelial compartment is not understood for all of these malignancies, the function of MSP/Ron in tumor inflammation is likely to be conserved. MSP and matriptase are also upregulated in many cancers [30, 38, 102, 103], further supporting the idea that both autocrine and paracrine pathways could contribute to tumorigenesis and/or progression of malignancy.

Ron is overexpressed in small cell lung carcinoma (SCLC) cell lines, a pulmonary carcinoid cell line, and in non-small cell lung carcinoma (NSCLC) [102, 103]. While MSP expression is low to undetectable in both SCLC and pulmonary carcinomas, MSP is expressed in NSCLC primary tumors and cell lines [103, 104]. Addition of MSP to NSCLC cell lines expressing Ron resulted in increased cell motility [103]. In addition, overexpression of Ron in distal lung epithelial cells results in the development of lung adenomas *in vivo* [105].

Expression of full length Ron, as well as various isoforms of Ron, has been demonstrated in human colon cancer cell lines as well as primary adenocarcinomas [97, 106, 107]. Ron is highly expressed in 60% of colorectal adenocarcinomas and its expression correlates with the degree of differentiation of these tissues [106, 107]. The constitutively active splice variants RONΔ155, RONΔ160, and RONΔ165 are most notably expressed in colon cancers. Expression of RONΔ155 or RONΔ160 in NIH3T3 cells lead to tumor formation *in vivo* [97, 106-108], and expression of full length Ron in colon epithelial cells results in an increase in cell motility and invasiveness, while protecting the cells from apoptosis [108]. Silencing Ron expression by RNAi in colon cancer cell lines led to decreased cell proliferation and motility, with an increase in apoptosis [109]. Silencing of Ron also reduced tumorigenesis *in vivo*, suggesting that Ron expression is required to maintain the tumorigenic phenotypes of colon cancer cells [109].

Table 1. Expression of Ron in Primary Human Cancer Tissues

Cancer Type	% of Tumors Expressing Ron	Reference(s)
Breast	50	[87]
	32	[32]
	100	[18]
	8-20*	[38]
Lung	93	[18]
	50 (NSCLC# only)	[103]
Colon	60	[106, 107]
	65	[18]
Pancreatic	93	[114]
	79-93	[115]
	69	[18]
Bladder	33	[116]
Ovarian	56-60	[118]
Prostate	92	[18]
Liver	29 (HCC† only)	[123]
Gastric	73	[18]
Glioblastoma	82	[121]

* MSP/matriptase/Ron co-overexpression
# Non small cell lung carcinoma
† Hepatocellular carcinoma

While mutations in Ron have not been identified in cancers other than in a single lung tumor [110], two alterations have been identified which may have a role in Crohn's disease [111]. A genome wide linkage study performed with a cohort of Crohn's disease patients identified strong linkage disequilibrium with two non-synonymous single nucleotide polymorphisms (SNPs) within the *RON* gene [111]. The first SNP, rs2230590, results in an Arg523Gln substitution while the second, rs1062633, results in a Gly1335Arg substitution. Further evidence for a role for the MSP/Ron pathway in inflammatory bowel diseases (IBD) comes from another genome wide linkage study performed on a cohort of IBD patients. This study identified significant linkage disequilibrium with a SNP located within the *MSP* gene [112]. This nonsynonomous SNP, rs3197999, results in an Arg698Cys coding variant, which is predicted to interfere with the ability of MSP to bind to Ron [112]. Importantly, this coding variant showed association with both Crohn's disease and ulcerative colitis, suggesting that the MSP/RON pathway

may have an important role in multiple forms of inflammatory bowel disease - though the mechanisms for this role have yet to be elucidated [112]. Associations between *RON* and *MSP* SNPs in inflammatory bowel diseases, which predispose patients to colon cancer (for a recent review, see [113]), provide further support for a connection between MSP/Ron function in inflammation and tumor progression (Fig. **1**).

Ron is overexpressed in 79-93% of human pancreatic tissue samples, and in 83% of metastatic lesions [114, 115]. Activation of Ron by MSP in pancreatic cell lines leads to activation of Erk and Akt pathways, as well as induction of EMT characteristics such as increased cell migration, invasion, and loss of E-cadherin [114, 115]. Inhibition of Ron by a neutralizing antibody resulted in inhibition of the cell migratory and invasive phenotypes [115].

Ron is overexpressed in 33% of primary bladder tumors, where Ron levels correlated with poor grade as well as tumor size and stage [116]. Overexpression of Ron in a uroepithelial cell line led to proliferation, motility, and increased survival [116]. Ron also cooperated with Met and EGFR in these cells; co-expression of Ron and Met was significantly associated with decreased survival and metastasis-free survival in 19% of patients [116]. Co-expression of Ron and EGFR was found in 33% of patients and significantly associated with invasion, risk of recurrence, and decreased patient survival [117].

Ron expression was detected in 56% of ovarian cancers and 60% of borderline ovarian tumors [118]. The level of Ron expression also significantly correlated with decreased survival in ovarian cancer patients [119]. A correlation between overexpression of Ron and concomitant expression of Met was demonstrated, and stimulation of ovarian cancer cell lines *in vitro* by MSP and/or HGF lead to increased motility and invasion [118, 120].

Full length Ron, and several splice variants, were expressed in primary human glioblastomas and glioblastoma cell lines. Of the glioblastoma patient samples analyzed, 82% expressed some form of Ron, while 100% of the glioblastoma cell lines analyzed demonstrated Ron expression [121]. MSP was also expressed in glioblastoma cell lines, where it functions to increase cell migration [121]. A novel splice variant, RONΔ90 was also identified, which inhibited MSP-induced phosphorylation of Ron as well as cell migration.

Ron was expressed in 92% of prostate tumor tissues and is overexpressed in prostate cancer cell lines [18]. Ron expression correlates with the stage of disease in the primary tumor and is expressed in prostate metastases. Levels of angiogenic chemokines correlate with Ron expression, and knockdown of Ron resulted in a decrease in angiogenic factors, NF-kB, and endothelial cell migration *in vivo*. Knockdown of Ron also resulted in decreased tumor growth and microvessel density, indicating that Ron may play an important role in the angiogenic process in prostate cancer [122].

Ron has been shown to be overexpressed in two out of seven hepatocellular carcinoma (HCC) tissue samples. The cytokines IL-1α, IL-6, and TNFα, as well as the growth factor HGF were shown to increase Ron expression in a HCC cell line. These factors are commonly upregulated in liver disease and may therefore play a role in liver carcinogenesis through the upregulation of Ron [123]. Notably, the liver is the primary site of MSP production and therefore may contribute to increased Ron activity in the liver.

CONCLUSIONS

In summary, the MSP/Ron pathway appears to be active in a large number of solid tumors from various organs, and MSP/Ron activity correlates with aggressive disease and poor outcome. The known roles for this pathway strongly suggest that MSP/Ron could play a significant, dual role in tumor progression by acting directly on tumor cells and indirectly through inflammatory cells. Thus, inhibition of Ron may provide a promising new avenue for cancer treatment by simultaneously affecting at least two critical aspects of tumor progression. In breast cancer, blockade of Ron function may succeed in decreasing tumor growth, metastasis, and destruction of bones through MSP-driven osteolysis.

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CHAPTER 4

INHIBITION OF RON KINASE BLOCKS CONVERSION OF MICROMETASTASES TO OVERT METASTASES BY BOOSTING ANTI-TUMOR IMMUNITY

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Inhibition of Ron Kinase Blocks Conversion of Micrometastases to Overt Metastases by Boosting Antitumor Immunity

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RESEARCH BRIEF

Inhibition of Ron Kinase Blocks Conversion of Micrometastases to Overt Metastases by Boosting Antitumor Immunity

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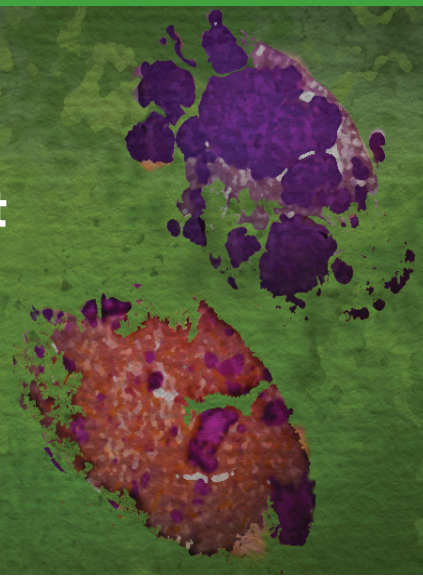

ABSTRACT Many "nonmetastatic" cancers have spawned undetectable metastases before diagnosis. Eventual outgrowth of these microscopic lesions causes metastatic relapse and death, yet the events that dictate when and how micrometastases convert to overt metastases are largely unknown. We report that macrophage-stimulating protein and its receptor, Ron, are key mediators in conversion of micrometastases to *bona fide* metastatic lesions through immune suppression. Genetic deletion of Ron tyrosine kinase activity specifically in the host profoundly blocked metastasis. Our data show that loss of Ron function promotes an effective antitumor $CD8^+$ T-cell response, which specifically inhibits outgrowth of seeded metastatic colonies. Treatment of mice with a Ron-selective kinase inhibitor prevented outgrowth of lung metastasis, even when administered after micrometastatic colonies had already been established. Our findings indicate that Ron inhibitors may hold potential to specifically prevent outgrowth of micrometastases in patients with cancer in the adjuvant setting.

SIGNIFICANCE: Our data shed new light on an understudied, yet critically important aspect of metastasis: the conversion of clinically undetectable micrometastatic tumor cells to overt metastases that eventually cause death of the patient. Our work shows that Ron inhibition can significantly reduce metastatic outgrowth, even when administered after metastatic colonies are established. *Cancer Discov; 3(7); 751–60. ©2013 AACR.*

INTRODUCTION

Metastatic tumor growth in secondary organs is the main cause of death from cancer. For example, 20% to 30% of people diagnosed with stage II–III breast cancer eventually develop metastatic disease, which typically occurs 3 to 16 years after the initial diagnosis (1). This clinical "dormancy" period followed by late relapse is also frequently observed in cancers of the prostate, kidney, and thyroid, and in B-cell lymphomas and melanoma (2), making this a critical issue in clinical cancer biology. The long latency between excision of the primary tumor and development of clinically detectable distant

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metastasis suggests that micrometastatic tumor cells are already seeded at other sites throughout the body at the time of diagnosis and surgery, but only "reawaken" after a period of inactivity or nonproductive growth.

The ability of micrometastatic tumor cells to convert into overt metastases is a key point in disease progression because, once detected, metastatic cancer is essentially incurable. Identifying pathways that can be targeted to prevent metastatic outgrowth is particularly important to understand from a therapeutic perspective, as prevention of very early tumor dissemination may not be clinically feasible. In fact, it has been suggested that "a new frontier" in cancer therapy will be to identify ways to revert or maintain cancers in an occult, minimal residual disease state (2, 3).

How tumor cells maintain and/or escape clinical dormancy is still largely unknown, but both tumor cell–intrinsic and –extrinsic mechanisms seem to contribute. For example, occult cancer cells are often senescent or arrested in G_0–G_1 phase, a process that may be mediated by the T-helper cell 1 cytokines IFN-γ and TNF-α (4). Failure to achieve sufficient angiogenesis, even in a proliferating lesion, can also induce dormancy (for review, see ref. 5). Escape from immune-mediated control has also been shown to contribute to outgrowth of micrometastases (6–8). A recent study of melanoma metastasis showed that dissemination of cancer cells occurs early in tumorigenesis—even before tumors are detectable; however, their outgrowth in metastatic sites was limited by cytostatic $CD8^+$ T lymphocytes (9). T lymphocytes have also been implicated in late metastatic outgrowth in other models (6), and key cytokines that regulate T-cell activity can contribute to maintenance of the dormant state (8). However, the pathways by which micrometastatic tumor cells suppress T-cell responses to facilitate outgrowth and give rise to overt metastases are very poorly understood.

$CD8^+$ CTLs destroy tumor cells using perforin- and granzyme-mediated cell death (10) as well as by secreting TNF-α, causing tumor cell apoptosis (11, 12). To survive, tumor cells evade the immune system through mechanisms such as downregulation of class I MHC molecules, production of anti-inflammatory cytokines, and/or recruitment of inflammatory-suppressor cells (13). However, most studies have focused on tumor–immune interactions in established primary tumors rather than in occult metastases. Identifying and targeting key mechanisms by which tumor cells mediate suppression of CTLs during metastatic outgrowth holds potential as a strategy to reduce or prevent escape from dormancy, and thereby block progression of metastasis.

Macrophage-stimulating protein (*MST1*; gene product commonly referred to as MSP), one of its activating proteases (*ST14*; gene product commonly referred to as matriptase), and the MSP receptor Ron (*MST1R*, gene product commonly referred to as Ron) become aberrantly overexpressed in around 40%, 45%, and 50% of human breast cancers, respectively (14), and are upregulated in many other cancers as well (15). We previously reported that overexpression of MSP/matriptase/Ron is a strong, independent, poor prognostic factor for outcome in human patients with breast cancer due to metastasis, and that expression of MSP in a mouse model of mammary cancer was sufficient to promote spontaneous metastasis to lung, lymphatics, and bone (14). However, the mechanisms by which MSP promotes metastasis were not understood.

MSP is constitutively secreted from the liver into serum as an inactive protein that is subsequently activated locally on macrophages by matriptase (16) or other extracellular serine proteases (17) in response to infection or injury. The processed MSP binds to Ron, which is selectively expressed on a subset of fully differentiated tissue macrophages, and also at low levels on various epithelial cells (18, 19). Ron is essential for protection from unregulated inflammation in several models of infection or injury; MSP/Ron signaling is responsible for regulation of several inflammatory mediators such as TNF-α, interleukin (IL)-12, IFN-γ, arginase, and inducible nitric oxide synthase (20–25). It is unknown, however, whether the role of MSP/Ron in inflammation also contributes to its function in cancer metastasis.

Here, we used both genetic and pharmacologic approaches to interrogate the mechanism by which MSP drives metastasis, and determined that MSP facilitates metastasis by suppressing antitumor immunity. Blocking MSP/Ron signaling, specifically in the host, selectively prevents conversion of pulmonary micrometastases to metastatic colonies by eliciting an effective $CD8^+$ CTL response. We found that inhibition of Ron with a selective tyrosine kinase inhibitor reduced outgrowth of metastasis, even when treatment was delayed until after metastatic colonies were established. Thus, inhibition of MSP/Ron signaling holds promise as an exciting new therapeutic approach to managing the problem of metastatic outgrowth in the adjuvant setting.

