Blocking short-form Ron eliminates breast cancer metastases through accumulation of stem-like CD4+ T cells that subvert immunosuppression

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Abstract

Immunotherapy has potential to prevent and treat metastatic breast cancer, but strategies to enhance immune-mediated killing of metastatic tumors are urgently needed. We report that a ligand-independent isoform of Ron kinase (SF-Ron) is a key target to enhance immune infiltration and eradicate metastatic tumors. Host-specific deletion of SF-Ron caused recruitment of lymphocytes to micro-metastases, augmented tumor-specific T cell responses, and nearly eliminated breast cancer metastasis in mice. Lack of host SF-Ron caused stem-like TCF1+ CD4+ T cells with type I differentiation potential to accumulate in metastases and prevent metastatic outgrowth. There was a corresponding increase in tumor-specific CD8+ T cells, which were also required to eliminate lung metastases. Treatment of mice with a Ron kinase inhibitor increased tumor-specific CD8+ T cells and protected from metastatic outgrowth. These data provide strong pre-clinical rationale to pursue small molecule Ron kinase inhibitors for prevention and treatment of metastatic breast cancer.

Statement of Significance

Discovery that SF-Ron promotes anti-tumor immune responses has significant clinical implications. Therapeutic antibodies targeting full-length Ron may not be effective for immunotherapy; poor efficacy of such antibodies in trials may be due to their inability to block SF-Ron. Our data warrant trials with inhibitors targeting SF-Ron, in combination with immunotherapy.

Introduction

Breast cancer is the second leading cause of cancer-related deaths in women in the United States (1). Although early-stage primary breast cancer can be cured with surgery and systemic therapy, metastatic breast cancer remains incurable. Metastasis, the deadly spread of cancer to other organs, occurs in 20-30% of breast cancer patients...
In most of those cases, metastasis occurs prior to the primary tumor being detectable, and metastatic recurrences happen years after initial diagnosis and treatment (3). Thus, spontaneous outgrowth of initially undetectable micro-metastatic disease is the largest contributor to breast cancer mortality. There is an urgent need to develop strategies that can prevent outgrowth of micro-metastases and/or effectively treat metastatic disease. Because the immune system is able to protect against cancer in its early stages (4), immune stimulation is an attractive strategy to potentially eliminate micro-metastatic and/or overtly metastatic cancer.

During tumor progression, the immune system becomes suppressed by multiple mechanisms, allowing tumor outgrowth. Continually evolving tumor-host interactions allow evasion from immune-mediated killing by cytotoxic T cells (CTL) or natural killer (NK) cells (4,5). Immune evasion is further promoted by other immune cells including monocytes, macrophages, dendritic cells (DCs), neutrophils, myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and B cells, all of which have been demonstrated to play an active role in promoting immune tolerance (6). Established tumors adopt features of chronic wounds that promote systemic inflammation, which in turn promotes distant metastasis (7,8).

Infiltration of different immune cell populations into primary tumors and metastases can serve as a prognostic factor in various cancers, suggesting that the presence of a certain immune milieu can have beneficial anti-tumor effects (9). Reviving the immune system's ability to kill tumor cells is an exciting area of research, so that new targets may be identified to prevent or treat deadly metastatic disease. Cancer immunotherapy has brought some success to the treatment of several advanced cancers, including melanoma, renal cancer, and lung cancers, all of which have relatively high somatic mutation burden and are therefore somewhat immunogenic (10-13). While breast cancer is recognized as poorly immunogenic and less responsive to immune checkpoint blockade, recent developments in high-throughput genomic and cellular analyses have revealed heterogeneous populations of immune cells in breast cancers and implied that breast cancers are not always "immune-cold" (14). Immunotherapies are not approved for most breast cancers; however, the FDA has approved atezolizumab, an anti-PD-L1 immune checkpoint inhibitor, in combination with nab-paclitaxel, for the treatment of advanced or metastatic triple-negative breast cancer (TNBC) (15). Despite this clinical advancement, the majority of overtly metastatic TNBC tumors do not respond to immunotherapy (16). The goal of our research is to identify
pathways that can boost the immune response to tumors in order to eliminate micrometastatic disease and prevent metastatic recurrences.

Overexpression of the receptor tyrosine kinase Ron occurs in various epithelial cancers, including breast cancer, and is significantly associated with tumor progression and metastasis (17,18). Ron is the cell surface receptor for macrophage-stimulating protein (MSP) (19). In addition to its expression on tumor cells, Ron is found on tissue-resident macrophages (20); hence Ron signaling has both tumor-intrinsic as well as tumor-extrinsic (host cell-mediated) effects on tumor progression and metastasis. Activation of Ron signaling specifically on tumor cells promotes tumor cell proliferation, survival, and invasion (21). Activation of Ron signaling on macrophages is reported to skew them towards an alternatively-activated (M2-like) phenotype (22,23). Genetically-engineered mice that lack Ron tyrosine kinase activity (Ron TK-/−) fail to downregulate pro-inflammatory immune responses in response to infection, inflammation, and injury (24-26). We previously showed that Ron functions specifically in the host to facilitate mammary tumor metastasis to lungs in the MMTV-PyMT mouse breast cancer model and that blocking Ron tyrosine kinase activity, either genetically or pharmacologically, protected mice from metastasis through stimulation of anti-tumor immunity (27). Our preclinical studies also showed that Ron inhibition was effective alone, and even better in combination with anti-CTLA-4 immune checkpoint blockade, to significantly shrink primary breast or colorectal tumors and to reduce lung metastatic tumor outgrowth (28). However, the mechanism by which Ron regulates the anti-tumor immune response is unknown and is an exciting area of investigation given the availability Ron inhibitors in clinical development.

More than a dozen small molecule inhibitors and monoclonal antibodies to target Ron are in clinical and preclinical development (29-32). Phase I trials have demonstrated that both categories of targeted therapeutics against Ron are well-tolerated (33-35). So far, only one trial reported on efficacy, and showed that the anti-Ron antibody narnatumab had only limited anti-tumor efficacy (33). This was perplexing given the strong preclinical genetic evidence that loss of Ron kinase activity protects from tumor growth and metastasis through both cell-intrinsic and immune-modulating effects.

Both breast cancer cells and macrophages, which are the only immune cells known to express Ron, express two isoforms of Ron with distinct functions: full-length Ron (FL-Ron) and short-form Ron (SF-Ron). SF-Ron is expressed from an alternative promoter located within intron 10 of the mouse Ron gene (Stk) (36,37). SF-Ron is also
produced in human cells in a similar manner, where its production is thought to be regulated by methylation of the main Ron promoter (38). Importantly, due to its N-terminally truncated nature, SF-Ron protein lacks most of the extracellular domain, including the entire ligand-binding domain. SF-Ron does contain the transmembrane and intracellular domains, hence retains the tyrosine kinase activity in a ligand-independent manner (39,40). It has been demonstrated that FL-Ron and SF-Ron have distinct tumor-intrinsic roles (41) and have non-redundant inflammatory functions in a non-cancer setting (42).

While FL-Ron’s function in macrophages has been studied extensively and its function in tumor immunity has been studied to some extent (22,27,28,43), the role of SF-Ron in anti-tumor immune responses is entirely unknown. Distinguishing the relative function of Ron isoforms in anti-tumor immunity is a critical gap in our knowledge because although both isoforms can be targeted with small-molecule kinase inhibitors, only FL-Ron can be targeted with antibodies such as narnatumab due to lack of the extracellular domain in the SF-Ron protein. Likewise, only FL-Ron is responsive to the Ron ligand MSP. Here, we sought to define the function of SF-Ron in breast cancer metastasis and anti-tumor immunity. We unexpectedly found that SF-Ron, not FL-Ron, is the key isoform that suppresses immune responses against breast cancer metastasis. Examination of mice specifically lacking SF-Ron also revealed new insight into the role of naïve, stem-like CD4+ T cells in control of micro-metastatic outgrowth of breast cancer.

