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

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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\textsuperscript{+} 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.

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
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 G0–G1 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

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**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,** quantification of tumor-cell seeding in the lung 36 hours after intravenous injection into WT or Ron TK−/− hosts (n = 3/group). **D,** 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). **E,** 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). 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**

<table>
<thead>
<tr>
<th>Host animal</th>
<th>Days to palpable tumor</th>
<th>Days to reach 2 cm</th>
<th>Spontaneous metastasis frequency</th>
<th>Survival (days to ethical endpoint)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVB WT (n = 15)</td>
<td>35</td>
<td>66</td>
<td>13/15 (87%)</td>
<td>40</td>
</tr>
<tr>
<td>FVB Ron TK−/− (n = 15)</td>
<td>53</td>
<td>71</td>
<td>1/15 (6.7%)</td>
<td>52</td>
</tr>
<tr>
<td>FVB Ron TK−/−;Prkdc−/− (n = 7)</td>
<td>41</td>
<td>71</td>
<td>5/7 (71.4%)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*P < 0.0001 vs. WT (Fisher exact test).

*P < 0.005 vs. FVB WT (Fisher exact test).

*Experimental metastasis assay; mice were euthanized when in respiratory distress.

*P < 0.05 (Mantel-Cox test).

*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. 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, naive 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, naive 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, naive 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, naive hosts) and TNF-α (Fig. 2G, naive 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 Prkdcsid mice, which lack functional lymphocytes. The double mutants, versus control Ron TK−/−;Prkdcsid littermates (all backcrossed to the FVB background), were used as hosts for orthotopically transplanted PyMT-MIG tumors. Tumors developed and grew with similar rates in both hosts; however, Ron TK−/−;Prkdcsid 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 populations.
increased proportion of Annexin V-positive tumor cells.

We next wanted to determine whether CD8+ T cells were sufficient to block metastasis in tumor-bearing Ron TK−/− mice 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-naive, syngeneic Ron TK−/+;Ptk/−/− 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 naive 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...
the CD8+ T cells in tumor-bearing WT mice were incapable of antimitastatic 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 (IC50 < 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.
Inhibition of Ron Kinase Blocks Metastatic Outgrowth

**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 anti-tumor 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 immuno-suppressive 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 polyanines, 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). Phä/− mice (The Jackson Laboratory) and Ron TK−/− or WT mice were backcrossed to generate Ron TK−/−Phä−/− mice and Ron TK−/−Phä−/− 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 GFPα−/− 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 Percoll (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 CD8e-FITC, CD98e-PE, CD45-PTC, CD11c-PerC7, CD11b-PerC7, 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 μg/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 Dil (Invitrogen) and resuspended in Hank’s Balanced Salt Solution at 10⁶ 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 image 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 Dil-labeled tumor cells.

**CD8e Killing Assays**

CD8e 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 (CD8e microbeads; MACS). Subsequently, the 50,000 CD8e 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).

**CD8e T-Cell Depletion and Adoptive Transfer**

For CD8e 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 CD8e-FITC antibodies and FACS sorted. A total of 500,000 donor CD8e T cells...
were injected into the lateral tail veins of recipientRon TK−/−,p53−/−/kid mice. Twenty-four hours later, the mice were injected with 250,000 Dil-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′-CTCCAAAACAGATACCCGCACAT ACT-3′ (forward) and 5′-GCTGGTCTGGTGCCGCTTTGCAGTAC-3′ (50). Thirty-five cycles of PCR for glyceraldehyde-3-phosphate dehydrogenase on the same samples was conducted using the following primers: 5′-ATGTTCCAGATGACTCCACT-3′ and 5′-CCACAAGCAGGTGCT-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).

**Disclosure of Potential Conflicts of Interest**

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