RESULTS

Loss of Host Ron Signaling Blocks Metastasis

We previously described a highly metastatic transgenic mouse model of breast cancer in which MSP expression drives widespread spontaneous metastasis to clinically relevant sites (14). MSP is a secreted protein, and both the tumor cells and host tissues express endogenous Ron, so the prometastatic function of MSP could be attributed to direct effects on tumor cells or to indirect effects on the host tumor microenvironment. To determine whether MSP/Ron promotes metastasis through cell-autonomous or non-cell–autonomous mechanisms, we transplanted polyomavirus middle T antigen (PyMT)-MSP tumor cells or PyMT–MSCV-IRES-GFP (MIG) control tumor cells into cleared mammary fat pads of immune-competent syngeneic wild-type (WT) mice or syngeneic mice lacking Ron activity through targeted deletion of the intracellular kinase domain (*Ron* $TK^{-/-}$; ref. 21; Fig. 1A). We found that knocking out host Ron function had no significant effect on primary tumor development, growth rates, proliferation, or apoptosis (Table 1 and Supplementary Fig. S1A and S1B). However, loss of host Ron activity nearly eliminated spontaneous lung metastasis ($P < 0.0001$; Table 1; Fig. 1B), suggesting that MSP/Ron functions through the host to promote metastasis. We also noted that, although not statistically significant, control PyMT-MIG tumors were also less metastatic in the *Ron* $TK^{-/-}$ hosts, suggesting that host Ron may promote metastasis even in the absence of overexpressed MSP from the tumor (Supplementary Table S1). In fact, MSP is constitutively produced by hepatocytes and present in the serum, where it can then

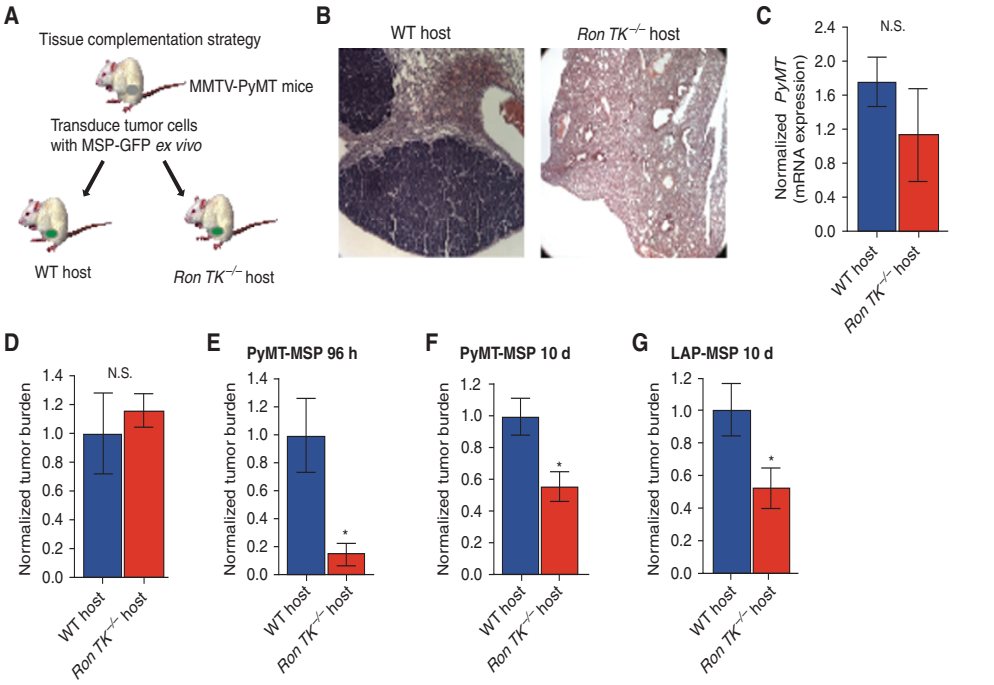

Figure 1. Loss of host Ron signaling attenuates metastasis specifically during the conversion of seeded micrometastasis to overt metastasis. **A,** schematic of the experimental strategy to determine whether host MSP/Ron signaling plays a role in mammary tumor development, initiation, and/or metastasis. **B,** representative image of spontaneous metastasis in lung sections from tumor-bearing WT and *Ron TK*$^{-/-}$ hosts. **C,** *PyMT* mRNA expression from peripheral blood (normalized to glyceraldehyde-3-phosphate dehydrogenase) as a surrogate marker for circulating tumor cells in WT and *Ron TK*$^{-/-}$ mice ($n = 6$ and 5, respectively). **D,** quantification of tumor-cell seeding in the lung 36 hours after intravenous injection into WT or *Ron TK*$^{-/-}$ hosts ($n = 3$/group). **E,** quantification of the metastatic tumor burden in the lung per field of vision 96 hours following intravenous tumor cell injection into WT or *Ron TK*$^{-/-}$ hosts ($n = 4$/group). **F,** quantification of metastatic tumor burden in the lung per field of vision 10 days following intravenous tumor cell injection into WT or *Ron TK*$^{-/-}$ hosts ($n = 5$ and 4, respectively). **G,** quantification of DiI-labeled tumor cells in the lung 10 days following intravenous injection of LAP-MSP (lung alveolar/bronchiolar carcinoma-P0297) lung cancer into WT or *Ron TK*$^{-/-}$ hosts ($n = 5$). Data are depicted as mean ± SEM. *, $P < 0.05$ (unpaired, two-sided *t* test). MMTV, mouse mammary tumor virus; N.S., not statistically significant.

be activated by macrophages in response to tissue injury or remodeling (26, 27).

Metastasis involves multiple steps: cell detachment from the primary tumor mass, local tissue invasion, entry into the circulation, extravasation into new tissues, colonization, and growth at the distant site (28). To test whether lack of metastasis in the *Ron TK*$^{-/-}$ hosts was due to a defect in invasion or intravasation, we analyzed the relative numbers of circulating tumor cells (CTC) in both groups of mice. CTCs were measured in blood from tumor-bearing WT and *Ron TK*$^{-/-}$ mice by quantifying levels of tumor-specific *PyMT* mRNA as a surrogate measure. Evidence for CTCs was found in both groups of mice, but we detected no significant difference between WT and *Ron TK*$^{-/-}$ tumor-bearing hosts (Fig. 1C). To determine

Table 1. Summary of the effect of host Ron on PyMT-MSP tumor growth, spontaneous metastasis, and survival following experimental metastasis

Host animal	Days to palpable tumor	Days to reach 2 cm	Spontaneous metastasis frequency	Survival (days to ethical endpoint)[c]
FVB WT ($n = 15$)	35	66	13/15 (87%)	40
FVB *Ron TK*$^{-/-}$ ($n = 15$)	35	53	1/15 (6.7%)[a]	52[d]
FVB *Ron TK*$^{-/-}$;*Prkdc*^{scid} ($n = 7$)	41	71	5/7 (71.4%)[b]	ND[e]

[a] $P < 0.0001$ vs. WT (Fisher exact test).
[b] $P < 0.005$ vs. FVB *Ron TK*$^{-/-}$ (Fisher exact test).
[c] Experimental metastasis assay; mice were euthanized when in respiratory distress.
[d] $P < 0.05$ (Mantel–Cox test).
[e] Not done.

whether host Ron plays a role in the later steps of metastasis, such as metastatic cell extravasation, seeding, and/or colonization of lungs, we conducted experimental metastasis assays. We injected equal numbers of identical tumor cells into the tail veins of WT or *Ron TK*$^{-/-}$ mice, and examined the ability of the cells to extravasate and seed the lung (36 hours later) and the ability of the cells to form colonies (5 days later; time points are based on ref. 29). Tumor burden was calculated using two different methods, which both consistently supported the same conclusions (see Methods for details). Both WT and *Ron TK*$^{-/-}$ hosts were equally competent for extravasation and metastatic seeding (Fig. 1D). However, *Ron TK*$^{-/-}$ hosts were defective in supporting the conversion of the seeded micrometastatic cells into metastatic colonies, resulting in less tumor burden in the lungs 5 days after injection (Fig. 1E and Supplementary Fig. S1C). This effect was sustained; tumor burden in the lungs was still significantly reduced 10 days after injection (Fig. 1F and Supplementary Fig. S1D), and *Ron TK*$^{-/-}$ hosts were able to survive about 50% longer than WT hosts before reaching the experimental endpoint of respiratory distress (Table 1). Similar results were obtained using syngeneic mouse lung cancer cells (LAP-MSP; ref. 30; Fig. 1G), as well as with PyMT-MIG control mammary tumor and LAP-MIG control lung tumor cells (Supplementary Fig. S2A and S2B). Thus, host Ron activity specifically facilitates the transition from micrometastasis to overt metastasis in multiple models of metastasis.

Ron TK$^{-/-}$ Hosts Mount a Robust CTL Response to Tumors, Which Is Critical for Preventing Metastasis

The expression pattern and known function of Ron (15) led us to postulate that the Ron-dependent host role in metastasis would be related to immune function. We first assessed splenic leukocyte populations in tumor-bearing WT or *Ron TK*$^{-/-}$ mice. We observed no significant differences between cohorts with respect to the proportion of splenic CD11b^{+} macrophages, Gr-1^{+} granulocytes, CD11b^{+}/Gr-1^{+} myeloid-derived suppressor cells, CD11c^{+} dendritic cells, CD4^{+} T cells, or CD4^{+}CD25^{+} T cells (Supplementary Fig. S3A-S3F). However, we detected a significant (~twofold) increase in the proportion of splenic CD8^{+} T cells in *Ron TK*$^{-/-}$ mice compared with WT hosts (Fig. 2A). Large clusters of CD8^{+} T cells were also detected around the margin of primary tumors in *Ron TK*$^{-/-}$ hosts compared with a general lack of CD8^{+} T cells around tumors in WT hosts (Fig. 2B). We could not detect CD8^{+} T cells within the core of the primary tumor in either group of mice (data not shown). WT and *Ron TK*$^{-/-}$ mice without tumors had similar numbers and proportions of splenic CD8^{+} T-cell populations (Fig. 2A, naïve hosts), indicating that the expansion of CD8^{+} T cells in *Ron TK*$^{-/-}$ hosts is tumor-dependent.

To specifically determine whether CD8^{+} T cells respond to the tumor challenge in the context of an experimental metastasis assay, we analyzed the immune milieu in the *Ron TK*$^{-/-}$ hosts and WT hosts in more detail. The initial stages of T-cell activation involve expansion of CD8^{+} T cells in response to antigen stimulation (31), so we postulated that an expansion of CD8^{+} T cells in the spleen or peripheral blood would precede an antitumor cytotoxic response in the lungs, which occurs between 36 and 96 hours after tumor injection (Fig. 1D and E). Therefore, we analyzed the CD8^{+} T-cell response at an intermediate time point (72 hours after tumor injection). *Ron TK*$^{-/-}$ hosts had an expanded CD8^{+} T-cell pool in the peripheral blood relative to WT hosts, in both the mammary and lung cancer models (Fig. 2C and D). Again, nontumor-bearing *Ron TK*$^{-/-}$ and WT mice had similar levels of CD8^{+} T cells in the blood (Fig. 2C, naïve hosts).

Despite increased expansion of CD8^{+} T cells in the periphery of *Ron TK*$^{-/-}$ mice 72 hours following tumor injection, we did not observe differences in the overall proportion of CD8^{+} T cells infiltrating the lungs at this time point (data not shown). To determine whether the CD8^{+} T cells were active, we profiled the inflammatory cytokines produced by CD8^{+} T cells isolated from both the lungs and peripheral blood of tumor-bearing animals. We found that the CD8^{+} T cells in the lungs of *Ron TK*$^{-/-}$ hosts produced more TNF-α than those from WT hosts (Fig. 2E), suggesting a stronger proinflammatory immune milieu in the lungs of *Ron TK*$^{-/-}$ hosts specifically following tumor challenge. CD8^{+} T cells from nontumor-bearing WT and *Ron TK*$^{-/-}$ hosts had similar low levels of TNF-α (Fig. 2E, naïve hosts). TNF-α is a potent antitumor factor secreted by immune cells that induces tumor cell apoptosis (12), and canonical MSP/Ron signaling is known to downregulate IL-12 and TNF-α to drive the switch from classical to alternative macrophage activation (20, 22, 23). Consistent with this, macrophages derived from lungs of *Ron TK*$^{-/-}$ mice 72 hours after tumor injection expressed increased IL-12 compared with macrophages isolated from WT hosts at the same time point (Fig. 2F and Supplementary Fig. S4). Moreover, macrophages from the spleen of *Ron TK*$^{-/-}$ hosts also expressed more TNF-α (Fig. 2G and Supplementary Fig. S4). Macrophages from nontumor-bearing WT and *Ron TK*$^{-/-}$ hosts had similar, low levels of IL-12 (Fig. 2F, naïve hosts) and TNF-α (Fig. 2G, naïve hosts). Thus, loss of host Ron activity enhanced tumor-dependent production of proinflammatory cytokines by macrophages, allowed expansion of the peripheral CD8^{+} T-cell population, and promoted infiltration of TNF-α–producing CD8^{+} T cells into the lungs 72 hours following tumor challenge. These events preceded the diminished tumor burden in the lungs of *Ron TK*$^{-/-}$ hosts (at the 96-hour time point; Fig. 1E), suggesting that enhanced antitumor immunity could be the cause of reduced metastasis in *Ron TK*$^{-/-}$ mice.

To test whether the improved CD8^{+} T-cell response in *Ron TK*$^{-/-}$ hosts was directly related to inhibition of metastasis, we asked if loss of T cells would restore metastasis in *Ron TK*$^{-/-}$ hosts. We crossed *Ron TK*$^{-/-}$ mice with *Prkdc*^{scid} mice, which lack functional lymphocytes. The double mutants, versus control *Ron TK*$^{+/+}$;*Prkdc*^{scid} littermates (all backcrossed to the FVB background), were used as hosts for orthotopically transplanted PyMT-MSP tumors. Tumors developed and grew with similar rates in both hosts; however, *Ron TK*$^{-/-}$;*Prkdc*^{scid} hosts displayed normal (restored) metastasis compared with the almost complete lack of metastasis in immune-competent *Ron TK*$^{-/-}$ hosts (Table 1; $P = 0.0043$).

To specifically determine whether CD8^{+} T cells were required to inhibit metastasis in *Ron TK*$^{-/-}$ hosts, we selectively depleted CD8^{+} T cells using anti-CD8 antibodies in the context of a 10-day lung colonization assay (as in Fig. 1F). Successful depletion of CD8^{+} T cells was confirmed by flow cytometry on splenic, lung, and peripheral blood cell

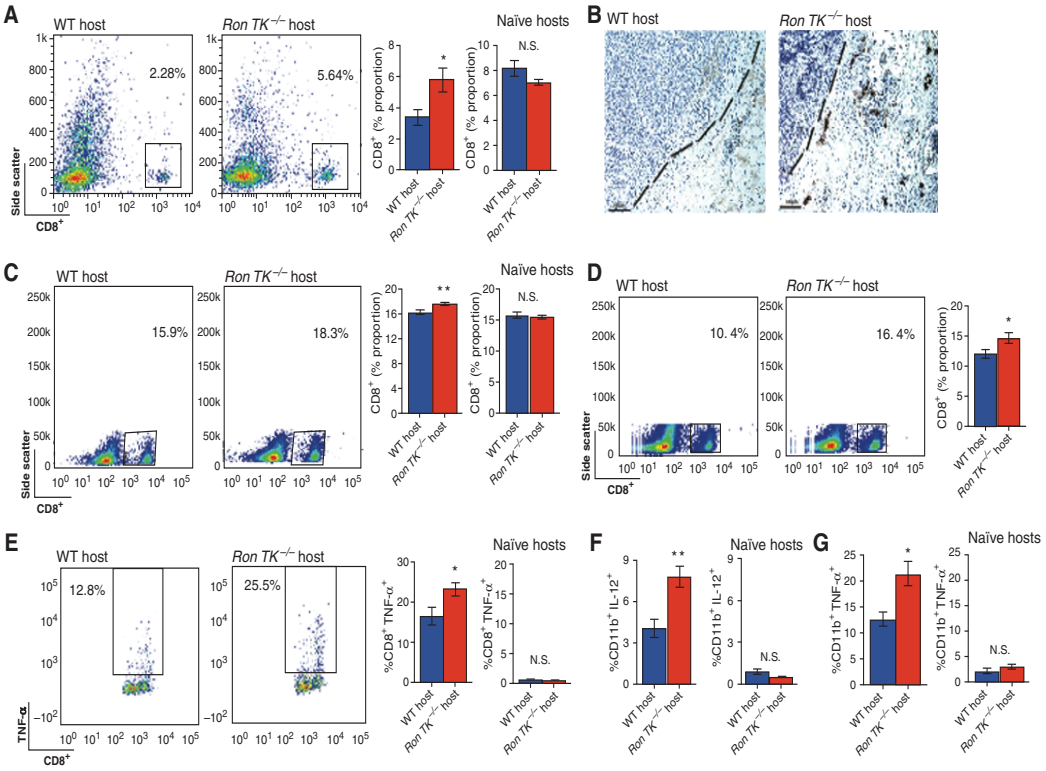

Figure 2. Loss of host Ron signaling results in expansion of CD8⁺ T cells and promotes production of proinflammatory cytokines. **A,** representative flow-cytometric analysis (left) and graph (right) depicting the population of CD8⁺ T cells in spleens of tumor-bearing WT and *Ron TK*$^{-/-}$ mice ($n = 6$ and 7, respectively), as well as naïve hosts. **B,** representative immunohistochemical analysis of CD8⁺ T-cell infiltration into the primary tumors of WT (left) and *Ron TK*$^{-/-}$ hosts (right). The dashed lines denote the tumor–stroma border. **C,** representative flow-cytometric analysis (left) and graph (right) depicting the population of CD8⁺ T cells in the blood of tumor-bearing WT and *Ron TK*$^{-/-}$ mice 72 hours after PyMT-MSP tumor cell injection ($n = 5$/group) as well as naïve hosts. **D,** Representative flow-cytometric analysis (left) and graph (right) showing the proportion of CD8⁺ T cells in the blood of tumor-bearing WT and Ron TK$^{-/-}$ mice 72 hours after LAP-MSP injection ($n = 5$/group). **E,** representative flow-cytometric analysis (left) and graph (right) depicting the population of TNF-α–expressing CD8⁺ T cells in the lungs of tumor-bearing WT and *Ron TK*$^{-/-}$ mice 72 hours after PyMT-MSP tumor cell injection ($n = 5$/group) as well as naïve hosts. **F,** graph depicting the population of IL-12–expressing CD11b⁺ cells in the lungs of tumor-bearing WT and *Ron TK*$^{-/-}$ hosts 72 hours after PyMT-MSP tumor cell injection ($n = 5$/group) as well as naïve hosts ($n = 4$). **G,** graph depicting the population of TNF-α–expressing CD11b⁺ cells in the spleen of tumor-bearing WT and *Ron TK*$^{-/-}$ hosts 72 hours after PyMT-MSP tumor cell injection ($n = 5$/group) as well as naïve hosts ($n = 4$). Data are depicted as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ (unpaired, two-sided *t* test). N.S., not statistically significant.

populations (Supplementary Fig. S5A–S5C). Depletion of CD8⁺ T cells resulted in a significant, approximately twofold increase in metastatic tumor burden in the lungs of *Ron TK*$^{-/-}$ mice (Fig. 3A and Supplementary Fig. S5D).