**Results**

**Mice lacking SF-Ron are protected from breast cancer lung metastasis.**

To determine if host SF-Ron plays a role in breast cancer progression, we used mice that were engineered to lack the SF-Ron isoform specifically (Ron SF-/-) (42) on a pure FVB background, which is syngeneic with the MMTV-PyMT mammary tumor model (44). To verify the relative expression of different Ron isoforms in our model, we performed RT-PCR and flow cytometry on Ron-expressing peritoneal macrophages isolated from wild type (WT), Ron tyrosine kinase deleted (Ron TK-/-) (24) and Ron SF-/- mice. Ron TK-/- mice are engineered to lack exons 13-18 of Ron, which encodes the entire tyrosine kinase domain and hence eliminates functional Ron tyrosine kinase activity from both isoforms (45). Using primers designed to specifically amplify cDNA encoding full length Ron or primers specific for SF-Ron (36) for RT-PCR analysis, we
verified that Ron SF-/− mice express FL-Ron mRNA but lack SF-Ron mRNA (Figure S1A). Analysis of FL-Ron protein by flow cytometry using an antibody against the extracellular domain of Ron (Supplemental Table 1) revealed that Ron SF-/− macrophages expressed FL-Ron protein at the cell surface at levels comparable to that of WT macrophages (Figure 1A, S1b-c). To ensure that FL-Ron was functional in Ron SF-/− macrophages, we examined three known downstream targets of Ron signaling, PD-L1, CD80, and CD86 (28). Stimulation of peritoneal macrophages from Ron SF-/− mice with MSP increased the expression of PD-L1 and CD80 and decreased the expression of CD86, similar to WT macrophages (Figure 1B and S1d). No significant change in any of these proteins was detected in Ron TK-/− macrophages in response to MSP. Together, these results verify that Ron SF-/− macrophages produce functional FL-Ron and specifically lack SF-Ron.

To test the role of host SF-Ron in spontaneous breast cancer metastasis, we orthotopically implanted MMTV-PyMT tumor cells into cleared mammary fat pads of WT or Ron SF-/− mice. Ron TK-/− mice were used as a positive control; we previously reported that lack of all host Ron tyrosine kinase activity reduces spontaneous and experimental metastasis (27). To control for any differences in primary tumor growth, we resected each primary tumor when they grew to 1 cm in diameter, then examined metastasis four weeks later. Loss of SF-Ron specifically (Ron SF-/−), or loss of function of both isoforms (Ron TK-/−), significantly delayed the primary breast tumor growth compared to WT mice (Figure 1C). We also found that both Ron SF-/− and Ron TK-/− mice developed significantly lower metastatic tumor burden than WT mice, even though all primary tumors were allowed to grow to the same size prior to resection (Figure 1D-E).

The most common scenario for human breast cancer metastasis is outgrowth of micro-metastatic tumor cells in distant sites, usually long after the primary tumor has been resected. To determine if SF-Ron plays a role in metastatic tumor outgrowth in the lungs, we performed experimental metastasis assays by injecting MMTV-PyMT tumor cells into the tail veins of WT, Ron TK-/− and Ron SF-/− mice. As expected from our previous results, Ron TK-/− mice were protected from metastasis. Interestingly, we found that there were almost no lung metastases in Ron SF-/− mice (Figure 1F). These results suggested that host SF-Ron, instead of FL-Ron, is required for lung metastasis of breast cancer.
To determine whether lack of SF-Ron prevented initial metastatic seeding versus metastatic outgrowth, we collected the lungs of WT, Ron TK-/-, and Ron SF-/- mice at an earlier time point, two weeks after tumor injection. Here, we found that Ron SF-/- mice had micro-metastatic nodules (Figure 1G), indicating that tumor cells are able to seed the lungs of Ron SF-/- mice and generate micro-metastatic lesions, but that they disappear by the later time point. Therefore, SF-Ron is required for the critical step of micro-metastatic outgrowth to overt metastasis in lungs, which is a disease stage amenable to metastatic tumor prevention in patients through use of adjuvant therapy. We next investigated the mechanism by which metastatic outgrowth was prevented in these mice.

Lack of SF-Ron stimulates robust systemic and local anti-tumor immune responses.

FL-Ron is well known to regulate inflammation through its function on macrophages (22,43,46) and there are some data indicating that FL-Ron and SF-Ron have similar but non-redundant roles in inflammation in response to injury (42). However, the role of SF-Ron in regulating immune responses in cancer is unknown, so we focused efforts on determining how this isoform regulates metastatic outgrowth. To determine whether Ron SF-/- mice mount anti-tumor immune responses, we performed multicolor flow-cytometry (Figures S2a-c) on splenocytes isolated from tumor-bearing Ron SF-/- mice and WT mice. These data revealed that Ron SF-/- mice produced significantly fewer CD4+ Tregs, F4/80+ macrophages and CD11b+ Ly6G+ myeloid cells but produced more CD4+ and CD8+ T cells than WT mice (Figure 2A-E). These data are consistent with the fact that Tregs, macrophages and CD11b+ Ly6G+ myeloid cells can be immunosuppressive, facilitating tumor progression and metastasis (47-49) and that higher frequencies of Tregs also correlate with poor prognosis of cancer patients (49,50). One of the mechanisms by which T cells exhibit anti-tumor immunity is by secreting cytokines like IFNγ and TNFα (51-54). We found that Ron SF-/- mice also have significantly more IFNγ-producing CD4+ and CD8+ T cells (Figure 2F-G). We found no significant differences in B220+ B cells, CD11c+ dendritic cells, or NK1.1+ natural killer cells between WT and Ron SF-/- mice (Fig S3a-c).

Infiltration of immune cells into tumors is critical for tumor control, and is prognostic for patient outcomes in multiple cancer types (9,55). To determine the tumor-infiltrating lymphocyte (TIL) landscape in the metastases in our model, we
performed immunohistochemical (IHC) analysis on the lungs of tumor-bearing mice at the 2-week time point, when the micro-metastases in Ron SF-/- mice were detected similar to those in WT mice. We found that TIL infiltration into metastases was much more robust in Ron SF-/- mice compared to WT mice (Figure 2H-J). The metastases in Ron SF-/- mice were swarmed by CD3+ TILs (both CD4+ and CD8+ T cells; Figure 2K).

To investigate the generality of the SF-Ron-regulated anti-tumor immune response, we utilized a second breast tumor model of a different subtype: the KPB1 (K14-Cre; p53<sup>f/f</sup>; Brca1<sup>f/f</sup>) basal-like mammary tumor model, also in the FVB/NJ background (56). We injected freshly isolated KPB1 tumor cells into tail veins of WT and Ron SF-/- mice, and the lung metastatic tumor burden was examined 24 days later. Again, lack of host SF-Ron significantly reduced the lung metastatic tumor burden when compared to WT mice (Figure S4a), although the lung metastases were not as efficiently cleared as in the MMTV-PyMT model. Immunofluorescence using lymphocyte markers (CD3e, CD4, and CD8a) and pan-cytokeratin as a tumor marker revealed a phenotype similar to what we found in the MMTV-PyMT metastasis model: significantly increased TIL infiltration in the tumor-bearing lungs of Ron SF-/- mice compared to WT mice (Figure S4b-e). Thus, loss of SF-Ron reproducibly promotes immune cell infiltration into micro-metastases. We next set out to determine specific differences in the metastatic tumor immune microenvironment of Ron SF-/- mice compared to WT mice, and whether the infiltrating immune cells were tumor-specific.

**Selective loss of SF-Ron results in expansion of stem cell-like T cell populations in metastatic lungs.**

Tumors attract a variety of immune cells, giving rise to a complicated and heterogenous tumor-immune microenvironment (57). To investigate the heterogeneity of the immune cell infiltrates resulting from the loss of SF-Ron, we performed single-cell RNA sequencing (scRNAseq) on flow-sorted CD45+ immune cells from tumor-bearing lungs of WT and Ron SF-/- mice (Figure 3A). 8-10 mice per group were pooled together as a single sample and around 16,000 sorted cells from each sample were loaded, to target 10,000 cells in the sequencing reaction. Expression data was recovered from 9,581 and 10,492 cells from WT and Ron SF-/- samples, respectively. The median number of genes detected in WT and Ron SF-/- samples were 1,511 and 1,515, respectively, which corresponds to 22,399 and 18,684 mean reads per cell, respectively. Unbiased cell-type classification can be achieved with as few as 10,000 reads per cell.
Unbiased clustering after merging both datasets revealed the presence of 16 cell type clusters in both WT and Ron SF-/- mice, numbered 0 to 15 (Figure S5a). The two major cell types present in the metastatic tumor microenvironment in both groups of mice were T cells expressing Cd3e and macrophages expressing Cd68, followed by neutrophils expressing S100a8, natural killer cells expressing Ncr1, and B cells expressing Cd19 (Figure 3B and S5b). To further classify the cell clusters in detail, and examine differences between genotypes, we utilized the web-based Cluster Identity Predictor (CIPR) tool, which compares the genome-wide expression signatures with those of known immune cell types from the publicly available Immunological Genome Project database (60,61). This revealed that T cells and macrophages each can be separated into four distinct populations, with other cell types spreading out to a lesser extent (Figure 3C). Using the expression of Cd4, Cd8a, and Sell, which encodes CD62L, the four clusters of T cells were further defined as conventional CD4+ and CD8+ T cells and their activated effector populations (Figure 3D, top plots). Likewise, the macrophage populations were divided into alveolar and interstitial macrophages based on the expression of Itgax, Siglecf, and Cx3cr1 (62). In our data, we identified two interstitial macrophage clusters and one alveolar macrophage cluster (Figure 3D bottom plots). A full list of genes differentially expressed in each individual cluster compared to the rest of the dataset is provided in Supplemental Table S2. Comparing immune cell clusters in metastatic lungs of WT and Ron SF-/- mice revealed significant differences in the immune microenvironment: Ron SF-/- lungs contained a large number of alveolar macrophages and T cells compared to WT lungs (Figure 3E and S5c), providing additional evidence that loss of SF-Ron modifies the immune microenvironment during metastatic outgrowth. These data, along with the swarming of early metastatic lesions by TILs (Figure 2K), suggested that T cells might be more effective at eliminating metastases in mice lacking SF-Ron. Therefore, we next investigated the nature of the T cells in Ron SF-/- metastatic lungs.

Analysis of the T cells in both mouse genotypes revealed the presence of 13 distinct T cell clusters (Figure 4A). Interestingly, the large proportion of CD4 T cells enriched in the Ron SF-/- lungs expressed Tcf7 (which encodes TCF1), Ccr7, and Lef1 (Figure 4B-C), which together represents a less-differentiated or stem cell-like T-cell signature (63). There were higher frequencies of T cells with the less-differentiated phenotype (CD8+ or CD4+ naïve and stem cell-like), and lower frequencies of T cells with a terminally differentiated phenotype (Tregs and exhausted CD8 T cells) in Ron SF-
lungs compared to WT lungs (Figure S5d). To validate the presence of these less-differentiated T cells in Ron SF-/- lungs, we isolated cells from the lungs of tumor-bearing WT and Ron SF-/- mice and performed flow cytometry analysis (Figure S6a). We found a significant enrichment of CD62L+CD44- CD4+ T cells in the lungs of Ron SF-/- mice (Figure 4D), while CD62L-CD44+ T cells were more abundant in WT mice, either CD44+ or CD44-, and indicating actively differentiated states (Figure S6b-c). CD62L+CD44+ resting central memory cells were rare and were not significantly different between the two groups (Figure S6d). To validate presence of the stem-like CD4+ T cells in the tumor microenvironment, we conducted 5-plex immunofluorescence staining and examined the expression of TCF1 in CD4+ or CD8+ T cells that had infiltrated into the metastatic tumor nodules at a 2-week time point when the tumor burden is comparable between WT and Ron SF-/- mice (Figure S7a-e). Though the proportion of both TCF1-expressing CD4 or CD8 TILs was higher in Ron SF-/- lungs compared to the WT lungs, TCF1+ CD4+ T cells were the predominant population (Figure 4E-G), consistent with the scRNAseq data (Figure S5d). Maintaining a T cell pool with a less differentiated phenotype in non-lymphoid tissues, such as the microenvironment that harbors a tumor, provides the ability to replenish the effector T cells that may become exhausted or apoptotic (64,65). A recently reported population of TCF1+ CD8+ stem-like T cells identified in human cancer was correlated with better treatment outcomes (66). Hence, we hypothesized that the presence of TCF1+ CD4+ T cells in Ron SF-/- lungs might be important in driving and/or maintaining the robust anti-tumor immune responses in Ron SF-/- mice during metastatic outgrowth.

**Recruitment of CD4+ T cells from lymph nodes is required for clearance of metastatic lesions in Ron SF-/- mice.**

To determine the importance of CD4+ T cell enrichment in Ron SF-/- mice during metastatic outgrowth, we depleted CD4+ T cells in vivo using an anti-CD4 antibody, and then injected tumor cells via tail vein. Compared to the isotype control, CD4+ T cell depletion significantly enhanced lung metastasis in Ron SF-/- mice but had no significant effect in WT mice (Figure 5A, Figure S8a). Given the high number of TCF1 expressing CD4+ TILs in Ron SF-/- lungs and their high expression of Ccr7 and Sell (Figure 4B-C), which are known markers for naïve or resting memory T-cells that home to lymph nodes (67), we hypothesized that cell trafficking between draining lymph nodes and lung was crucial for recruitment of those T cells into the tumor microenvironment and for
suppression of metastatic growth. To determine if circulating, rather than resident, T cells were important for controlling metastatic outgrowth, we treated WT and Ron SF-/- mice with the sphingosine-1 phosphate receptor inhibitor FTY-720, which prevents egress of T cells from the lymph node (68), beginning the day after tumor cell seeding. Inhibition of trafficking was verified by the significant decrease of T cells in the peripheral blood and spleens and retention of these cells in lymph nodes of tumor-bearing mice at the endpoint (Figure S8b-c). Sequestering T cells in the lymph node not only completely restored metastasis in Ron SF-/- mice but also resulted in increased metastasis burden in WT mice (Figure 5B). IHC staining of T cells verified loss of tumor-infiltrating T cells (Figure S8d). These data demonstrate that recruitment of T cells from lymph nodes to the site of metastasis, and the presence of CD4+ T cells, is crucial to control metastasis in the Ron SF-/- mice.