We next sought to determine if the tumor-induced expansion of CD8⁺ T cells also resulted in increased cytotoxic ability. We isolated CD8⁺ T cells from the blood of *Ron TK*$^{-/-}$ and WT hosts 96 hours following intravenous injection of PyMT-MSP tumor cells. We cocultured PyMT-MSP tumor cells with CD8⁺ T cells (1:1 ratio) for 24 hours. We observed that CD8⁺ T cells isolated from the blood of *Ron TK*$^{-/-}$ hosts had increased cytotoxic ability *in vitro*, as evidenced by the increased proportion of Annexin V⁺ propidium iodide (PI)⁺ double-positive tumor cells (Fig. 3B). This was tumor specific; CD8⁺ T cells isolated from naïve WT and *Ron TK*$^{-/-}$ hosts had similar, low levels of cytotoxicity (Fig. 3B, naïve hosts).

We next wanted to determine whether CD8⁺ T cells were sufficient to block metastasis in tumor-bearing *Ron TK*$^{-/-}$ hosts *in vivo*. We isolated tumor-educated CD8⁺ T cells from the spleens of tumor-bearing mice and conducted adoptive transfer of these cells into tumor-naïve, syngeneic *Ron TK*$^{+/+}$;*Prkdc*^{scid} mice, which lack endogenous lymphocyte function. One day later, we injected the tumor cells (derived from the same donor mice as the CD8⁺ T cells) into the tail veins (Fig. 3C). This strategy allowed us to determine if the CD8⁺ T cells that were educated and activated in tumor-bearing WT or *Ron TK*$^{-/-}$ mice were sufficient to affect metastasis in a naïve host in the absence of other functional lymphocytes. Adoptive transfer of CD8⁺ T cells from WT tumor-bearing mice did not have a significant effect on metastasis, whereas adoptive transfer of the same number of CD8⁺ T cells from tumor-bearing *Ron TK*$^{-/-}$ mice significantly reduced metastatic tumor burden in the lungs (Fig. 3D and Supplementary Fig. S5E). Collectively, these results showed that the expanded CD8⁺ T-cell population in tumor-bearing *Ron TK*$^{-/-}$ mice was both necessary and sufficient to reduce metastasis, whereas

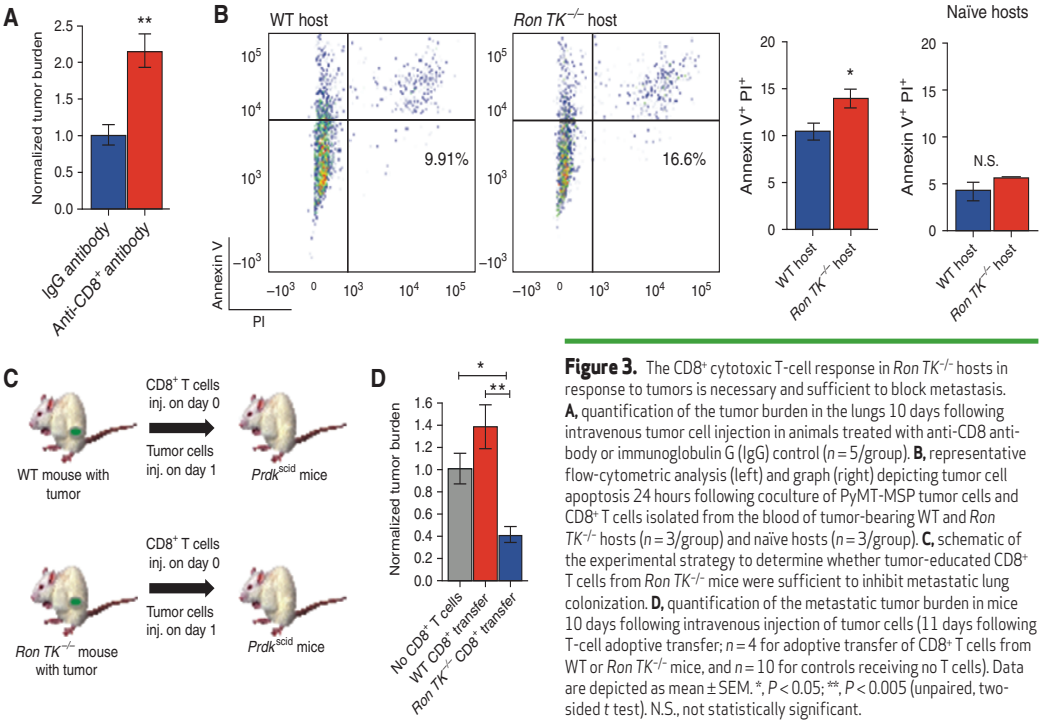

Figure 3. The CD8+ cytotoxic T-cell response in *Ron TK*$^{-/-}$ hosts in response to tumors is necessary and sufficient to block metastasis. **A,** quantification of the tumor burden in the lungs 10 days following intravenous tumor cell injection in animals treated with anti-CD8 antibody or immunoglobulin G (IgG) control ($n = 5$/group). **B,** representative flow-cytometric analysis (left) and graph (right) depicting tumor cell apoptosis 24 hours following coculture of PyMT-MSP tumor cells and CD8+ T cells isolated from the blood of tumor-bearing WT and *Ron TK*$^{-/-}$ hosts ($n = 3$/group) and naïve hosts ($n = 3$/group). **C,** schematic of the experimental strategy to determine whether tumor-educated CD8+ T cells from *Ron TK*$^{-/-}$ mice were sufficient to inhibit metastatic lung colonization. **D,** quantification of the metastatic tumor burden in mice 10 days following intravenous injection of tumor cells (11 days following T-cell adoptive transfer; $n = 4$ for adoptive transfer of CD8+ T cells from WT or *Ron TK*$^{-/-}$ mice, and $n = 10$ for controls receiving no T cells). Data are depicted as mean ± SEM. *, $P < 0.05$; **, $P < 0.005$ (unpaired, two-sided *t* test). N.S., not statistically significant.

the CD8+ T cells in tumor-bearing WT mice were incapable of antimetastatic activity. Our data shed light on how MSP/Ron signaling causes metastasis of breast cancer, at least in these animal models: through suppression of an effective antitumor CD8+ T-cell response. Blocking host Ron activity relieved this immunosuppression and effectively inhibited metastasis. Our next question centered on the potential clinical relevance of our findings.

Pharmacologic Inhibition of Ron Diminishes Metastatic Outgrowth

To test whether pharmacologic inhibition of Ron could decrease metastatic outgrowth in WT mice, we used BMS-777607/ASLAN002, a small-molecule inhibitor selective for Ron and, to a lesser extent, its homolog Met (32). We validated the ability of BMS-777607/ASLAN002 to inhibit mouse Ron activity by treating PyMT tumor cells, which express endogenous Ron, with MSP in the presence or absence of BMS-777607/ASLAN002. As expected from published data (32), this compound was effective in diminishing MSP-induced phosphorylation of Ron at submicromolar concentrations ($IC_{50} < 500$ nmol/L; Supplementary Fig. S6A).

To establish whether BMS-777607/ASLAN002 treatment could reduce metastatic colonization in a manner comparable with that seen in *Ron TK*$^{-/-}$ hosts, we first tested Ron inhibition in the prophylactic setting. WT mice were treated orally with 50 mg/kg BMS-777607/ASLAN002 (or vehicle control) once a day for 2 weeks (days 1–14). PyMT-MSP tumor cells were injected into the tail vein on day 3, and on day 14 the lungs were harvested and assessed for tumor colonization. The results showed that prophylactic treatment with BMS-777607/ASLAN002 significantly reduced metastatic outgrowth in the lungs by two- to threefold (Fig. 4A and Supplementary Fig. S6B). To determine if CD8+ T cells mediated the anticolonization effects of BMS-777607/ASLAN002, WT mice were treated orally with 50 mg/kg BMS-777607/ASLAN002 (or vehicle control) once a day for 7 days. We concurrently depleted CD8+ T cells with anti-CD8 antibodies. PyMT-MSP tumor cells were injected into the tail vein on day 3, and 96 hours later the lungs were harvested and assessed for tumor colonization. Treatment with BMS-777607/ASLAN002 resulted in two- to threefold more TNF-α–positive macrophages, similar to the increased proinflammatory milieu we had observed in *Ron TK*$^{-/-}$ mice (Fig. 4B and Supplementary Fig. S6C). However, treatment with BMS-777607/ASLAN002 in the absence of CD8+ T cells did not reduce tumor colonization, indicating that CD8+ T cells are key mediators of the anticolonization effect of BMS-777607/ASLAN002, phenocopying the genetic loss of Ron (Fig. 4C and Supplementary Fig. S6D).

To mirror the clinical situation more closely, however, where micrometastases may have been seeded before diagnosis, we next tested Ron inhibition in the "adjuvant" setting. We injected PyMT-MSP tumor cells into the tail veins of WT mice and waited 14 days for metastatic colonies to become fully established, then began daily treatment for 8 days (days 14–22). On day 22, the lungs were harvested and assessed for metastatic outgrowth by determining the percentage of lung area that was taken by tumor. Treatment with BMS-777607/ASLAN002 attenuated the formation of metastatic nodules by approximately fourfold, even when administered after metastatic colonization had occurred (Fig. 4D and E). Thus, inhibition of Ron kinase activity carries promising potential as a novel therapeutic option to inhibit metastatic outgrowth when given in the adjuvant setting.

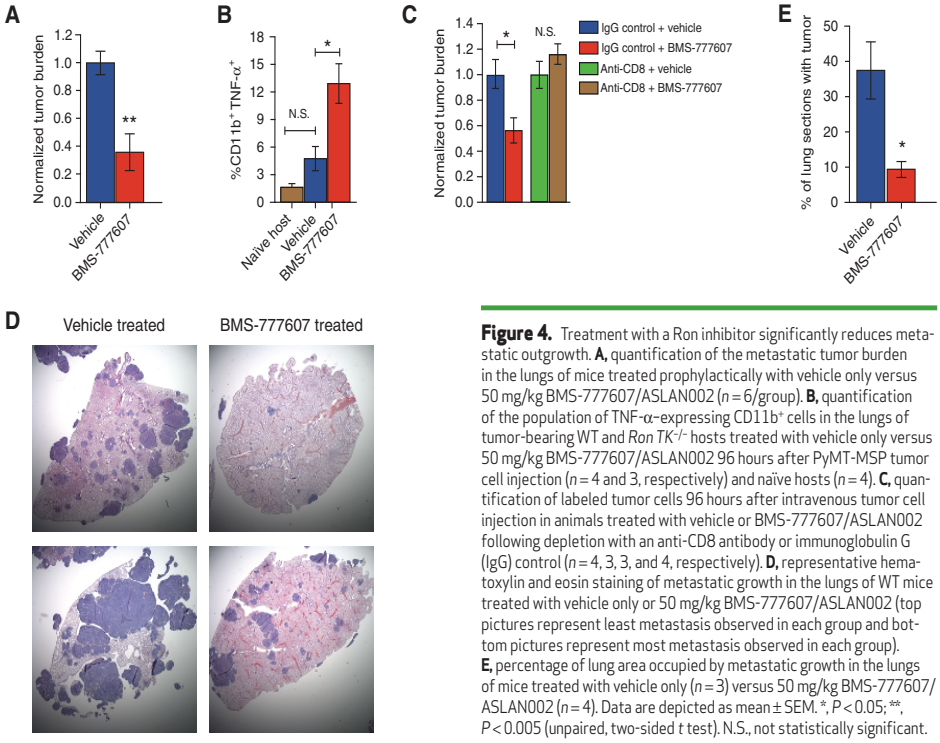

Figure 4. Treatment with a Ron inhibitor significantly reduces metastatic outgrowth. **A,** quantification of the metastatic tumor burden in the lungs of mice treated prophylactically with vehicle only versus 50 mg/kg BMS-777607/ASLAN002 ($n = 6$/group). **B,** quantification of the population of TNF-α–expressing CD11b$^+$ cells in the lungs of tumor-bearing WT and *Ron TK*$^{-/-}$ hosts treated with vehicle only versus 50 mg/kg BMS-777607/ASLAN002 96 hours after PyMT-MSP tumor cell injection ($n = 4$ and 3, respectively) and naïve hosts ($n = 4$). **C,** quantification of labeled tumor cells 96 hours after intravenous tumor cell injection in animals treated with vehicle or BMS-777607/ASLAN002 following depletion with an anti-CD8 antibody or immunoglobulin G (IgG) control ($n = 4$, 3, 3, and 4, respectively). **D,** representative hematoxylin and eosin staining of metastatic growth in the lungs of WT mice treated with vehicle only or 50 mg/kg BMS-777607/ASLAN002 (top pictures represent least metastasis observed in each group and bottom pictures represent most metastasis observed in each group). **E,** percentage of lung area occupied by metastatic growth in the lungs of mice treated with vehicle only ($n = 3$) versus 50 mg/kg BMS-777607/ASLAN002 ($n = 4$). Data are depicted as mean ± SEM. *, $P < 0.05$; **, $P < 0.005$ (unpaired, two-sided t test). N.S., not statistically significant.

DISCUSSION

Ultimately, our ability to reduce cancer mortality depends on identifying ways to prevent or treat distant metastatic disease over long periods of time. The data presented here reveal that the Ron ligand, MSP, which is aberrantly overexpressed in 40% of human breast cancers and many other cancers (14, 15), promotes metastasis by inhibiting an effective antitumor immune response through activation of Ron signaling in the host. Although there are clearly many ways that tumors achieve metastasis, we propose that some tumors upregulate MSP as one way to effectively evade the immune system. Furthermore, our data show that host Ron is also important for immune suppression when tumors themselves do not overexpress MSP, presumably through activation of endogenous serum-derived MSP by macrophage- and/or tumor-derived serine proteases (16). Together, our data suggest that blocking Ron kinase activity allows for reactivation of the antitumor immune response and reduces metastatic outgrowth.

It has long been known that infiltration of CD8$^+$ CTLs into tumors is a useful prognostic indicator for various types of tumors (33–35). Recent evidence suggests that immunosurveillance by CD8$^+$ T cells keeps melanoma metastasis in check by promoting tumor dormancy (9). However, suppression of the immune system, also known as immunosubversion, is a critical step in tumor development (36). Tumors downregulate MHC molecules and overproduce arginase-1 and indoleamine 2,3-dioxygenase, both of which inhibit CD8$^+$ T-cell function. Hypoxia also suppresses T-cell activity through expression of hypoxia-inducible factor in macrophages (36). These effects likely cooperate, ultimately leading to a strong immunosuppressive environment in tumors. Such redundancy may explain why primary tumor growth is similar in WT and *Ron TK*$^{-/-}$ mice despite increased CD8$^+$ T-cell infiltration around the periphery of primary tumors in *Ron TK*$^{-/-}$ hosts. Conversely, tumor cells that have seeded a new environment or are just beginning to effectively colonize the distant organ may be more vulnerable to immune-mediated control. Indeed, our results show that cells that are in the process of converting from seeded tumor cells to overt metastases are vulnerable to CD8$^+$ T cells. Here, we describe a novel pathway that, when inhibited, is sufficient to activate the CTL response, reducing metastasis and extending life—at least in immune-competent mouse models. These results warrant additional studies focused on whether Ron inhibitors could be tested for antimetastatic effects in the clinical adjuvant setting.

The precise molecular role for MSP/Ron in suppressing antitumor immunity is still unknown and will be the focus of important future studies. Tumors are sites of chronic inflammation and are reminiscent of unhealed wounds, where tumor-associated macrophages (TAM) seem to be skewed toward an M2 alternative activation state (37, 38). Although M2 macrophages are important for wound healing, they are thought to contribute inadvertent advantages to tumors by stimulating angiogenesis and producing polyamines, growth factors, and cytokines that favor tumor growth. Several factors, most notably colony-simulating factor 1, have been implicated in the recruitment of macrophages into tumors where they promote metastasis (39, 40), but little is known about the specific signaling pathways in tumors that drive the M2 state of TAMs. On the basis of published studies and our results, it is tempting to speculate that MSP/Ron signaling

simply favors conversion of TAMs to an M2 state, resulting in suppression of CTL responses (41). In support of this hypothesis, subcutaneous growth of several mouse tumor types is regulated extrinsically through Ron function in TAMs, which affects CTL responses (25, 42). In addition, Ron-deficient mice clearly exhibit amplified inflammatory responses upon challenge with infection or injury due to unregulated production of proinflammatory cytokines (21, 43, 44). A similar mechanism could be involved in the tumor setting, whereby increased production of IL-12 and TNF-α from *Ron* $TK^{-/-}$ macrophages is either causal to or symptomatic of a broad proinflammatory cytokine milieu that results in improved $CD8^+$ T-cell responses, including production of TNF-α. However, the immune milieu of tumors (and the resulting effects on tumor progression) is extremely complicated; detailed genetic and immunologic studies will be required to determine the precise role of Ron in antitumor immunity.

Cancer immunotherapy carries strong appeal because the immune response is individualized, it is effective against diverse antigens, and it is potentially able to evolve and retain immunologic memory for long-term control of disease. A major challenge, however, is that by the time tumors are clinically detectable they are already "invisible" to the immune system. Strong natural selection exists to favor tumor cells that can escape immune control by promoting immune tolerance and/or by fostering a strong immunosuppressive environment that renders effector cells inactive (45). These same issues have also been barriers to effective antitumor immune therapies, and the clinical results of immunotherapy for breast cancer have been, at most, only moderately effective (46). Drugs that block the inhibitory signals on T cells, such as CTLA-4 inhibitors, are now being used in combination with immunotherapy to generate a more productive antitumor immune response (47). Future work will be important to determine whether Ron inhibitors may function in a similar immune-modulatory role to boost the clinical response to immunotherapy and whether CTL activity is a good clinical biomarker of Ron inhibition.

METHODS

Mice and Cells

All procedures were reviewed and approved by the University of Utah Institutional Animal Care and Use Committee. FVB mice with a deletion in the Ron tyrosine kinase domain ($TK^{-/-}$) have been described previously (21). *Prkdc*^{scid} mice (The Jackson Laboratory) and *Ron* $TK^{-/-}$ or WT mice were backcrossed to generate *Ron* $TK^{-/-}$;*Prkdc*^{scid} mice and *Ron* $TK^{+/+}$;*Prkdc*^{scid} mice on the FVB background. Tumors were generated from mouse mammary tumor virus (MMTV)-PyMT transgenic mice engineered to express MSP-IRES-GFP or IRES-GFP (pMIG), and 100,000 GFP^+ cells were orthotopically transplanted as described previously (14). LAP-0297 lung cancer cells (30) were engineered to express MSP-IRES-GFP (LAP-MSP) using the same method, and 250,000 cells were injected into the tail vein. These cells were obtained from Dr. Peigen Huang (Harvard/Massachusetts General Hospital, Boston, MA) without additional authentication.

Immunohistochemistry

Tissues were processed, sectioned (5 μm), and stained using standard procedures. Apoptosis was assessed with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays (Roche). Antibodies used for immunohistochemistry were phosphohistone H3 (1:100; Cell Signaling Technology) and CD8 (1:100; Abcam). The Envision+System HRP Detection Kit (DAKO) and Vector M.O.M. and horseradish peroxidase (HRP) kits were used according to manufacturers' instructions.

Lymphocyte Isolation and FACS

Splenocytes were isolated by disrupting spleens over a wire mesh, followed by red blood cell (RBC) lysis. Lung lymphocytes were isolated following digestion of lungs in Collagenase IV (Sigma) for 1 hour, followed by Percol (Sigma) separation. Peripheral blood lymphocytes were isolated as previously described (48). Briefly, blood was harvested from WT and *Ron* $TK^{-/-}$ mice into anticoagulant citrate dextrose solution Formula A followed by incubation with dextran solution for 20 minutes at 37°C. The upper layer of RBC-depleted fluid was harvested, and lymphocytes were collected. Antibodies used for fluorescence-activated cell sorting (FACS) were CD8α-FITC, CD8β-PE, CD4-FITC, CD45-FITC, CD11c-PeCy7, CD11b-PeCy7, Gr-1-APC, CD25-PE (all 1:400; BD Pharmingen) for cell surface staining. Intracellular FACS staining was done with TNF-α-APC and IL-12-eFluor450 (1:400; eBioscience). For cell surface staining, cells were incubated in 2% FBS in PBS for 20 minutes. For intracellular staining, cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate/ionomycin in the presence of Brefeldin A (1 μL/mL). Cells were stained with cell surface staining antibodies, permeabilized, and stained with anticytokine antibodies as per manufacturer's instructions (BD Biosciences). Cells were analyzed using FACScan and FACSCanto II cytometers (BD Biosciences) and results analyzed using FlowJo Software (Treestar).

Experimental Metastasis Assays

Tumor cells were stained with DiI (Invitrogen) and resuspended in Hank's Balanced Salt Solution at 10^6 cells/mL, and 250 μL (250,000 cells) was injected into the lateral tail veins of *Ron* $TK^{-/-}$ or WT mice. At experimental endpoints, mice were euthanized, and lungs were prepared by perfusion with 4% paraformaldehyde and frozen in optimum cutting temperature compound. Images of 16-μm sections were captured using ×10 magnification. Fluorescent cells/colonies were quantified using ImageJ software. Tumor burden was calculated by multiplying the colony count by the colony size for each section. Alternatively, as a secondary quantitative measure, epithelial cells from freshly harvested lungs were collected following Percol (Sigma) separation and analyzed with a FACSCanto II cytometer to calculate percentage of DiI-labeled tumor cells.

$CD8^+$ Killing Assays

$CD8^+$ T cells were magnetically sorted from the blood of WT and Ron $TK^{-/-}$ hosts 96 hours after intravenous injection of PyMT-MSP tumor cells and control nontumor-bearing hosts (CD8α microbeads; MACS). Subsequently, the 50,000 $CD8^+$ T cells were cultured with plate-bound anti-CD3 antibody (BD; 5 μg/mL) and cocultured with 50,000 PyMT-MSP tumor cells (12, 49). Twenty-four hours later, cell pellets were collected for apoptosis analysis using Annexin V–APC and PI per the manufacturer's instructions (eBioscience).

$CD8^+$ T-Cell Depletion and Adoptive Transfer

For $CD8^+$ T-cell depletion, mice were injected with 100 μg anti-CD8 or immunoglobulin G (IgG) control antibodies (Bio-X-Cell) once a day, intraperitoneally, for 3 days before tumor injection. A total of 250,000 tumor cells were injected into the tail vein on the fourth day (day 0). Antibodies were reinjected on day 2 and 7. On day 10, mice were euthanized and metastatic burden quantified as described earlier. For adoptive transfer experiments, splenocytes from tumor-bearing WT and *Ron* $TK^{-/-}$ mice were stained with CD8α-FITC antibodies and FACS sorted. A total of 500,000 donor $CD8^+$ T cells

were injected into the lateral tail veins of recipient *Ron TK*$^{+/+}$;*Prkdc*^{scid} mice. Twenty-four hours later, the mice were injected with 250,000 DiI-labeled tumor cells that were isolated from the same mice as the donor T cells.

Circulating Tumor Cells

Blood was harvested by cardiac puncture on freshly euthanized WT and *Ron TK*$^{-/-}$ mice with tumors. Whole blood RNA was isolated using manufacturer's instructions (Qiagen RNeasy kit). Reverse transcription followed by 35 cycles of PCR for *PyMT* RNA was conducted using the following primers: 5′-CTCCAACAGATACACCCGCACAT ACT-3′ (forward) and 5′-GCTGGTCTTGGTCGCTTTCTGGATAC-3′ (50). Thirty-five cycles of PCR for glyceraldehyde-3-phosphate dehydrogenase on the same samples was conducted using the following primers: 5′-ATGTTCCAGTATGACTCCACT-3′ and 5′-CCACAAT GCCAAAGTTGTCAT-3′ (51) and served as a control for normalization. Ethidium bromide–stained gels were quantified according to pixel density analysis using ImageJ software.

Drug Treatment

For "prophylactic" treatment, mice were administered 50 mg/kg BMS-777607/ASLAN002 (or 70% PEG-400 vehicle) orally once a day for 3 days before intravenous injection of 250,000 tumor cells (day 0 of the experiment). Treatment continued for 10 more days. On day 11, mice were euthanized and metastasis quantified as described earlier. For "adjuvant" treatment, 250,000 tumor cells were injected intravenously (day 0 of the experiment). Beginning on day 14, mice were treated with 50 mg/kg BMS-777607/ASLAN002 or vehicle orally once a day for 8 days. On day 22, mice were euthanized and lungs were fixed and paraffin-embedded. The extent of metastasis was quantified using ImageJ and calculated as the average tumor area versus total lung area on each hematoxylin and eosin–stained section. *In vitro* activity of BMS-777607/ASLAN002 against murine Ron was measured by growing mouse tumor cells (MMTV-PyMT) until 80% confluent, incubating overnight in medium with 0.5% serum, and then stimulating the cells with 1 ng/mL recombinant human MSP and 0.5, 2.0, or 5.0 μmol/L BMS-777607/ASLAN002. Cells were harvested 60 minutes later and analyzed by Western blot analysis with phospho-Ron and total-Ron antibodies (1:400; Santa Cruz Biotechnology).

To determine whether the BMS-777607/ASLAN002 mechanism of action was dependent on CD8$^+$ T cells, mice were injected with 100 μg anti-CD8 or IgG control antibody (Bio-X-Cell), intraperitoneally, 7 days before tumor injection and every 5 days until euthanasia. Tumor cell injection and drug treatment were identical to the "prophylactic" treatment protocol. Epithelial cells from the lungs were collected following Percol (Sigma) separation and analyzed with a FACSCanto II cytometer to calculate percentage of DiI-labeled tumor cells.

Disclosure of Potential Conflicts of Interest

A.L. Welm has received a commercial research grant from Astellas/OSI Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: H. Eyob, H.A. Ekiz, M.A. Williams, A.L. Welm
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Eyob, H.A. Ekiz, Y.S. DeRose, S.E. Waltz, A.L. Welm
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Eyob, H.A. Ekiz, A.L. Welm
Writing, review, and/or revision of the manuscript: H. Eyob, H.A. Ekiz, A.L. Welm
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Eyob, Y.S. DeRose, A.L. Welm
Study supervision: A.L. Welm

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Supplementary Figures

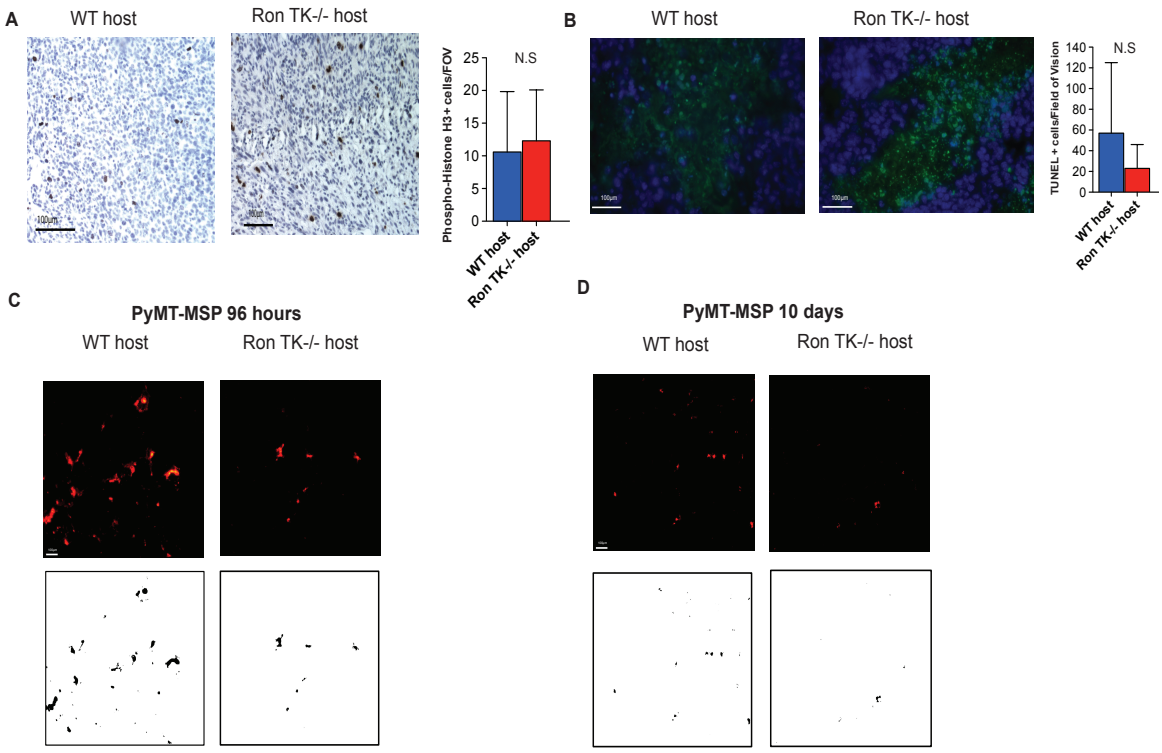

Figure S1. Proliferation and apoptosis of tumor cells was not significantly changed in WT versus Ron TK-/- hosts. A. Representative images showing immunohistochemical analysis of phospho-histone H3 protein in tumors growing in WT or Ron TK-/- hosts, with quantification on the right. **B.** Representative images showing immunofluorescence analysis of TUNEL staining in tumors growing in WT or Ron TK-/- hosts, with quantification on the right. **C.** Representative images of PyMT-MSP, 96 hours following i.v injection, showing DiI labeled tumor cells **(top)** in the lungs of WT or Ron TK-/- hosts and subsequent Image J image modification for analysis **(bottom)**. **D.** Representative flow cytometric analysis **E.** Representative images of PyMT-MSP 10 days following i.v injection, showing DiI labeled tumor cells **(top)** in the lungs of WT or Ron TK-/- hosts and subsequent Image J modification for analysis **(bottom)**. Data are depicted as mean+/- s.e.m. N.S (not statistically significant).

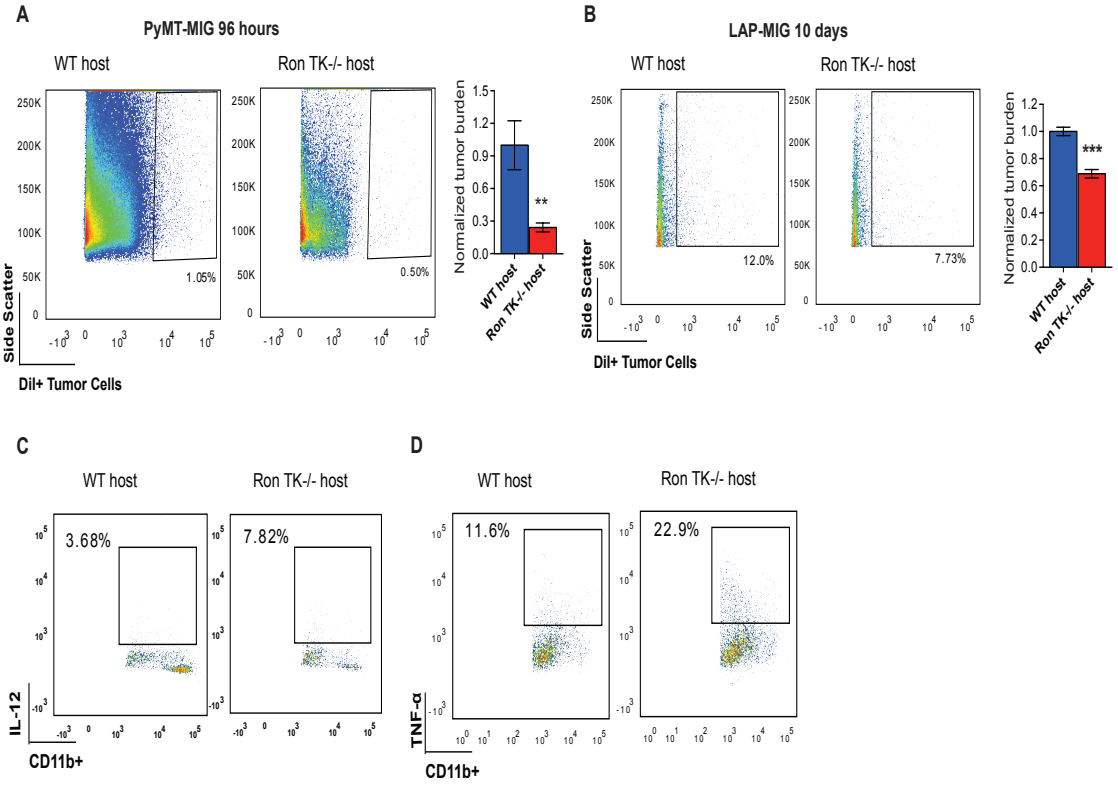

Figure S2. Loss of host Ron signaling attenuates growth of seeded micro-metastasis to overt metastasis in a tumor derived MSP-independent manner.