**CD4+ T cells are skewed toward a Th1 phenotype in Ron SF-/- mice during metastasis elimination.**

Upon antigen encounter, naïve CD4+ T cells, which express TCF1, differentiate into at least four subsets to execute different immune functions: type 1, type 2, and type 17 helper T cells (also known as Th1, Th2, and Th17, respectively), as well as Tregs. This differentiation is determined by the signals received during antigen encounter (69-71). Expression of TCF1 on CD4+ T cells is also required for the generation of follicular helper T cells (Tfh) in response to viral infection and the development of immunological memory (72). How each of these CD4+ T cell subsets contributes to anti-tumor immunity is still controversial. To determine the differentiation status of the CD4+ T cells present systemically in WT and Ron SF-/- mice, we isolated CD4+ T cells from the spleens of mice during metastatic outgrowth and performed flow cytometry to identify CD4+ T cell subsets based on the expression of their master transcription factor proteins: T-BET for Th1; GATA3 for Th2; RORγT for Th17; and FOXP3 for Tregs (Figure 5C, S9a). We found that Ron SF-/- mice had significantly more Th1 and fewer Treg cells than WT mice; there was no significant difference in Th2 or Th17 cells (Figure 5D). These data suggest that the increased anti-tumor immunity resulting from loss of SF-Ron may be associated with an increased systemic Th1 CD4+ T-cell response.

In influenza virus-infected animals and in autoimmune encephalomyelitis, TCF1+ CD4+ T cells are capable of differentiating into either Th1 cells in the presence of IL-2 and IL-12, or Th17 cells in a manner dependent on mTORC1 activity (73,74). Because
we observed robust enrichment of Tcf7/TCF1 in the naïve and stem cell-like CD4+ T cells in the lung of Ron SF-/- animals, which correlated with the elimination of metastasis, and because CD4+ T cells were required to suppress metastasis, we tested the differentiation potential of CD4+ T cells isolated from spleens and tumor-bearing lungs of WT and Ron SF-/- animals. Sorted CD4+ T cells from each organ were labeled with cell proliferation tracking dye before stimulation with plate-bound anti-CD3ε and anti-CD28 antibodies together with cytokines that are known to induce the four main T helper subsets (75). We monitored both proliferation and differentiation of CD4+ T cells under each condition using the expression of T-BET, GATA3, IL17A, and FOXP3 as markers of Th1, Th2, Th17, and Treg differentiation, respectively. Cells that were positive for each marker were determined with no-antibody-control stains (FMI, Figures S9b-c). Relative to the control condition with only anti-CD3ε/CD28, CD4+ T cells isolated from the lungs of tumor-bearing WT mice proliferated dramatically and produced appropriately differentiated T cells under Th2-, Th17-, and Treg-inducing conditions, but not under Th1-inducing conditions (Figures 5E and S9d-e). Expansion was most apparent in the Th17-inducing condition (Figures 5E and S9d-e). On the other hand, CD4+ T cells from tumor-bearing Ron SF-/- lungs were poised toward Th1 differentiation at baseline (in the anti-CD3ε/CD28 control setting) and, with cytokine treatment, differentiation could be further enhanced to the Th1 state and induced to the Th17 or Treg state, but not to the Th2 state (Figures 5E and S9d-e). The strong expansion of the Th17 subtype seen in cells isolated from WT animals was also not apparent in cells isolated from Ron SF-/- mice (Figures 5E and S9d-e). Resistance to Th17 differentiation was also observed using splenic CD4+ T cells from Ron SF-/- tumor-bearing mice (Figure S9f, top panel), suggesting that reduced Th17 differentiation of Ron SF-/- T cells might not depend on direct exposure to the tumor microenvironment. To address this question further, we also examined splenic CD4+ T cell differentiation from WT or Ron SF-/- mice with no tumors. We found that splenic CD4+ T cells isolated from naïve WT and Ron SF-/- mice were all able to differentiate appropriately; however, Th17 differentiation of CD4+ T cells from Ron SF-/- spleens was again reduced compared to WT (Figure S9f, bottom panel). These data suggest that loss of SF-Ron reduces Th17 differentiation of naïve CD4+ T cells, and this phenotype is enhanced in the presence of tumors, where Th1 differentiation is strongly favored in Ron SF-/- mice.

Taken together, these data show that Ron SF-/- animals effectively recruit T cells from lymph nodes into the lung metastatic microenvironment, that these T cells are
predominantly a TCF1+ CD4+ population with increased potential for Th1 differentiation, and that CD4+ T cells are required to eliminate metastatic outgrowth.

**Ron SF-/- mice promote CD8+ T cell-dependent, tumor-specific immune responses to eliminate lung metastasis.**

A major role of Th1 CD4+ helper T cells is to activate CD8+ cytotoxic T cells, which are the central effector cells for tumor surveillance due to their capacity for direct tumor cell killing (76). We therefore investigated the requirement for CD8+ T cells for clearance of metastasis, and their tumor-specificity, in Ron SF-/- animals. To examine the tumor-specific CD8+ T cell responses, we evaluated the response of splenocytes from tumor-bearing mice to a pool of three MHC-I (H-2q) PyMT-specific peptides (77) using IFNγ ELISPOT assays. These peptides allow us to examine the PyMT tumor antigen-specific response without the need of exogenously expressed model antigen such as ovalbumin (78). Our results demonstrated that splenocytes isolated from tumor-bearing Ron SF-/- mice have significantly more tumor-specific, IFNγ-producing cells compared with those from WT mice (Figure 6A-B). To further assess PyMT tumor-specific CD8+ T cells, we stained splenocytes with PyMT peptide-bound MHC-I tetramers (77) by flow cytometry. The results revealed that PyMT-specific T cells were rare among the entire CD8+ T cell population in the spleen of WT mice, but the frequency was increased in the spleens of Ron SF-/- mice (Figures 6C and S10a-b). Interestingly, approximately 35% of the PyMT-specific (tetramer+) splenic CD8+ T cells from Ron SF-/- mice were CD62L low and CD44 high, which define effector memory T cells known to be associated with anti-tumor response and responses to chronic infections (Figures 6D and S10c).

We next asked whether CD8+ T cells were necessary for eliminating metastasis in Ron SF-/- mice. We depleted CD8+ T cells from WT and Ron SF-/- mice then performed experimental metastasis assays. Flow cytometry confirmed complete depletion of CD8+ T cells in the spleen (Figure S10d). We analyzed the metastatic tumor burden at the endpoint and found that CD8+ T cell depletion rescued metastasis in Ron SF-/- mice, while not significantly altering metastasis in WT mice (Figure 6E-F).

T cell memory is required to induce long-term protective immunity and rapid response upon re-encounter with tumor antigens (79). To understand whether the lack of SF-Ron promotes T cell memory development after the robust anti-tumor immune
response seen in these mice, we performed a tumor re-challenge experiment. In this experiment, the lung metastases were first induced by tail vein injection of MMTV-PyMT tumor cells, followed by implantation 14 days later of the same batch of tumor cells into the inguinal mammary fat pads. Mice were harvested 4 weeks later, and the mammary tumor burden was assessed. While all rechallenged WT mice had visible mammary tumor growth at the site of injection, rechallenged Ron SF-//- mice were almost completely protected. No palpable tumors were present or found by histology in Ron SF-//- mice, but IHC staining with PyMT antibody identified one small cluster of tumor cells remaining in one animal (Figures 6G, H and S10e). IFNγ ELISpot of splenocytes also showed a significant increase in the number of PyMT tumor-specific T cells in rechallenged Ron SF-//- mice compared with the rechallenged WT mice (Figure 6I). Taken together, these data demonstrate that Ron SF-//- mice are better than WT mice at generating robust anti-tumor CD8+ immune responses, with effective immunological memory that protect them from tumor outgrowth.