A. Representative flow cytometric analysis **(left)** and quantification **(right)** of DiI labeled tumor cells in lung 96 hours following intravenous PyMT-MIG tumor cell injection into WT or Ron TK-/- hosts (n=7 and 5 respectively)*. **B.** Representative flow cytometric analysis **(left)** and quantification **(right)** of DiI labeled tumor cells in lung 10 day following intravenous LAP control lung cancer line injection into WT or Ron TK-/- hosts (n=5)**. **C.** Representative flow cytometric analysis of CD11b+ macrophages expressing IL-12 isolated from lungs of WT and Ron TK-/- hosts 72 hours after i.v PyMT-MSP injection. **D.** Representative flow cytometric analysis of CD11b+ macrophages expressing TNFα isolated from lungs of WT and Ron TK-/- hosts 72 hours after i.v PyMT-MSP injection**.** Data are depicted as mean +/- s.e.m. *p<0.05 (unpaired, two-sided t-test); **p<0.0002 Mann Whitney test.

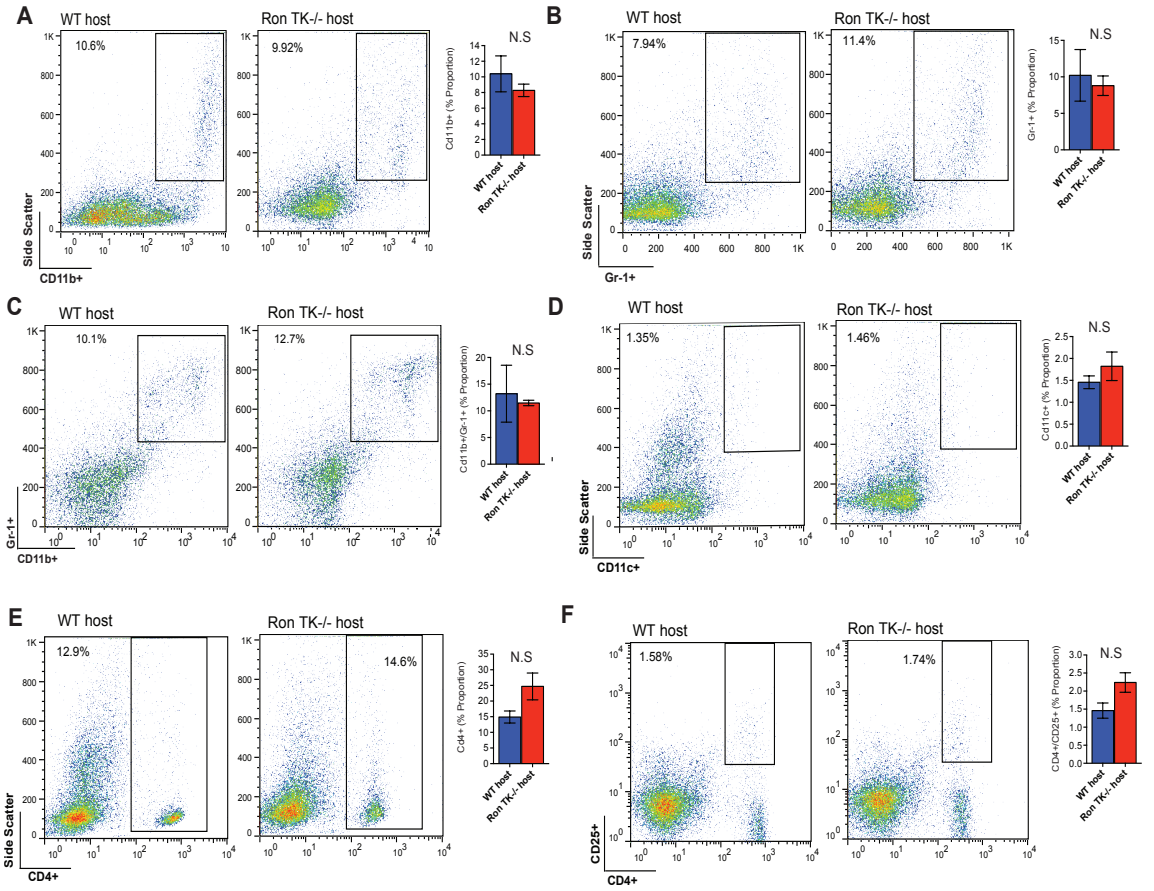

Figure S3. Most splenic immune cells are present in similar proportions in tumor-bearing mice WT and Ron TK-/- hosts. A-F. Flow cytometric analysis of macrophages (**A**), granulocytes (**B**), myeloid-derived suppressor cells (**C**), dendritic cells (**D**), $CD4^+$ T cells (**E**), and regulatory T cells (**F**). Data are represented as mean+/- s.e.m to the right of each plot (n=5 per group). N.S (not statistically significant).

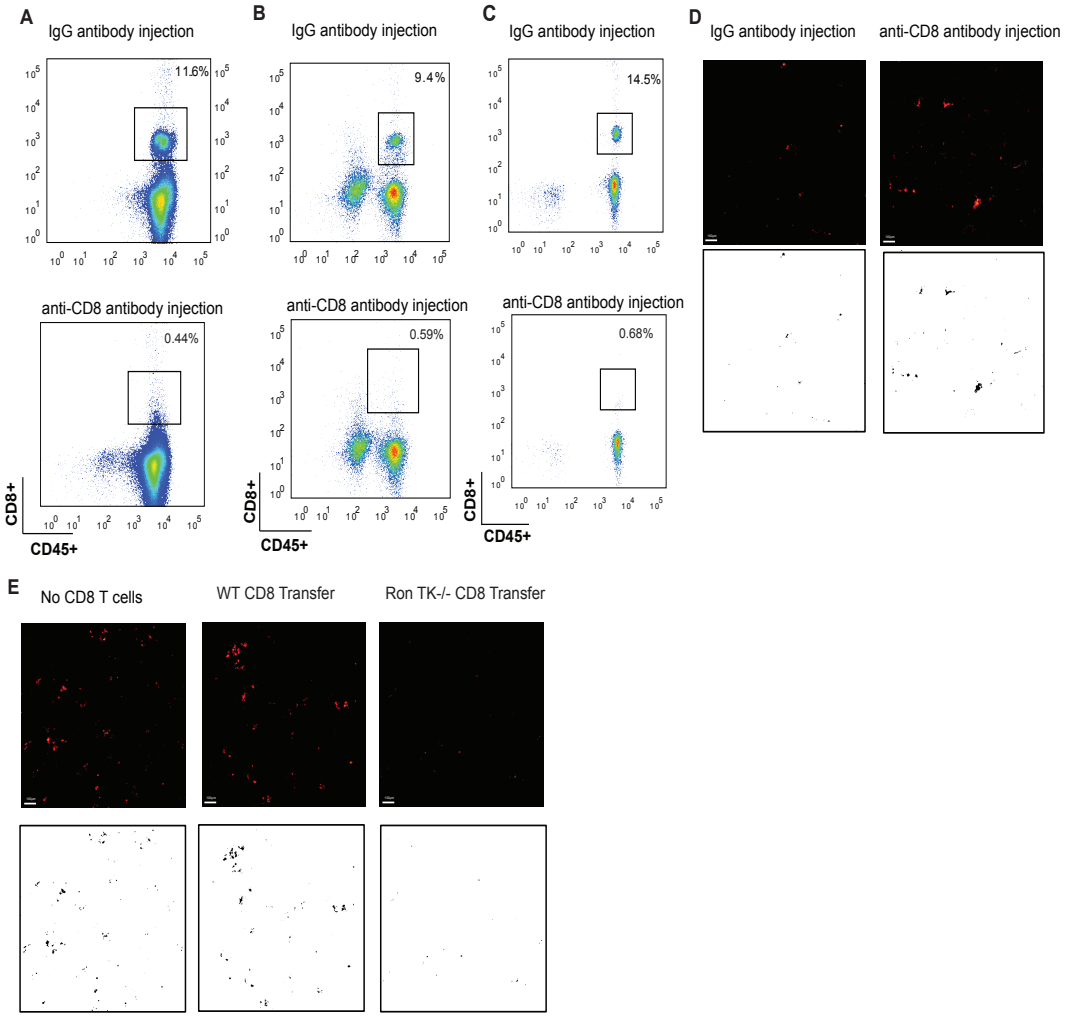

Figure S4. Immunodepletion of CD8$^+$ T cells following injection of anti-CD8 antibody. Flow cytometric analysis of CD8$^+$ T cells in spleens (**A**), lung (**B**) and peripheral blood (**C**) of anti-CD8 antibody or control (IgG) antibody injected mice. **D.** Representative images of lung colonization in Ron TK-/- hosts that have been treated with control antibody **(left)** and anti-CD8 antibody **(right),** with the image J quantification on the bottom. **E.** Representative images of lung colonization in *Prkdc*^{scid} hosts that have been injected with CD8$^+$ T cells from WT or Ron TK-/- hosts.

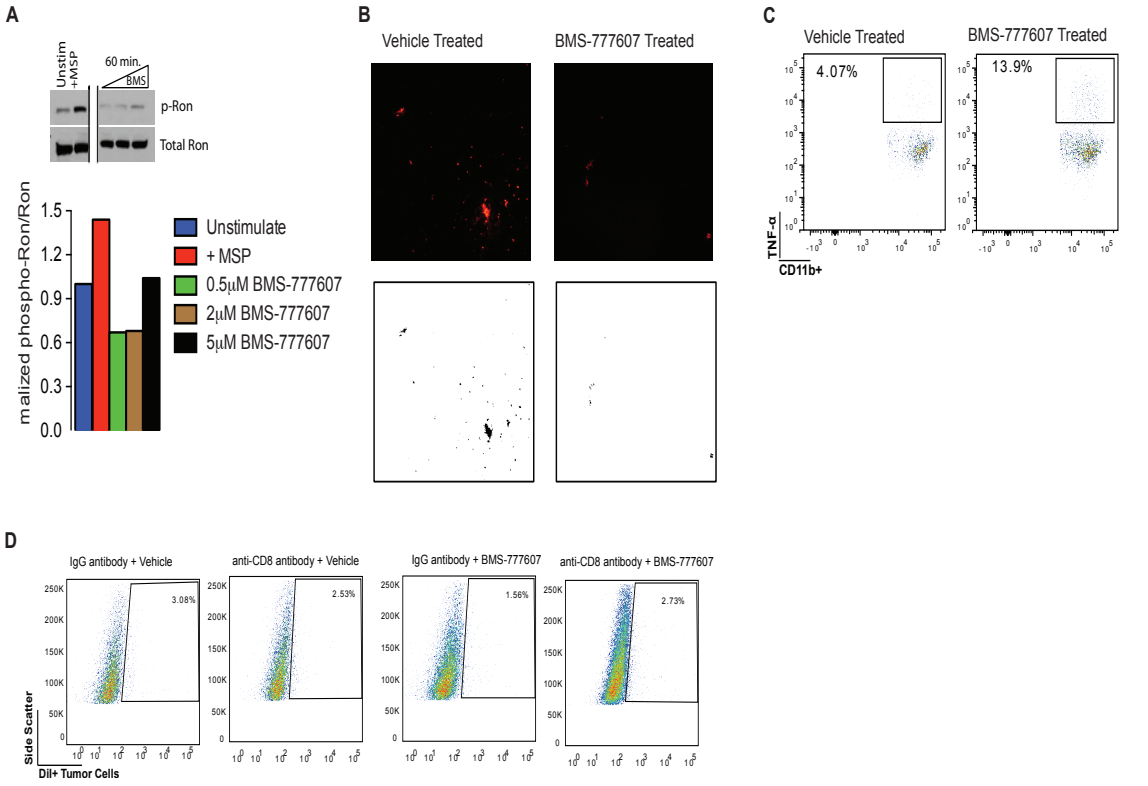

Figure S5. Treatment with a Ron inhibitor, BMS-777607, reduces metastatic outgrowth. **A**. Immunoblots (**upper**) and densitometric analysis (**lower**) using the indicated antibodies on MMTV-PyMT tumor cells (as a source of murine Ron) treated with increasing concentrations of BMS-777607 for 60 minutes. Data were calculated as ratio of phosphor-Ron to total Ron. **B.** Representative images of metastatic burden in WT hosts that have been treated with vehicle or BMS-777607. **C.** Representative flow cytometric analysis of CD11b+ macrophages expressing TNFα , isolated from the lungs of tumor bearing WT and Ron TK-/- hosts treated with vehicle only versus BMS-777607 96 hours after i.v PyMT-MSP injection. **D.** Representative flow cytometric analysis of labeled tumor cells 96 hours after intravenous tumor cell injection in animals treated with vehicle or BMS-777607 following depletion with an anti-CD8 antibody or IgG control. (n=3-4 per group).

Supplementary Table 1. Summary of the effect of host Ron on PyMT-MIG tumor spontaneous metastasis

Host animal	Spontaneous metastasis frequency
FVB wild type (n=12)	6/12 (50%)
FVB Ron TK-/- (n=10)	2/10 (20%)*

* n.s

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

My dissertation demonstrates that host MSP/Ron signaling potentiates metastasis by suppressing the immune system. In this chapter, I will discuss the unpublished data that we have been working on to understand the mechanisms of MSP/Ron-mediated immunosuppression. Additionally, I will discuss the future directions and potential clinical applications of my dissertation.

Unpublished data on mechanism of MSP/Ron mediated metastasis

My dissertation has revealed that the MSP/Ron pathway is a key mediator of conversion of micrometastases to bona fide metastatic lesions in lungs, through its role in suppressing antitumor inflammation (1). Loss of Ron function allows an effective antitumor $CD8^+$ T cell response, which is necessary and sufficient to inhibit outgrowth of seeded metastatic tumor colonies (1). However, $CD8^+$ T cells do not express Ron; we have yet to identify the cell type that responds to MSP, and the subsequent molecular mechanisms that suppress $CD8^+$ T cells. Based on previous studies on MSP/Ron signaling in the noncancer setting (2), we initially hypothesized that Ron signaling in macrophages (MΦs) mediates immunosuppression and ensuing metastasis.

Mature MΦs are the only immune cell reported to express Ron, other than hematopoietic stem cells (3). We hypothesized that MΦs infiltration would be increased in tumors growing in wild-type (WT) hosts, since MSP can function as a MΦs chemoattractant (4,5). However, immunohistochemical analysis for F4/80+ MΦs in tumors derived from WT and Ron tyrosine kinase knockout (Ron TK-/-) hosts revealed no significant difference in infiltration of MΦs into the primary tumors (Appendix). Although there was no quantitative difference in MΦs infiltration, this did not preclude a qualitative

difference between the infiltrating MΦs. These data also did not rule out that another cell type might contribute.

To ensure that cells of the hematopoietic lineage mediated the host-dependent metastatic difference, we transplanted bone marrow from WT hosts into irradiated Ron TK-/- hosts, and vice versa, followed by orthotopic transplantation of PyMT-MSP tumor cells. Bone marrow transfer from WT to Ron TK-/- mice was sufficient to restore metastasis (Appendix Table A1), demonstrating that the hematopoietic cell compartment contains the cell type that mediates immunosuppression. However, transplant of Ron TK-/- bone marrow into WT hosts was unable to prevent metastasis. Since resident MΦs are not efficiently depleted with irradiation (6), we hypothesized that the WT MΦs may have been insufficiently undepleted and still able to promote metastasis. We therefore attempted to deplete WT resident MΦs using a transgenic mouse with an inducible MΦs depletion system. Briefly, in this model, the promoter for CD11b, a cell surface molecule expressed on myeloid cells such as MΦs, drives the human diphtheria toxin receptor (DTR), which is 10^3 to 10^5 times more sensitive to diphtheria toxin (DT) than the mouse DTR (7). The cells expressing the fusion CD11b-DTR (mostly MΦs) die when exposed to DT. Because the metastatic studies required long term MΦs depletion, and because the CD1b promoter construct was slightly leaky, causing liver and lung toxicity (7), we had first expand CD11b-DTR BM into irradiated hosts to ensure WT hematopoietic-specific expression. We then depleted the MΦs using DT injection once a day for 10 days. Subsequently, we intravenously injected PyMT-MSP into the MΦs depleted mice, and assayed for metastatic colonization. Depletion of MΦs in this setting did not significantly impact metastatic colonization (Appendix). However, DTR-mediated MΦs depletion may be incomplete (8).