**Ron SF-//- T cells are sufficient to protect from metastatic outgrowth when transferred to WT mice.**

Although FL-Ron expression in the immune system is restricted to resident macrophages (Figure S11a-b), expression of SF-Ron has not been characterized. There are no antibodies that are specific for SF-Ron, so we used a SF-Ron specific RT-PCR primer pair that takes advantage of the unique 5'UTR of SF-Ron, derived from intron 10 (36), to examine SF-Ron expression in T cells. As expected, we found FL-Ron mRNA expressed in macrophages but not in T cells; however, SF-Ron mRNA was detected in both CD4+ and CD8+ T cells (Figure S11c). This raised the possibility that SF-Ron in T cells could be directly responsible for promoting increased anti-tumor activity. To test this idea, we examined whether T cells isolated from naïve Ron SF-//- mice could prevent metastasis when adoptively transferred to WT mice. We magnetically-sorted T cells from either Ron SF-//- or WT mice and confirmed that the purity of magnetic sorted donor T cells from both genotypes was over 97% (Figure S12a). These cells were adoptively transferred into WT hosts, and tumor cells were injected 2 days later. We isolated lungs 3 weeks after tumor injection, and assessed metastasis burden and immunophenotype. As shown in Figure 7A, WT mice that received T cells from naïve Ron SF-//- donor mice, and thus had both WT and Ron SF-//-
T cells, had a significantly lower metastatic tumor burden compared to mice that received T cells from naïve WT donor mice. Moreover, we found more effector memory (CD62L-CD44+) and IFN-γ-producing CD4+ T cells in spleens of tumor-bearing mice that received T cells from Ron SF-/- donors versus from WT donors (Figure 7B-C and Figure S12b-d). No significant differences were observed in other CD4 subsets (Figure S12d). These data provide the first indication that SF-Ron functions in T cells to facilitate anti-tumor immune responses, and can do so in the context of a WT host.

**Pharmacologic inhibition of Ron activity boosts tumor specific CD8+ T cell activity and reduces metastatic outgrowth.**

The activation status and number of T cells found in the tumor microenvironment have been shown to dictate whether immunotherapy is effective (14). Since loss of SF-Ron significantly promoted robust T cell infiltration and enhanced anti-tumor activity of T cells, targeting SF-Ron alone or in combination with immune checkpoint blockade to augment immunotherapy is an attractive approach. We previously found that a small molecule kinase inhibitor of all Ron isoforms cooperates with anti-CTLA4, but not anti-PD1, immunotherapy to abrogate breast cancer progression (28). The data presented here, which for the first time specifically implicate SF-Ron in the regulation of the anti-tumor T cell response, provide a mechanistic explanation for how a Ron inhibitor might boost tumor immunity. We therefore tested whether specific anti-tumor CD8+ T cell responses and prevention of metastatic outgrowth could be achieved with a Ron kinase inhibitor. We established lung metastasis in WT mice via tail vein injection to mimic the clinical setting of already-seeded breast cancer metastases then, one week later, initiated treatment with either the Ron inhibitor (Ronı) BMS-777607 (80) or vehicle control. After 4 weeks of treatment, mice were euthanized, and lung metastasis was quantified. Treatment of mice with BMS-777607 significantly reduced metastatic burden, to about half of that in control mice (Figure 7D), and generated approximately 10-fold more IFNγ-producing T cells in response to stimulation with PyMT tumor-specific peptides (Figure 7E). Tetramer staining with PyMT-specific MHC-I tetramers (77) also showed significantly more tumor-specific CD8+ T cells in mice treated with BMS-777607 (Figure 7F). Together, these data show that Ron inhibitor treatment can elicit tumor-specific CD8+ T cell responses, and results in reduced progression of already-seeded metastatic lesions.
Discussion

In this study, we provide the first evidence that deletion of host SF-Ron alone is sufficient to reverse the metastatic tumor microenvironment from a pro-tumorigenic, immune-suppressive state to an immune-activated state in mammary tumor models that are classically recalcitrant to immunotherapy. Specifically, lack of SF-Ron redirected CD4+ T cell differentiation and enhanced anti-tumor CD8+ T cell activity. Enhanced immune activity in mice lacking SF-Ron culminated in robust infiltration of TILs into metastatic lesions, followed by clearance of lung metastases. Importantly, pharmaceutical inhibition of Ron kinase activity also manifested in enhanced tumor-specific immune responses and resulted in significantly less metastatic outgrowth. Our results provide rationale for using Ron kinase inhibitors, at least two of which have completed Phase I clinical trials, as a new form of immunotherapy to treat metastatic breast cancer.

We showed that loss of SF-Ron protects from metastatic outgrowth in two different mammary tumor types, representing the FVB genetic background. Our previous study showed that the combination of a Ron kinase inhibitor and anti-CTLA4 can shrink breast tumors, block metastatic outgrowth of breast cancer, and slow the growth of colorectal tumors in a host Ron-dependent manner (28), in two different models and two different mouse genetic backgrounds. Taken together, these data suggest that our findings can be extended to multiple cancer models and may have relevance for human cancer. Indeed, we showed in a Phase I clinical trial in cancer patients that a Ron kinase inhibitor blocked downstream effects of Ron in bone turnover (81). Although the latter study does not address the role of SF-Ron in immune responses to metastasis, it demonstrates that future studies to address this issue are feasible and can have impact.

One particularly interesting finding was accumulation of less-differentiated naïve and stem cell-like CD4+ T cell precursors that express Sell and Ccr7, but not traditional activation markers such as Pdcd1, in the lungs of Ron SF-/- mice with metastases. One possibility is that as metastases are eliminated in Ron SF-/- mice, the activated tumor-specific T cells may return to a resting phase; these T cells may be destined to become resident memory T cells, return to the lymph node, or patrol the periphery to prevent future relapse. It was recently reported that resident memory T cells (TRM) and stem cell-like memory T cells (TSCM) have superior anti-tumor immune responses compared to classic effector T cells, which are usually short-lived because of their terminal differentiated status (79). TRMs arise from a subset of antigen-experienced T cells and can be reactivated if the tumor relapses. Our data showing that Ron SF-/- mice are
almost completely protected from tumor rechallenge supports the notion of a robust memory response when SF-Ron function is absent.

A similar T cell population with self-renewal capacity and stem cell-like features, defined by the expression of Tcf7 (encoding TCF1), Lef1, and Ccr7 (66), was recently reported in tumors, albeit in the CD8+ subset. In kidney cancer, it was shown that this population of TCF1+ CD8+ TILs are present in regions dense with MHCII+ antigen presenting cells (APCs), referred to as the APC niche, within the tumor. T cells in this APC niche continually differentiate to effector cells that express higher levels of immune checkpoint molecules and eventually become exhausted, which can be reversed by immune checkpoint inhibitor treatment (66). Likewise, patients with more APC niches in their tumors had better responses to immunotherapy (66,82). Although the less-differentiated T cells identified in our study are CD4+ T cells that have not been previously described in tumors, we speculate that the TCF1+ CD4+ T cells present in the Ron SF-/- mice may have similar functions. In an influenza virus infection model, antigen-experienced TCF1+ CD4+ T cells were able to divide asymmetrically to give rise to a TCF1-negative Th1 effector cell population and maintain a TCF1+ CD4+ T cell population for self-renewal (74). Moreover, in an experimental autoimmune encephalomyelitis (EAE) mouse model, a similar subset of TCF1+ cells are able to differentiate into Th17 cells that produce IL-17 when the mTORC1 pathway is blocked (73). Our data show that CD4+ T cells are required to suppress metastasis in Ron SF-/- mice. Ron SF-/- mice have more Th1 cells and fewer Tregs compared to WT mice, which were skewed toward the Th2 subset. Importantly, although CD4+ T cells from metastatic lungs of both WT and Ron SF-/- mice were able to differentiate to Th17 cells in vitro, only CD4+ T cells from Ron SF-/- mice were capable of differentiation to Th1 cells that can enhance CD8+ cytotoxic T-cell activity.