As a second approach to determine the role of MΦs in MSP-mediated metastasis, we utilized clodronate-liposome mediated MΦs depletion. Liposomes are artificially prepared lipid vesicles that encapsulate clodronate, a hydrophilic toxin (9). After injection into the mouse, liposomes are ingested by MΦs, followed by intracellular release and accumulation of clodronate, causing apoptosis of the MΦs (9).

Liposomal-mediated depletion of MΦs also did not significantly impact metastatic colonization (Appendix). Despite the caveats of the MΦs depletion experiments, mainly that depletion was never complete, we obtained no evidence to support a role for MΦs as the Ron-dependent signal to suppress $CD8^+$ T cells.

These results highlight our difficulty in discovering mechanisms and pathways of MSP/Ron-mediated immunosuppression *in vivo* using our current mouse and tumor models. For example, both DT-and liposome-mediated MΦs depletion can cause cell death of a wide range of mononuclear phagocytes, including neutrophils, monocytes, eosinophils, MΦs and dendritic cells, due to some expression of CD-11b in non-MΦs cells or broad phagocytosis, respectively (10). Such effects could themselves be inflammatory (10), confounding interpretation of our results. Moreover, the diversity and plasticity of myeloid cells (12), coupled with the redundancies of tumor microenvironmental signaling (13), make it very difficult to dissect the molecular mechanisms of immunosuppression. More versatile and precise mouse models and tools will be required to dissect the cellular and molecular mechanisms by which Ron-expressing immune cells suppress the $CD8^+$ T cell response.

A critical tool to dissect cell-specific Ron functions *in vivo* is a conditional Ron knockout and reporter mouse. In this system, the loxP recombinase recognition sites will flank the exons encoding the tyrosine kinase domain of *Ron* (14). Cre recombination will

result in deletion of the Ron tyrosine kinase domain, and replace it with a fluorescent reporter. The resulting allele will express a functionally deficient Ron and a reporter. This reporter will mark Ron-expressing cells for fluorescent activated cell sorting (FACS) analysis and subsequent molecular profiling and expression analysis. Development of a Ron "floxed" reporter mouse will facilitate discovery of functions of Ron in different cell types using different Cre drivers (10).

In this regard, the role of Ron in myeloid cells can be interrogated by using a number of different Cre drivers. The intrinsic plasticity of myeloid cells, and the similarity between MΦs, dendritic cells, and granulocytes (15) make it difficult to generate a cell specific Cre driver (10). However, even with this caveat, such models can be used to interrogate the function of myeloid cells versus lymphoid or non-hematopoietic components. The LysM-Cre model is driven by lysozyme 2, an antimicrobial and cationic protein present in MΦs and granulocytes (16). Although widely used, this model does not distinguish between myeloid cell types, but does exclude CD11c-positive dendritic cells (17). Additionally, lysozyme 2 is also expressed in nonhematopoietic cells, including myocardial precursor cells (18). It was recently published that Ron deletion using LysM-Cre impacts primary prostate tumor growth, suggesting that Ron-specific functions in myeloid cells during metastasis could be analyzed using that Cre driver (19). A second potential driver is the mouse-colony stimulating factor receptor (m-CSF1R, also known as c-fms) Cre mouse (20). This promoter is present on dendritic cells, MΦs and granulocytes (21,22). Importantly, CSF1R mutations cause a selective loss of tissue MΦs and monocytes (23), suggesting that this Cre driver may be best suited to test hypotheses concerning the role of MSP/Ron signaling in tumor-associated MΦs. A third possibility involves use of CD11c-Cre, a component of the complement receptor (24), which is used

to drive Cre in antigen-presenting dendritic cells, and may therefore enable us to understand the role of Ron in antigen-presentation (25).

In addition to a Ron conditional knockout mouse model, a second mouse model for temporal analysis of Ron-specific functions *in vivo* will be a doxycycline inducible re-expression system. Our lab has developed a tetracycline response element- Ron (TRE-Ron) transgenic mouse and crossed it onto the Ron TK-/- background. An important next step is to cross these mice with a mCSF1R-reverse tetracycline-controlled transactivator (mCSF1R-rtTA) (26) mouse to generate a TRE-Ron;mCSF1R-rtTA mouse. In this model, treatment of mice with doxycycline will result in rescued expression of Ron in Ron TK-/- host CSF1R expressing cells, which include MΦs, granulocytes and dendritic cells.

Another key tool will be a well-defined tumor immunology model. Such a model will entail expression of an antigenic protein in tumors that is coupled with an engineered matching transgenic T cell receptor (TCR). One of the most widely used models is the OT-1 transgenic model (27). In this model, $CD8^+$ T cells express a transgenic TCR that specifically recognizes the SIINFEKL peptide of ovalbumin (ova) (27). OT-1 $CD8^+$ T cells are activated when presented with this peptide (27), making it a powerful tool for analyzing immune responses of $CD8^+$ T cells. For tumor studies, PyMT tumor cells would need to be moved to a C57/BL6 genetic background and engineered to express the ova peptide. Subsequent transplantation into WT or Ron TK-/- hosts that are also transgenic for the OT-1 TCR would result in a defined activation of $CD8^+$ T cells in response to the tumors. This model will enable us to address basic questions about T cell activation dynamics, cytokine production, clonal expansion and other aspects of the anti-tumor response of WT and Ron TK-/- hosts.

Concurrently with these complicated mouse models, we need to develop a way to identify and isolate Ron-expressing hematopoietic cells. It has so far been reported that only terminally differentiated resident tissue MΦs express Ron (2). Thus, results from experiments to detect Ron-expressing cells following FACS sorting with general cell surface markers (e.g., CD11b) may be uninterpretable due to the lack of Ron in the majority of these cells. Therefore, another important tool will be identifying or developing a Ron-specific FACS antibody that binds to the extracellular domain of Ron. This type of antibody will minimize the permeabilization and fixation processes, allowing for isolation of cells for analysis. Although we have tested multiple Ron antibodies, we have found none so far that are suitable for FACS.

Following generation of these tools, we should be able to answer critical "next-step" questions that will lead us to discover novel pathways and mechanisms of Ron function.

1. Which immune cells express Ron? A comprehensive analysis of Ron expression, using both FACS as well as Ron-specific reporters, will allow us to design appropriate assays for Ron function *in vivo*.

2. Which molecular signaling pathways are changing in Ron-expressing immune cells during metastasis? Given our limited understanding of Ron in the immune system, an unbiased approach will be most appropriate. PyMT-MSP tumor cells could be injected into Ron conditional knockouts and control hosts. Subsequently, Ron-expressing immune cells from tumor bearing mice will be FACS sorted at different time points, and from different anatomical locations (i.e., lymph nodes, lungs, and primary tumor). These cells will be analyzed by RNA sequencing to identify pathways that may contribute to $CD8^+$ T cell activation or suppression.

3. Is the T_H1 immune response that occurs in Ron TK-/- hosts constitutive, or does it require sustained stimulation by myeloid cells? This question can be addressed by re-expressing Ron at different time points during the tumorigenic process using the tet-regulated system. These data could have implications for how patients are selected and treated for clinical trials for Ron inhibitors.

4. How broadly applicable is Ron-mediated immunosuppression? Does loss of Ron contribute to chronic inflammatory conditions? Crohn's disease and ulcerative colitis are chronic inflammatory bowel diseases (IBD) that affect the digestive tract (28). The most common symptoms of IBD are abdominal pain, weight loss, fever and diarrhea. IBD is a complex genetic disease where environmental factors (e.g., cigarette smoking or diet) and infectious microbes interact with genetic susceptibility, resulting in a mysregulated immune system that can result in mucosal inflammation (28). Genome wide association studies have shown that single nucleotide polymorphisms in both Ron and MSP are associated with IBD (29,30). Although the exact nature of MSP/Ron signaling in IBD is unclear, it is plausible that MSP/Ron immune function may play a role in IBD pathogenesis.

Despite the lack of tools to make a definitive conclusion, we can speculate on how MSP/Ron signaling suppresses $CD8^+$ T cells. Based on our current understanding of the MSP/Ron signaling pathway, MSP/Ron inhibition activates several cytokines that are known to boost the activation and priming of $CD8^+$ T cells. These cytokine-signaling pathways may also be interdependent, feeding back on each other. In the following sections, I will briefly discuss the hypothetical mechanisms that may explain Ron-mediated immunosuppression.

Potential mechanisms of MSP/Ron mediated immunosuppression

Firstly, MSP/Ron signaling may enhance the antigen presentation process (Figure 5.1.). One intriguing possibility in this regard is the intersection of Ron signaling with the Signal transducer and activator of transcription (STAT) pathway (31,32). Stat proteins play key roles in tumor immunity, with Stat1 increasing antitumor immunity and Stat3 suppressing antitumor immunity (33). Importantly, Ron deletion has been demonstrated to increase Stat1 activation and concomitantly inhibit Stat3 activation (19). Stat3 signaling in APCs is a critical pathway that influences the functional outcome of antigen-specific T cells (34,35). Stat3-ablated MΦs inhibit antigen-specific T cell anergy, making T cells more responsive (36). Although this mechanism is unclear, it is hypothesized that, in the absence of a functional Stat3 protein, APCs exhibit increased $CD8^+$ T cell activating capacity. Stat3-ablated MΦs are better than wild-type MΦs at acquiring and processing antigen for effective cross-presentation to $CD8^+$ T cells (37).

Since Ron TK-/- myeloid cells have decreased levels of Stat3 (2,19) they may more effectively cross-present antigen when compared to WT MΦs. It would be interesting to determine if Ron TK-/- myeloid cells maintain this enhanced ability to capture and cross-present antigens in the tumor suppressive microenvironment. This hypothesis can be tested utilizing *in vitro* antigen presenting assays. Ron-expressing myeloid cells from WT mice or Ron TK-/- mice will be sorted by FACS. The sorted myeloid cells will be pulsed with ova peptide and cocultured *in vitro* with ova-specific $CD8^+$ T cells from an OT-1 mouse. The $CD8^+$ T cell response will be analyzed by FACS for TNF-α, IFN-γ, Grz-B, as well as $CD8^+$ T cell proliferation. This assay will determine if Ron TK-/- myeloid cells robustly activate $CD8^+$ T cells when compared to WT myeloid cells. *In vivo*, this hypothesis can be tested by the viral infection model, such as

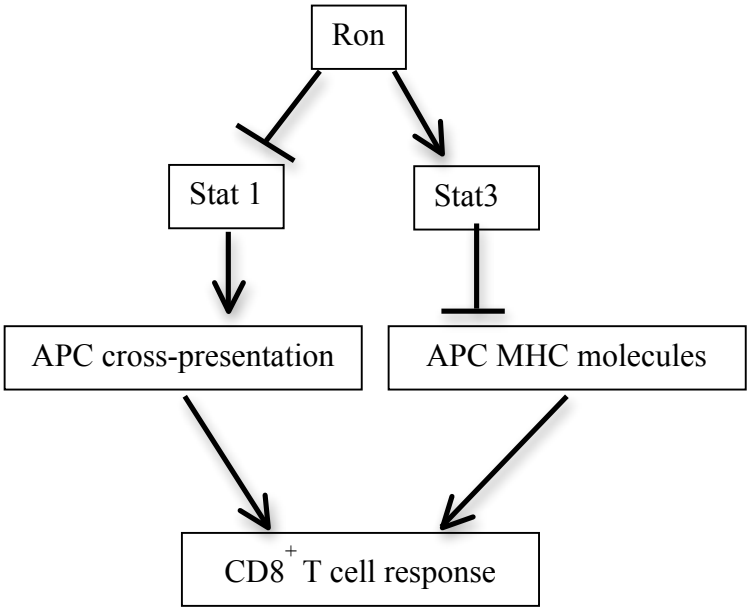

Figure 5.1. MSP/Ron signaling may enhance the antigen presentation process. MSP/Ron signaling may result in suppression of $CD8^+$ T cell response by preventing stat1 activation and activating Stat3 signaling. Stat1 has been shown to play a role in activating $CD8^+$ T cells by the process of cross-presentation, while Stat3 downregulates MHC molecules and suppresses $CD8^+$ T cell response.

lymphocytic choriomeningitis virus (LCMV) (39). Ron-expressing myeloid cells from WT or Ron TK-/- hosts will be sorted, pulsed with an LCMV peptide, and adoptively transferred into naïve mice. These naïve mice will then be challenged with LCMV. The mice that have been injected with the more effective antigen presenting APCs will mount a stronger immune response as assessed by the same parameters as described for the *in vitro* assays. To determine if Stat signaling proteins are downstream of Ron in antigen-presentation, the presence or absence of Stat proteins will be analyzed by fluorescence activated cell sorting (FACS). More detailed genetic studies, where Ron TK-/- mice and WT mice are crossed with Stat 1 (38) and Stat 3 knockout mice (37), will need to be undertaken to dissect the interaction of Ron and Stat proteins.

In addition to cross-presentation, Stat3 deficient MΦs have enhanced expression of MHC class II, a molecule involved in $CD4^+$ T cell activation (34). Loss of Stat3 results in decreased expression of arginase (40,41), whose expression in APCs down-regulates MHC class II molecules (42). L-arginine is required for MHC class II expression on APCs and under L-arginine–deficient conditions, as is the case when Stat3 is activated, APCs have decreased MHC II antigen-presenting ability to $CD4^+$ T cells (42). Like Stat3-activated MΦs, MSP activity on MΦs increases arginase expression (43,44), and decreases MHC class II molecule expression (31). Thus, Ron-expressing immune cells may be skewed toward less decreased $CD4^+$ T cell activation, resulting in less effective $CD8^+$ T cell activation. Accordingly, Ron ablation may also cause APCs to present more effectively to $CD4^+$ T cells, thereby leading to efficient $CD8^+$ T cell activation. Although the proportion of $CD4^+$ T cells in the spleen of tumor-bearing WT and Ron TK-/- hosts were not statistically significant, we observed a trend of increased $CD4^+$ T cells in Ron TK-/- hosts (1). This hypothesis can initially be tested in the experimental metastasis

setting, depleting $CD4^+$ T cells in Ron TK-/- hosts and testing the expansion of $CD8^+$ T cells and resulting effect on metastatic colonization.

Stat3 inhibitors that directly inhibit Stat3 phosphorylation (45), or, peptidomimetics that inhibit dimerization (46), are currently in preclinical development (33). However, there are few Stat3 inhibitors nearing clinical trials, mainly due to the difficulty of identifying a suitable inhibitor that targets the large surface area of the protein-protein interactions (47). It will be interesting to determine if inhibition of Ron by BMS-777607 results in inhibition of the Stat3 pathway and/or promotes Stat1 signaling, thus skewing the immune system toward a T_H1 response.

Secondly, MSP/Ron signaling may skew the production of cytokines (Figure 5.2). Antigen presentation is not the only mechanism that activates $CD8^+$ T cells. The potency of an immune response is dictated by the particular APC as well as the context (inflammatory versus noninflammatory) in which the APC acquires the antigens for processing and presentation (48,49). $CD8^+$ T cells that are exposed to strong inflammatory signals, such as interleukin-12 and interferon-γ (IFNγ), undergo strong proliferation and activation (50). Conversely, in the absence of a strong inflammatory signal, $CD8^+$ T cells undergo tolerance (51). Thus, APCs isolated from tumors (which are naturally selected for lack of strong inflammatory signals) are inefficient at priming adaptive immune responses, thereby inducing T cell tolerance (52,53). Indeed, tumor-associated MΦs display activated Stat3 (54–56), which upregulates immunosuppressive factors, including interleukin-10 (57) and vascular endothelial growth factor (VEGF) (58), and do not exhibit antitumor effects in the tumor microenvironment (59).