Interestingly, a preclinical study in a melanoma model indicated that a subset of long-lived, less-differentiated Th17 cells that expressed TCF1, LEF1, and CCR7 are endowed with stem-like self-renewal potential and are able to repolarize to a Th1-like state and facilitate anti-tumor immune responses to eradicate tumors (83). The ability of less-differentiated Th17 cells to transdifferentiate to IFNγ-producing Th1-like cells was also shown in an EAE mouse model (73). Thus, the accumulation of naïve, stem cell-like CD4+ T cells in the lungs of Ron SF-/- mice with metastases, along with their ability to differentiate into Th17 cells but with a strong skew toward the Th1 phenotype, may contribute to elimination of metastasis. Our results raise the intriguing possibility that
SF-Ron might act specifically to block the trans-differentiation of IL-17 producing Th17 cells to anti-tumorigenic IFNγ-producing T-BET+ Th1-like effector cells that eradicate metastatic tumors. Indeed, CD4+ T cells from Ron SF−/− lungs were able to differentiate to a Th17 state but failed to accumulate as seen in the WT setting. These results warrant future studies to address the role of SF-Ron in mediating the differentiation and trans-differentiation potential of CD4+ T cells.

Our previous study investigating the combination of Ron kinase inhibition with immunotherapy (28) revealed that the Ron kinase inhibitor combined with anti-PD-1 immunotherapy provided no benefit, whereas the combination of Ron kinase inhibitor with anti-CTLA-4 was beneficial in two different models and two different genetic backgrounds. However, it has been unclear whether, or how, SF-Ron contributes to this synergistic effect. The immune activation mechanisms of anti-CTLA4 and anti-PD-1 are distinct (84). CTLA4 blockade not only is important for early priming of T cells in the lymph node, but also affects differentiation of CD4+ T cells. It has been shown that anti-CTLA-4 monotherapy increased the frequency of CD4+ T cells and promoted them to differentiate to Th1-like ICOS1+ CD4+ T cells, while anti-PD-1 monotherapy only expanded the PD-1+ but not PD-1- CD8+ T cells. This indicates PD-1 blockade acts at later stages of T cell activation, mainly in peripheral tissues, and has no effect on T cell differentiation (85-87). The combination of both CTLA-4 and PD-1 blockade on treating murine colon cancer increased Th1-like effectors and expanded both PD-1+ and PD-1 CD8+ T cell effectors contributing to tumor elimination (88). Our new data reinforces the notion that inhibition of Ron, specifically SF-Ron, enriches naïve, stem-like CD4+ T cells that are then primed and kept from exhaustion when combined with anti-CTLA4. Adoptive transfer of Ron SF−/− T cells into WT hosts was sufficient to significantly reduce metastasis burden, further suggesting that T cells are the target of SF-Ron-associated immunosuppression. Interestingly, we found that SF-Ron, but not FL-Ron, is expressed in T cells, hinting that SF-Ron may dampen T cell function by preventing differentiation of CD4+ T cells and therefore failing to recruit and revitalize CD8+ effectors. Inhibition of SF-Ron may maintain stem-like T cell pools in the metastatic microenvironment with the potential to differentiate to tumoricidal effectors. However, pharmaceutical inhibition of Ron alone under the conditions we used did not provide complete protection from metastasis (Figure 7) (28), while mice genetically lacking SF-Ron nearly eliminated lung metastasis. Future work might entail how to achieve optimal inhibition of SF-Ron with the various Ron kinase inhibitors that are available.
Regulation of the expression of FL- and SF-Ron isoforms is not well studied. One study found that differences in the methylation of CpG islands in the main promoter region are responsible for the switch in the expression of FL- and SF-Ron isoforms (38). Importantly, many studies have identified non-redundant functions of FL- and SF-Ron in oncogenic processes and in regulating host immune responses to infection. In cancer cells, SF-Ron, but not FL-Ron, promotes EMT, invasive ability, anchorage-independent growth in vitro, tumor growth, and metastasis of human breast cancer cell lines (40,41). Expression of SF-Ron activated the PI3K pathway and suppressed the MAPK pathway whereas exogenous expression of FL-Ron activated the MAPK pathway in breast cancer cells (41,89). Constitutively active SF-Ron, but not FL-Ron activated by MSP, was shown to induce resistance to MET inhibitor treatment in gastric cancer (90). Moreover, mice that specifically lack SF-Ron expression due to naturally occurring genetic polymorphisms are resistant to Friend virus-induced erythroleukemia (36) due to SF-Ron's ability to interact with viral protein gp55 in erythroid cells to induce an erythropoietin-independent signaling cascade (91). Moreover, mice genetically engineered to express only FL-Ron and lack SF-Ron, the same mouse strain used in our study, are more susceptible to concanavalin A-induced acute liver injury marked by enhanced IFNγ production (42). Thus, there is accumulating evidence in a variety of disease settings that FL-Ron and SF-Ron are functionally non-redundant and differentially orchestrate host immune responses. Although the role of host Ron in promoting breast cancer metastasis has been studied previously (27,28), those experiments were carried out in mice that either express or lack both isoforms of Ron. Hence, the specific contribution of each of these isoforms for breast cancer progression was unclear and required further investigation. Our results reveal that SF-Ron is the major isoform regulating immune responses during breast cancer metastatic outgrowth: metastases are nearly eliminated from mice that lack SF-Ron, even though they still express functional FL-Ron.

The dual role of Ron and SF-Ron signaling in both tumors and host reinforces the potential of Ron kinase inhibitors for the treatment of metastatic breast cancer. Although monoclonal antibodies (mAbs) can have fewer off-target effects, our data suggests that mAbs that target FL-Ron may not be effective for immunotherapy. Indeed, poor efficacy of anti-Ron mAbs like narnatumab and others in clinical and preclinical studies (29,33,92) may be due to their inability to block SF-Ron. Our data warrants clinical investigations using small-molecule Ron kinase inhibitors that inhibit SF-Ron.
signaling, perhaps in combination with immunotherapy, for the prevention and treatment of metastatic breast cancer.

**Methods**

**Mice and tumor cells**

All animal procedures were reviewed and approved by the University of Utah Institutional Animal Care and Use Committee. Ron TK-/- and Ron SF-/- mice were described previously (24,42) and were backcrossed to the FVB genetic background. 6–8 week old female WT, Ron TK-/- and Ron SF-/- mice were used in all experiments unless otherwise specified. Spontaneous metastasis experiments were carried out in 3-5 week old female WT, Ron TK-/- and Ron SF-/- mice. Tumor cells were derived from spontaneous mammary tumors of transgenic MMTV-PyMT mice on the FVB background (44). Tumor cells were cultured short term (not as cell lines) in DMEM:F12 medium (Gibco, Invitrogen) supplemented with fetal bovine serum (FBS, 10%) (Gibco, Invitrogen), insulin-transferrin-selenium-ethanolamine (1x) (Gibco, Invitrogen), recombinant murine EGF (10 ng/ml) (Invitrogen), hydrocortisone (1 μg/ml) (Sigma), penicillin-streptomycin-gentamycin (Gibco, Invitrogen) (1x) for a maximum of 2 days before injection into mice. 500,000 MMTV-PyMT-cells in 200ul HBSS were injected via the lateral tail vein of mice for all experimental metastasis experiments. Lung metastasis were allowed to develop for three to four weeks unless otherwise specified. 100,000 MMTV-PyMT-cells in 20ul HBSS were injected orthotopically into the cleared mammary fat pads of mice for spontaneous metastasis experiments. For tumor re-challenge experiments, lung metastasis was firstly induced via tail vein injection of tumor cells as described above, followed by orthotopic implantation of the same tumor line as described above after 2 weeks. Mice were harvested 2 weeks after mammary tumor implantation for subsequent examination and analysis.

The basal-like KBP1 tumor line was kindly provided by Dr. Charles M. Perou of Lineberger Comprehensive Cancer Center at the University of North Carolina (56). In this model, freshly harvested cells from orthotopically grown mammary tumors were utilized for experimental metastasis assay. Briefly, tumors were digested with collagenase for 45 minutes at 37°C and passed through the 40μm cell strainer to obtain a single-cell suspension. Experimental metastasis assay was then carried out by intravenously injecting 150,000 KPBP1 tumor cells resuspended in 200μl of 2% FBS containing HBSS via the lateral tail vein.
Macrophage isolation, RT-PCR, and in vitro MSP stimulation

Peritoneal lavage fluid was collected by injecting 8ml of ice-cold DMEM+GlutaMAX (Gibco) medium supplemented with 10% heat-inactivated FBS into the peritoneal cavity of the WT, Ron TK/- and Ron SF/- mice. The collected fluid was centrifuged for 5 min at 1800 rpm, and the cell pellet was used for in vitro MSP stimulation, RT-PCR, and flow cytometry analysis. For FL-and SF-Ron mRNA expression analysis, total RNA was isolated using RNeasy Plus Mini Kit (Qiagen), 1 µg of RNA was used to generate cDNA using SuperScript™ IV VILO™ Master Mix kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. PCR primers and conditions for SF-Ron expression analysis was described previously by Persons et al. (36). PCR primers for for FL-Ron (which amplify the cDNA region between exons 9 and 13) are 5’–GATGGACAAAGTACAGTGGAGAG and 5’–GCAGCAGTGGGACACTTGTCC–3’; and SF-Ron specific PCR primers are 5’ TCTGGCTGATCCTTCTGTCTG–3’ and 5’–GCAGCAGTGGGACACTTGTCC–3’. The PCR products were resolved using 2% agarose gel. For the determination of FL-Ron protein expression, flow cytometry was performed as described in the Flow cytometry section. For FL-Ron function analysis, 500,000 peritoneal lavage cells per well were cultured for 18 hours in 12 well-plates in 1ml of MSP- (100ng/ml, recombinant human MSP, R&D Systems) or vehicle-containing DMEM medium supplemented with 10% FBS and penicillin/streptomycin (Gibco). At the endpoint, the wells were washed three times with 1X phosphate buffered saline (PBS, pH 7.4) and incubated for 30 min in 5mM EDTA in PBS (pH 7.4) at 37°C to dislodge and collect the cells. Flow cytometry staining was then performed as described in the flow cytometry section.

Tissue processing

For quantification of metastatic lung tumor burden, mice were euthanized at their respective endpoints, and lungs were harvested and fixed for 48 hours in formalin-free zinc fixative (BD Pharmingen). Lung images were then captured and imported to Image J for quantification. The percentage of lung area occupied by the tumor was quantified Image J software. For processing lungs for flow cytometry and single-cell RNA sequencing, lungs were perfused with 5ml of 1X PBS (pH 7.4). Lung lobes were then dissected, transferred to 5 ml of Accumax cell/tissue dissociation solution (STEMCELL technologies), and physically dissociated between frosted microscope slides. The
suspension was then incubated at room temperature for 30 min with continuous agitation for enzymatic dissociation. After enzymatic dissociation, contaminating red blood cells were lysed using ammonium-chloride-potassium (ACK) lysis buffer and filtered through 100 μm nylon mesh filter to obtain a single-cell suspension. For obtaining splenocytes for flow cytometry and in vitro differentiation assays, spleens harvested from mice were physically dissociated by pressing between frosted microscope slides. Contaminating red blood cells were then lysed using ACK buffer. The splenocytes were then filtered through a 100 μm nylon mesh filter to obtain a single-cell suspension. The single-cell suspension of lungs and spleens thus obtained were used for down-stream processing accordingly (see below).

**Flow cytometry**

Single-cell suspensions, as described above, were used to analyze several surface or intracellular markers, such as transcription factors (TFs). For surface marker staining, cells were stained with fluorophore-conjugated antibodies on ice, in PBS supplemented with 2% FBS. For intracellular marker staining, cells were re-stimulated with PMA (50 ng/ml) and ionomycin (0.5 μg/ml) for 5 hours and stained with BD Cytofix/Cytoperm Kit plus kit according to manufacturer’s instructions. For TF staining, cells were stained with eBioscience™ FoxP3/Transcription Factor staining buffer set according to manufacturer's protocol. Prior to surface antibody staining in all surface/intracellular/TF staining protocols, cells were first stained with fixable viability dye in PBS to exclude dead cells and then incubated with anti-mouse CD16/32 Fc-blocking antibodies (Clone 93, Biolegend) to reduce non-specific binding. For the list of the antibodies used, please refer to Supplementary Table 1. The stained samples were acquired using the BD LSRFortessa flow cytometer, and the analysis of different immune populations was carried out using FlowJo software. Briefly, for analysis, forward scatter vs. side scatter was first used to exclude cellular debris and doublets. The single cells were then gated to exclude dead cells followed by gating on lineage-specific subpopulations. Frequencies of immune cell populations were compared using the student’s T-test or one-way ANOVA, where applicable.

**Chromogenic immunohistochemistry (IHC)**

The fixed lung tissues, as described above, were embedded in paraffin blocks. Four-micron sections were obtained by manual rotary microtome (Leica Biosystems) and
baked at 62°C for one hour. Deparaffinization and rehydration were carried out with CitriSolve solution and serial dilutions of ethanol followed by heat-inactivated epitope retrieval (HIER) with 10mM Sodium Citrate, pH 6.0. Tissue sections were incubated in 3% hydrogen peroxide diluted in methanol for ten minutes to block endogenous peroxidase. Non-specific blocking was performed by incubating the sections with PBS containing 5% bovine serum albumin (BSA), 10% normal goat serum, together with mouse FcR blocking reagent (Miltenyi Biotec, GmbH). For antibodies raised from rat, the Mouse-On-Mouse blocking reagent (Vector Laboratories) was added into the blocking solution with the additional steps of incubating the sections with avidin/biotin blocking reagents (Vector Laboratories) to block endogenous avidin and biotin activity. After blocking, the sections were incubated with primary antibody overnight at 4°C, followed by corresponding secondary antibodies. EnVision+ goat anti-rabbit horseradish peroxidase (HRP) polymer reagent (Dako/Agilent) is used against primary antibodies raised in rabbit; biotinylated secondary antibodies were used for primary antibodies raised from rat, followed by the avidin-biotin HRP amplification system (Vector Laboratories). The chromogenic revelation was conducted with the 3,3'-Diaminobenzidine (DAB) system, and the nuclear counterstain was performed with Mayer’s hematoxylin (Sigma-Aldrich). Stained tissue sections were then dehydrated with ethanol, infiltrated with CitriSolve solution, and mounted with Cytoseal Mounting medium (VWR). Image acquisition was performed with either Olympus BX-50 microscope equipped with the Nikon camera, or the Zeiss Axioscan Z1 slide scanner for whole slide scan at 20X magnification. Quantification of TILs with IHC stained samples was performed on five samples from each group, and at least ten separate tumor fields-of-vision containing visible tumors were extracted from the whole section image. Computer-assisted quantification of CD3e, CD4, or CD8a signals from the extracted images was performed by setting a standard threshold for all images for the same markers by using ImageJ based FIJI software. Primary antibodies used for histology analysis in this study were all unconjugated, and includes: CD3e (D4V8L, Cell Signaling), CD4 (4SM95, eBioscience/ThermoFisher), CD8a (4SM15, eBioscience/ThermoFisher), B220 (CD45R) (RA2-6B2, Santa Cruz), pan-cytokeratin (Agilent Technologies), TCF1 (C63D9, Cell Signaling), and PyMT (PyMT, Novus Biologicals).