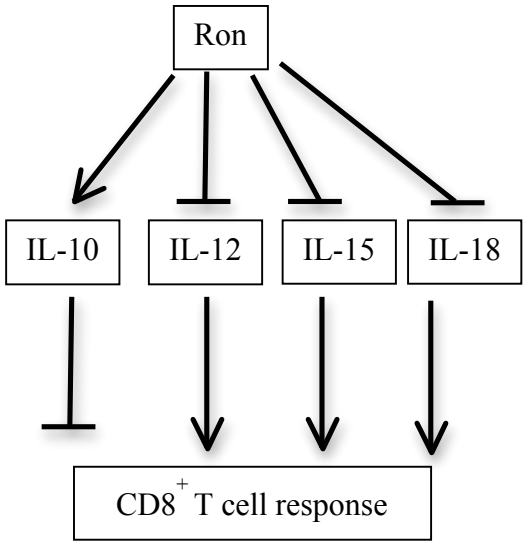

Figure 5.2. MSP/Ron signaling may skew the production of cytokines. MSP/Ron signaling in the tumor microenvironment may result in a skewing of cytokine production, thereby impacting $CD8^+$ T cell activation and response. Notably, MSP has been reported to upregulate IL-10, an immunosuppressive cytokine, and downregulate several cytokines, such as IL-12, IL-15 and IL-18, which have been shown to activate $CD8^+$ T cells.

Conversely, Stat3-deficient MΦs and neutrophils have increased levels of pro-inflammatory mediators, including IL-12, IFNγ, and nitric oxide, thereby leading to more potent antitumor responses (54).

WT MΦs that are stimulated with MSP have a consistently immunosuppressive cytokine profile, expressing higher levels of IL-10, and decreased levels of IL-12 (Appendix). Conversely, loss of Ron results in increased levels of proinflammatory cytokines including IL-12 and TNF-α (1,2,31,32,60). Thus, an alternative hypothesis is that loss of Ron simply causes a proinflammatory microenvironment that increases the potency of the resulting $CD8^+$ T cell response, rather than directly influencing the antigen presenting capacity of the APCs. This hypothesis can also be addressed in a viral infection model (61). Under this system, ova-specific $CD8^+$ T cells derived from the OT-1 transgenic model are cultured for 3 days *in vitro, together with* WT or Ron TK-/- APCs pulsed with ova. The $CD8^+$ T cells are then labeled with fluorescent dye, and transferred into naïve mice. In the presence of a robust inflammatory signal during the *in vitro* stimulation, the $CD8^+$ T cells will continue to expand for several days following transfer, and then undergo a contraction phase. The surviving cells will have a "memory" phenotype and will rapidly re-expand and protect against challenge with *Listeria* that is engineered to express ovalbumin peptide. In the absence of IL-12 or IFNα/β during the initial response to antigen, T cells will proliferate at low levels. Thus, if Ron TK-/- APCs cause an inflammatory microenvironmental cytokine milieu, $CD8^+$ T cells will have more robust proliferation. To differentiate the ensuing proliferation from antigen-presentation, adding conditioned media from Ron TK-/- cocultures to WT cultures, or adding exogenous IL-12 to the WT cultures should rescue the proliferation phenotype. Inhibition

of IL-12 in the Ron TK-/- cocultures using anti-IL12 antibodies is hypothesized to abrogate the robust proliferation.

In addition to IL-12, the expression of IL-15 and IL-18 are also inhibited by MSP (32). Interestingly, IL-15 plays a significant role in T-cell activation and effector functions (62), including T cell proliferation, TNF-α production (63) and cytotoxicity (64,65). Blocking IL-15 or IL-15R on dendritic cells in the context of a viral infection resulted in increased $CD8^+$ T-cell apoptosis in the lung and inefficient viral clearance (66). $CD8^+$ T-cell survival was dependent on IL-15 activity. Intriguingly, engineering tumor-specific T cells to express IL-15 enhanced T-cell survival, and resulting in improved antitumor effects (67).

In addition to IL-15, MSP suppresses IL-18 production (32). IL-18 is produced by many cell types including MΦs (68), dendritic cells (69), epithelial cells (70), and osteoblasts (71). The IL-18 receptor is upregulated on T cells and B cells in response to IL-12 (72). IL-18 is a chemoattractant for $CD8^+$ T cells (73), and contributes to the expansion and effector responses of antigen-specific $CD8^+$ T cell populations (74), and enhances the killing activity of NK cells (75,76). Treating tumor-bearing mice with IL-18 results in tumor growth inhibition (77). Additionally, IL-18 as an adjuvant enhances the antitumor efficacy of a DNA-based vaccine (78).

Thirdly, MSP/Ron signaling may alter the metastatic niche (Figure 5.3). In addition to potential roles in priming and activating $CD8^+$ T cells, loss of host MSP/Ron may impact $CD8^+$ T cell proliferation and/or apoptosis within the metastatic niche. MSP/Ron signaling controls the balance of inducible nitric oxide signaling versus arginase 1 production(2). MSP/Ron signaling inhibits the expression of inducible nitric oxide synthase (iNOS), resulting in decreased levels of nitric oxide (NO) (43,79). Likewise,

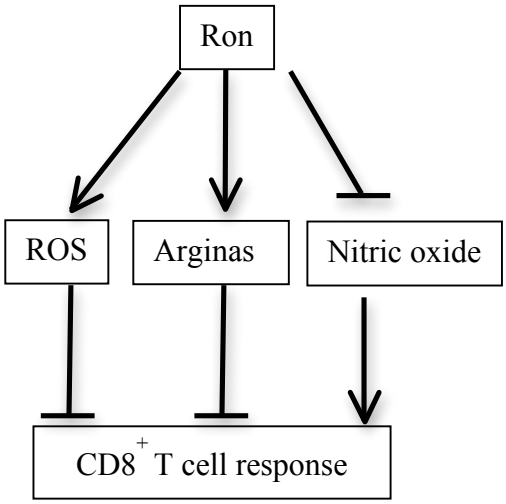

Figure 5.3. MSP/Ron signaling may alter the metastatic niche. MSP/Ron signaling has been shown to regulate the production of factors that remodel the tumor microenvironment. Specifically, MSP/Ron signaling upregulates arginase and downregulates nitric oxide. This signaling pathway has been shown to play a role in $CD8^+$ T cell response. Moreover, MSP/Ron activity results in increased ROS, which have been shown to result in $CD8^+$ T cell apoptosis.

Ron-ablated MΦs have increased levels of NO both *in vitro* and *in vivo*, rendering Ron TK-/- mice more susceptible to LPS-induced endotoxic shock (43,80). Conversely, peritoneal MΦs stimulated with MSP upregulate arginase, which competes with iNOS for their common substrate L-arginine.

iNOS catalyzes the synthesis of NO and citrulline from L-Arginine (81) and is a hallmark of classically activated MΦs (82). By contrast, Arg1 catalyzes the hydrolysis of L-Arginine to L-ornithine and urea (83), and is a hallmark of alternatively activated MΦs (82). In addition to upregulation by Ron, Arg1 expression is induced in myeloid cells by several cytokines including TGF-β, GM-CSF, and IL-4 (84–87).

Increased arginase expression causes depletion of extracellular L-Arginine concentration, which causes T cells hyporesponsiveness, such as decreased proliferation and cytokine synthesis (88–90). Although the mechanism is unclear, arginine downregulates the CD3ζ T cell receptor (91), which subsequently inhibits interleukin 2 receptor signaling pathway (involved in $CD8^+$ T cell proliferation) (92). Thus it is plausible that MSP/Ron signaling induced skewing of the arginase balance results in increased $CD8^+$ T cell apoptosis, and loss of MSP/Ron signaling relieves this apoptotic stress. Ron signaling could also impact $CD8^+$ T cell proliferation and activation by controlling the production of reactive oxygen species (ROS). ROS are a class of molecules including superoxide anion (O_2) and hydrogen peroxide (H_2O_2), which cause $CD8^+$ T cell apoptosis *in vitro* and *in vivo* (93,94). For example, in a viral infection model, increasing the levels of ROS caused a 10-fold decrease of antigen specific $CD8^+$ T cells due to increased T cell apoptosis (95). In the tumor microenvironment or metastatic niche, myeloid cells produce high levels of ROS that can inhibit antigen-induced proliferation and response of $CD8^+$ T cells.

Accordingly, inhibiting ROS in myeloid cells removes the inhibition on $CD8^+$ T cells (96). MSP signaling has been reported to induce a dose-dependent superoxide anion production in human alveolar and peritoneal MΦs as well as in monocyte-derived MΦs, but not in circulating human monocytes (97). Superoxide itself is very unstable and is converted to H_2O_2 and O_2 (96). This is consistent with data suggesting that ROS accumulates primarily in form of H_2O_2, but not superoxide in the tumor microenvironment. Thus, we can speculate that WT myeloid cells release ROS, which subsequently kill the $CD8^+$ T cells. This hypothesis predicts that inhibiting ROS using small molecules in WT hosts may prevent $CD8^+$ T cell apoptosis. Conversely, increasing ROS in Ron TK-/- hosts using small molecules would be predicted to result in immunosuppression and increased metastasis.

Finally, MSP/Ron signaling may result in direct upregulation of cytolytic $CD8^+$ T cell activity. Loss of MSP/Ron signaling relieves immunosuppression of $CD8^+$ T cell and results in production of TNF-α in $CD8^+$ T cells (1). TNF-α regulates multiple aspects of the antitumor response (98). For example, TNF-α mediated killing of infected cells by $CD8^+$ T occurs after cross-presentation (99). Moreover, there is a feedback loop between T cells and APCs, whereby TNF-α produced by $CD8^+$ T cell binds to the TNF-α-receptor on APCs (100). This results in upregulation of positive costimulatory signals such as CD40 and CD86 costimulatory molecules on APCs, thereby increasing the potency of the immune response. Consequently, TNF-$\alpha^{-/-}$ and TNF-α receptor $^{-/-}$ APCs have an impaired ability to mature in response to limited or suboptimal inflammatory conditions, such as those encountered in the tumor microenvironment (100). Thus, inhibition of Ron signaling may initiate an inflammatory signaling loop, strengthening the immune response.

Crossing the Ron TK-/- with TNF-α knockout mice will test if this feedback loop is necessary for the decreased metastasis and immunosuppression.

$CD8^+$ T cells also can kill cells via the production of granzyme B, IFN-γ, and TNF-α (101). We have assayed for the expression of each of these cytolytic molecules in our models, but observed no significant differences in IFN-γ or Granzyme B (Appendix A5). The population of antigen-specific $CD8^+$ T cells at any given time is low (49). Thus, without restimulating these $CD8^+$ T cells with a specific antigen, it is difficult to detect these lytic molecules. Thus, development of tumor models with a defined antigen, (e.g., OT-1 transgenic mice) will allow us to specifically measure the expression of these molecules in response to specific antigens. Genetic models, in which granzyme B, or other lytic molecules are ablated, will also enable us to functionally determine the contribution of particular $CD8^+$ T cell lytic pathways.

In summary, published data suggest that MSP/Ron signaling can impinge on $CD8^+$ T cell activity in several ways; by impacting antigen presentation, by skewing cytokine production in the tumor microenvironment, by altering the metastatic sites or by directly upregulating cytolytic molecules in $CD8^+$ T cells. These various known mechanisms downstream of MSP/Ron that I have described were originally defined in infection or inflammation models, but may also be relevant to the tumor setting. Moreover, it is plausible that many of these pathways are acting in concert during tumor-mediated immunosuppression. Additionally, the pleiotropic effect of MSP/Ron signaling suggests that there may be broader applications to inhibiting this pathway, including in viral infections and autoimmune diseases.

Translational applications

Cancer immunotherapy utilizes the anticancer immune response or components of the immune system as cancer treatment (102). The history of cancer therapy shows waxing and waning of enthusiasm for cancer immunotherapy (102). Although there are multiple reasons why immunotherapy been disappointing, a key indicator is that cancer progression is, in itself, a sign that the complicated and interconnected immune regulatory system has failed. Thus, given the enormous complexity of the system and the numerous unresolved regulatory pathways, it has been difficult to understand which signaling nodes are critical for this immune failure, and whether they can be manipulated to design effective therapies. We consider MSP/Ron signaling to be an immune system "rheostat," tempering the immune response to various insults. Our data suggests that tumors can subvert this built-in regulatory pathway to evade the immune system. Thus, inhibiting MSP/Ron signaling in the host, if it in fact works to reactivate the immune system, could have immense clinical impact.

Historically, a major roadblock in immunotherapy and metastatic drug development is the unsuitability of the Response Evaluation Criteria In Solid Tumors (RECIST) parameters to assess efficacy (103). RECIST measures the efficacy of a therapy depending on tumor shrinkage, whereby an increase in tumor size and/or the appearance of new lesions is considered to be a treatment failure (104). However, these criteria do not readily apply to immunotherapy (104). Immunotherapy-induced tumor regressions can occur after initial tumor progression, and even after the appearance of new lesions. For example, in some patients receiving ipilimumab, an inhibitor of the immune-suppressive CTLA-4, metastases grew or new lesions developed before there was a decline in total tumor burden (105).

Thus, compared to classic chemotherapy, successful immunotherapy may have a variable clinical response, with four patterns of response (106). First, some patients show immediate response and tumor size decreases. Second, some patients show durable stable disease with a possible slow decrease in tumor burden. Third, there are responses after an initial tumor burden increase (possibly due to lymphocyte infiltration). Finally, there is a response in the appearance of new lesions, with some lesions decreasing and others increasing. In addition to the variable clinical response, it is difficult to define the optimal treatment schedule for immunotherapies, due to an insufficient correlation between the maximal tolerated dose and the maximal effective dose (102). Clinical responses following immunotherapy have several implications for bringing Ron inhibitors to the clinic, namely clinical trial design and patient selection.

Appropriate patient selection is crucial for immunotherapeutic success (103). Although our data shows that loss of Ron can affect metastasis in an MSP-independent manner (1), expression of MSP/Ron/Matriptase and its association with poor prognosis (107) suggests that this population may be most suitable for clinical trials with Ron inhibitors. Overexpression of MSP, in particular, may indicate that the tumor may be evading immune response through host Ron signaling. Selection of patients with appropriately staged tumors may also be critical for success in immunotherapeutic settings (108). Immunotherapy is ineffective in patients with a large tumor burden, both due to the correlation of tumor burden and immune suppression, and the delay in the time taken to translate immune responses into a survival benefit (109–113). Thus, Ron inhibitors for immunotherapy would ideally be tested in a low volume and/or microscopic adjuvant disease setting.

Appropriate choice of response criteria will also be critical for successful assessment of the efficacy of Ron inhibitors in the clinic. The immune-related response criteria (irRC) was adopted to capture the spectrum of clinical patterns of antitumor response for immunotherapeutic agents (106). irRC calculates tumor burden as a continuous variable. Accordingly, the percent change in tumor burden between assessment time points describes the size and growth kinetics of total measurable tumor burden over time (106). Using irRC, the appearance of new lesions alone does not constitute progressive disease, if it does not add to the tumor burden by at least 25% (106). Importantly, early increase in the size of lesions, which may be due to the infiltration of lymphocytes, does not mean that the treatment has failed, as a response may be obtained at the next time point (106). These features of immunotherapeutic responses make it important to use different statistical methods for trial design and analysis of survival outcomes (114).

Appropriate statistical analysis of Ron inhibitor clinical trials will be key to assess efficacy. Unlike chemotherapy, for which an early clinical effect is possible, immunotherapies demonstrate delayed clinical effects. The current statistical methods used to analyze clinical trials are unable to calculate the delay in survival benefits that may occur due to immunotherapies (115–117). This may increase the chance of a negative early analysis, and of concluding ineffectiveness because of projected results without a delayed separation (114). This can be avoided by conducting randomized Phase II clinical trials to assess survival curves, thereby allowing better planning of statistical analyses in phase III clinical trials.

Immunotherapies also have a different range of toxicities than cytotoxic drugs (118). For example, ipilimumab is associated with the development of immune-related

adverse reactions such as colitis, dermatitis, hepatitis and endocrinopathies. Although MSP-/- mice and Ron TK-/- mice are viable and developmentally normal, they exhibit increased inflammation in response to exogenous challenges (119,120). Thus, a potential side effect of Ron inhibition could include the inability to downregulate the inflammatory response following a severe infection or injury, suggesting that Ron inhibitors may have to be temporarily withdrawn during serious injury or infection.

Tracking immune responses is critical during immunotherapies, and should be done with immune-specific biomarker assays (114). There bioassays include enzyme-linked immunosorbent spot assays and FACS for intracellular cytokines (114). Our results show that loss of Ron increases expression of TNF-α in $CD8^+$ T cells (1). Thus, one potential biomarker for drug response would be a cytokine panel for TNF-α and potentially other T cell activation markers. Additionally, profiling our tumors for cytokine differences uncovered that tumors from WT hosts express significantly more interleukin 1-β (IL-1β) when compared to Ron TK-/- hosts (Appendix). Additionally, culturing MΦs in the presence of MSP for 72 hours results in increased levels of IL-1β (Appendix). This suggests that downregulation of IL-1β in tumors or MΦs may be a biomarker for Ron inhibition. IL-1β is an "alarm cytokine" that is secreted by MΦs, initiates inflammation and may play a role in promoting immune tolerance (121). Secretion of IL-1β, by both tumor cells and stromal cells, has been reported to induce T cell anergy (122), and correlates with the accumulation of myeloid-derived suppressor cells in the peripheral blood, spleen, and tumor (122). However, antibody mediated depletion of IL-1β (Appendix) in our model did significantly affect metastatic outgrowth. Thus, although IL-1β may not be a mechanistic driver of Ron-dependent metastasis, it may potentially serve as a biomarker for Ron activity.

Synergy of Ron inhibitors with chemotherapeutic agents and vaccines would theoretically increase the potency of the immune response. Vaccines effectively prevent many pathogen infections (123). This success, as well as the recent approval of sipuleucel-T (124), an antigen-presenting cell vaccine for prostate cancer, has generated immense interest in using vaccines to treat or prevent cancer patients (125). However, the etiological agents for most human cancers remains unknown (126); therefore, vaccines may be more useful in the short term as therapeutics rather than preventatives. Therapeutic vaccines attempt to stimulate the patient's immune system to respond to an existing cancer (125). Unfortunately, the endogenous T cell repertoire lacks high-avidity clones due to thymic negative selection (127). Moreover, those T cells that have escaped thymic negative selection are subjected to highly regulated peripheral tolerance mechanisms (128). These mechanisms, combined with the negative influence of the tumor microenvironment and other immunosuppressive factors, have contributed to the limited success of vaccines (125,129,130). However, preclinical studies show that vaccine combination with either immune stimulants or inhibitors of immune suppression, greatly enhances antitumor responses (131,132). Our results demonstrate that because Ron inhibition breaks immunosuppression, combination therapy with a Ron inhibitor and antitumor vaccine may be effective. However, preclinical proof-of-concept experiments are needed to validate this.

How would one design and validate a vaccine/Ron inhibitor combination? Vaccination is often grounded on identifying an antigen that will stimulate T cell activity in a tumor-specific manner (133). Preclinically, a proof of concept experiment would involve pulsing APCs with the ovalbumin peptide, for example, followed by adoptive transfer into a PyMT-ovalbumin tumor-bearing Ron TK-/- hosts or WT hosts treated with

Ron inhibitors. If we determine that Ron inhibitors significantly impact metastasis and tumor growth by synergizing with vaccines, the next step would be to combine Ron inhibitors with vaccines in current clinical trials (125). These vaccines are based on peptides from known tumor-associated antigens, and are administered with an adjuvant and/or other immune modulators. For example, current vaccines are targeted to overexpressed HER2/neu (134), and oncofetal antigens such as carcinoembryonic antigen (CEA) (135), and mucins (136). Although these approaches may prove clinically beneficial, there are currently no approved vaccines that are in clinical use for breast cancer.

Chemotherapy-induced immune activation (102,137) opens up the exciting possibility that Ron inhibitors could potentially synergize with chemotherapy to impact tumor growth and metastasis. It is currently unknown which chemotherapy combinations have the most impact when combined with Ron inhibitors. Thus an important experiment will be to determine which combinations are most effective.

Although some chemotherapy treatments may have immunosuppressive effects, seen with high-dose cyclophosphamide (138) and folate antagonists (139), response to chemotherapy and targeted therapies is, in part, regulated by the immune system (137,140–142). Chemotherapies used in breast cancer can induce various tumor cell death pathways, leading to the release of tumor-associated antigens that can activate immune cells. Oxaliplatin and anthracyclines such as doxorubicin induce “immunogenic tumor cell death,” which enhances cross-presentation of tumor-associated antigens by DCs, and subsequent activation of T cells (143,144). Doxorubicin has been shown to increase antigen-specific $CD8^+$ T cell proliferation in tumor-draining lymph nodes, and promotes T cell infiltration into tumors (145). Taxane microtubule inhibitors are a class of cytotoxic

agents that are considered to be the standard of care for treatment of metastatic disease (146). Paclitaxel, a taxane, inhibits regulatory T cells, thereby rendering tumor cells susceptible to $CD8^+$ T cell-mediated lysis (147,148). Docetaxel, used for the treatment of anthracycline-resistant breast cancer, has been reported to decrease splenic MDSC levels in tumor-bearing mice (149). Gemcitabine, a nucleoside analog (150), increases the expression of class I human leukocyte antigen (HLA) on tumor cells, (151) and augments the cross-presentation of tumor associated antigens to $CD8^+$ T cells (140). Platinum-based chemotherapies (including cisplatin and carboplatin) also increase HLA expression, and can relieve immunosuppression by limiting expression of programmed death ligand 2, a T cell inhibitor (152).

A significant hurdle to developing and testing Ron inhibitors and combinations as immunotherapies is the absence of human immune-competent preclinical mouse models (153). Current mouse models may not predict the clinical response to immunotherapies (154). Although experiments in mice with tumors expressing xenogeneic proteins that are coupled with transgenic T cells are used to address questions about the antitumor immune response (155–157), they may be inadequate for modeling the human immune response to immunotherapy strategies due to the foreign nature of the antigens (158). Thus, a mouse model with human tumors and human immune system (159) may provide a better method to test Ron inhibitors, alone and in combination. In this model, immunodeficient mice are being engrafted with human hematopoietic cells to generate a functional human immune system (159). Consequently, these mice would have both a human immune system and human cancers. This type model would be ideal to test drugs for Ron and check for chemotherapy combinations.

In summary, our finding that MSP/Ron signaling enables metastasis by suppressing the immune system has revealed many questions about mechanism and function. To answer these questions will require the development of more versatile and precise animal models. Moreover, our finding that MSP/Ron signaling may function as an immunotherapy in the metastatic setting has important implications for clinical trial design and analysis.

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APPENDIX

UNPUBLISHED DATA

WT hosts Ron TK-/- hosts

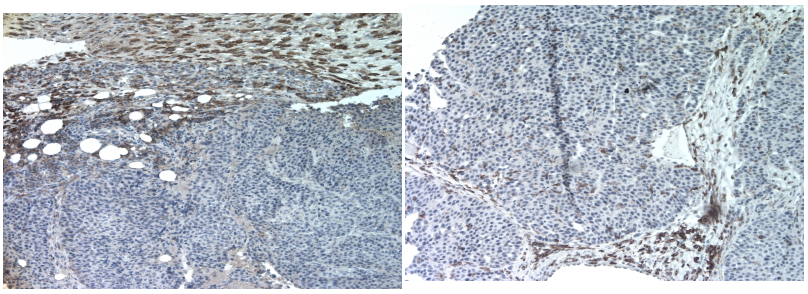

Figure A.1. Levels of macrophage infiltration in PyMT-MSP tumors in WT and Ron TK-/- hosts are similar. Representative immunohistochemistry analysis of macrophage infiltration into WT or Ron TK-/- hosts. Paraffin fixed- PyMT-MSP tumors were stained with F4/80 (1:100) 1 hour at room temperature. There was not a significant difference in the level of infiltration of macrophages.

Donor/Recipient	METASTASIS
A. WT bone marrow into WT	3/3 (100%)
B. Ron TK-/- bone marrow into Ron TK-/-	0/3 (0%)
C. WT bone marrow into Ron TK-/-	2/3 (67%)
D. Ron TK-/- bone marrow into WT	2/3 (67%)

Table A.1. The myeloid compartment is responsible for the metastatic effect.
WT and Ron TK-/- hosts were irradiated with 900 rads, followed by tail vein injection of $1x10^6$ bone marrow cells from WT or Ron TK-/- hosts. Three months later, 100,000 PyMT-MSP expressing cells were injected orthotopically and metastasis to the lungs was analyzed. Our results show that WT bone marrow is sufficient to restore the metastatic effect in Ron TK-/- hosts.

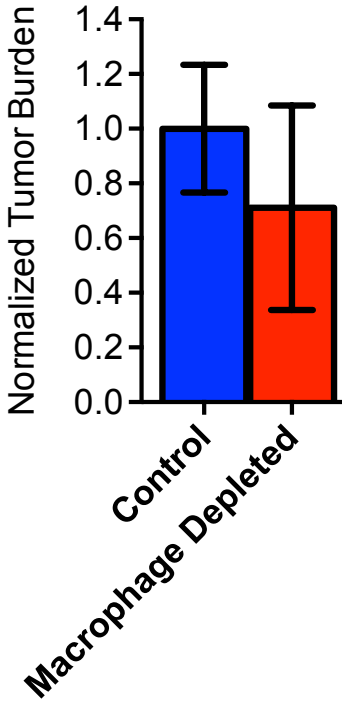

Figure A.2. Diphtheria-toxin mediated macrophage depletion does not significantly decrease metastatic colonization. WT hosts were irradiated with 900 rads. Subsequently, $1x10^6$ bone marrow cells from Diphtheria-toxin receptor mice were injected into the tail vein. One month later, the mice were injected with diphtheria toxin. 250,000 PyMT MSP tumor cells were injected into the tail vein. Ten days later, the animal was sacrificed, and lungs sectioned and colonies quantified. Depletion of macrophages did not decrease metastatic colonization.

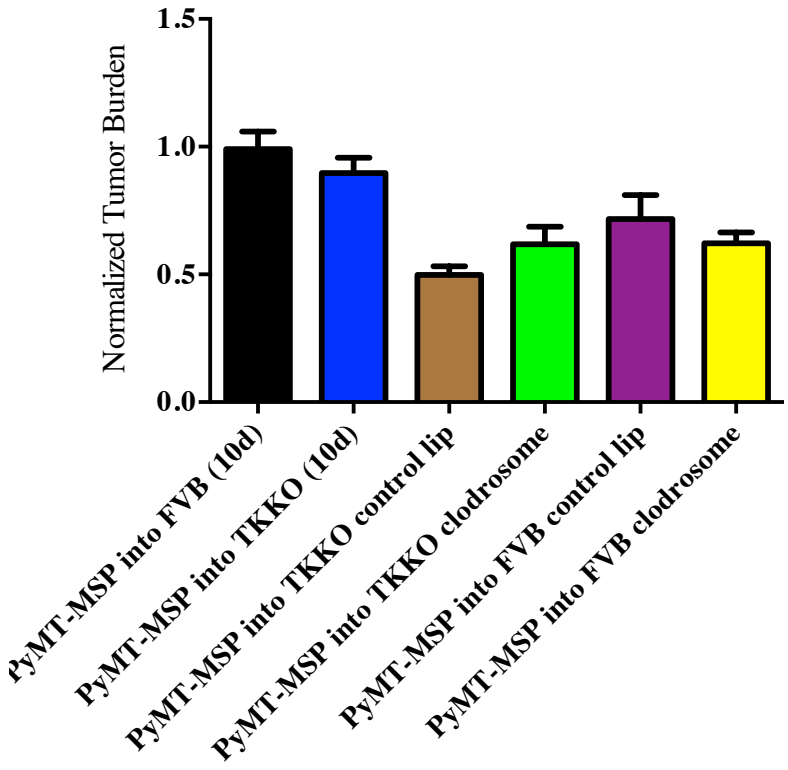

Figure A.3. Liposome-clodronate mediated macrophage depletion does not significantly decrease metastatic colonization. WT and Ron TK-/- hosts were treated with lipsome-clodronome or control clodrosome. Subsequently, 250,000 PyMT MSP tumor cells were injected into the tail vein. Ten days later, the animal was sacrificed, and lungs sectioned and colonies quantified. Depletion of macrophages with liposome did not decrease metastatic colonization.

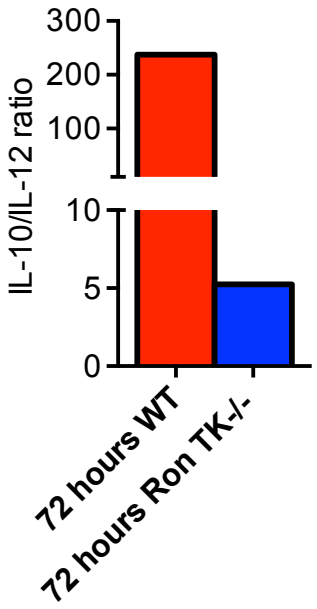

Figure A.4. WT macrophages have an immunosuppressive cytokine profile. WT macrophages and Ron TK-/- bone marrow derived macrophages were stimulated MSP (100ng/mL) for 72 hours. WT macrophages had an increased IL10/Il-12 ratio, indicative of immunosuppressive macrophages.

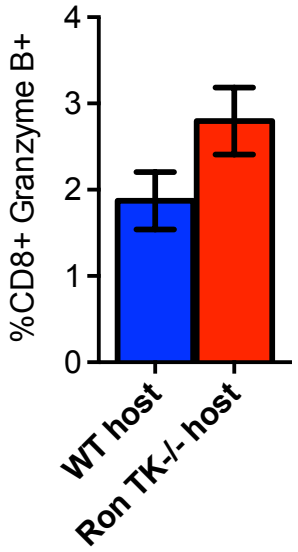

Figure A.5. CD8$^+$ T cell expression of Granzyme B+ is similar between WT and Ron TK-/- hosts. Graph depicting the population of Granzyme B+ expressing CD8$^+$ T cells in lungs of tumor bearing WT and Ron TK-/- mice 72 hours post-PyMT-MSP tumor cell injection.

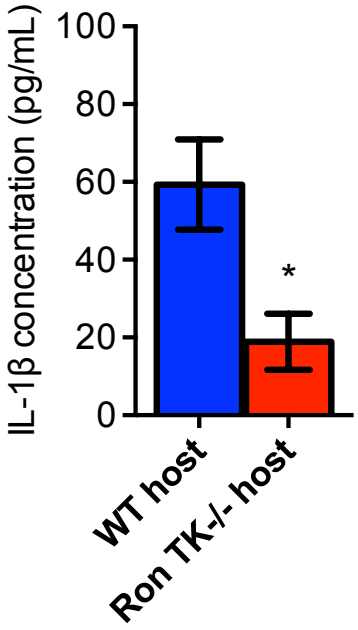

Figure A.6. IL-1β protein is significantly decreased in PyMT-MSP tumors from Ron TK-/- hosts. PyMT-MSP tumor lysates were isolated using our previously published protocol. The tumors were subsequently analyzed using the MSD cytokine profile as per manufacturers instructions. The results show that IL-1β levels are decreased in tumors derived from Ron TK-/- hosts (p=0.01).

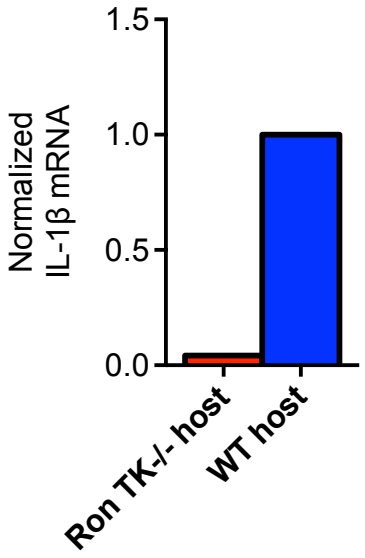

Figure A.7. IL-1β mRNA levels in WT macrophages are increased 72 hours after MSP stimulation. Bone marrow derived macrophages were differentiated using Macrophage Colony Stimulating Factor for 10 days. Subsequently, they were transferred to a serum free media, and stimulated with MSP for 72 hours (100ng/mL). RNA was isolated using Quiagen RNA isolation protocol, and expression of IL-1β analyzed using RT-PCR. IL-1β is significantly increased with MSP stimulation.

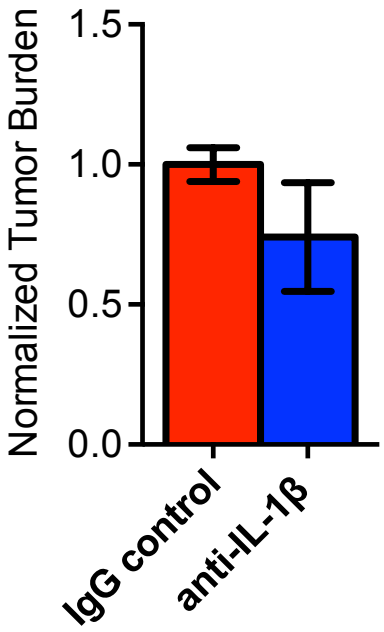

Figure A.8. IL-1β inhibition does not decrease metastatic colonization. WT hosts were treated with an anti-IL-1β antibody. Subsequently, 250,000 PyMT-MSP tumor cells were intravenously injected, and metastatic colonization analyzed. IL-1β inhibition does not prevent outgrowth of metastatic outgrowths.