**Multiplexed immunofluorescence (mIF)**
After deparaffinization and rehydration of the slides, HIER was carried out with 10mM Tris-EDTA, pH9.0 containing 0.05% Tween-20 followed by endogenous peroxidase blocking and non-specific blocking as described above for IHC. Sections were then subjected to the consecutive staining procedure with primary antibodies and corresponding HRP conjugated secondary antibodies followed by signal detection with TSA-Opal detection system including OPAL480, OPAL520, OPAL570, OPAL620, and OPAL690 (Akoya Bioscience). The primary antibody conditions used were the same as described for IHC. Antibody stripping with boiling 10mM Sodium Citrate, pH6.0 was performed after signal detection of each primary antibody and before the application of the next primary antibody. Autofluorescence was removed by TrueBlack Lipofuscin Autofluorescence Quencher according to the manufacturer’s protocol (Biotium). Following this, sections were stained with DAPI for nuclear counterstain and mounted in VECTASHIELD Vibrance Anti-fade mounting medium (Vector Laboratories). The staining panel in this study includes a 6-marker panel (B220, CD3e, CD8a, pan-CK, CD4, and DAPI) and a 5-marker panel (CD8a, TCF1, CD4, CD8a, and DAPI), and the best sequence of staining with each antibody was established as addressed above. Upon completion of the staining process, the entire slides were scanned using the Vectra Polaris Multispectral Imaging system (Akoya Bioscience) and processed with either the Phenochart Whole Slide Viewer (Akoya Bioscience) or the QuPath open-source software for pseudo coloring of each marker. The QuPath was also used for quantification of TILs in the tumor regions of the whole sections after defining the tumor regions by PyMT or pan-cytokeratin signal using the function of Object Classification by pixels, followed by Cell Detection with DAPI for cell number measurement and the Single Measurement Classifier function for defining each marker by thresholding. Once the parameters were set, machine-assisted automated quantification was performed for all slides.

**Single-cell RNA sequencing and analysis**

The graphic workflow of single-cell RNA sequencing is illustrated in Figure 3A. Briefly, the lungs were harvested from mice and processed to obtain single-cell suspension as described above. Lungs from at least eight mice were pooled together per group. Single-cell suspensions were stained with anti-CD45 (clone 30-F11, BD Bioscience) conjugated with PE in 1:200 dilution for 30 minutes on ice and resuspended in PBS supplemented with 2% FBS. These samples were then incubated with DAPI (5 µg/ml) for five minutes.
before sorting with the BD FACSARia flow cytometer to obtain live CD45+ DAPI- immune cells. Sorted cells were washed 3 times with PBS and resuspended in 0.04% BSA containing PBS. Around 16,000 cells per sample were processed with droplet-based 3’ end single-cell RNA sequencing using Chromium Single Cell 3’ Gene Expression Library Prep Kit V3 according to manufacturer’s protocol (10x Genomics), by which the sorted single cells were loaded into a Chromium Chip B along with partitioning oil, the reverse transcription reagents, and a collection of gel beads that contain 3,500,000 unique 10X Barcodes. Only the droplets contain single cell and a gel bead with reverse transcription reagents will be subjected to libraries preparation. RNA libraries in paired-end format were then sequenced by the Illumina NovaSeq 6000 sequencer, and the sequence reads were pre-processed using the 10x Genomics CellRanger V3 pipeline and further analyzed with the Seurat R package V3. The output data from the CellRanger pipeline was processed to regress out mitochondrial genome representation and unique molecular identifier (UMI) counts variance followed by integrating the two datasets, WT and Ron SF-/-, into a combined dataset. The Seurat pipeline (93,94) was then applied to the combined dataset. Principle component analysis (PCA) and the t-distributed stochastic neighbor embedding (tSNE) dimensional reduction was performed using the first 30 PCA components to obtain a two-dimensional representation of the cell states. Cell clusters were identified using the Seurat’s FindCluster function, which implements the shared nearest neighbor (SNN) modularity optimization-based algorithm with a resolution of 0.4 to 0.7, leading to 16 to 24 clusters. A resolution of 0.4 was chosen for the analysis. The biological identities of cell clusters were annotated by utilizing the web-based Cluster Identity Predictor (CIPR) tool, which compares the cell cluster signatures with the publicly available Immunological Genome Project (ImmGen) database (60,61). To examine T cell subsets, clusters that are expressing Cd3e (Figure S4b) were extracted from the combined dataset, and Seurat’s pipeline with the resolution of 0.7 was re-applied on 30 PCA components for clustering. The mean expression of markers in each cluster was used for heatmap representation.

**CD4+, CD8+ T-cell depletion and drug treatment**

For CD4+ and CD8+ T cell depletion, mice were injected with 100 μg of anti-mouse CD4 (clone GK1.5, BioXCell) or anti-mouse CD8a (clone 53-6.7, Bio X Cell), or immunoglobulin G (IgG) isotype control antibodies (BioXCell). Antibodies were injected intraperitoneally, once daily for three days before tumor cell injection. 500,000 MMTV-
PyMT tumor cells were injected via the lateral tail vein of mice on the fourth day (day 0). Antibodies were then injected twice weekly until the endpoint. Mice were euthanized on day 28, and metastatic tumor burden was quantified as described earlier. For the treatment of the mice with Ron inhibitor in the adjuvant setting, 500,000 MMTV-PyMT tumor cells were first injected via the tail vein on day 0 and are allowed to establish for seven days. Starting on day 8, the mice were treated with either 50mg/kg BMS777607/ASLAN002 or 70% PEG-400 vehicle control, 5 days per week until the endpoint. The mice were euthanized on day 32 and lungs and spleens were harvested for tumor burden quantification (as described above) and tumor-specific T cell analysis (see below). For blocking lymphocyte egress, FTY-720 (Cayman Chemical) was dissolved in absolute ethanol to make a 100 mg/ml stock solution. The working solutions were then prepared in saline. Tumor cells were injected via tail vein on day 0 and, beginning day 1, 25µg of FTY720 or saline vehicle control was injected intravenously for three days, followed by oral administration of 1 mg/kg daily until the endpoint.

**in vitro CD4+ T-cell differentiation**

Lungs and spleens were harvested 4 weeks after tumor cell injections via the tail vein and processed as described above. Tissues from at least 5 mice per group were pooled together. CD4+ T cells were sorted by magnetic cell separation using EasySep Mouse CD4 Positive Selection Kit II according to manufacturer’s protocol (STEMCELL technologies). Sorted CD4+ T cells were labeled with cell tracing violet dye (Invitrogen/ThermoFisher) before adding to the subtype-specific differentiation medium. CD4+ T cell *in vitro* differentiation protocol was described previously by Sekiya and Yoshimura (75). In brief, 200,000 CD4+ T cells per well were cultured for 96 hrs in 24-well plates that were precoated with 2 µg/ml of anti-CD3e and 0.5 µg/ml of anti-CD28 in the presence of cytokines and antibodies described below, designed to facilitate differentiation of the sorted CD4+ T cells to four major subtypes. The differentiation media for specific subsets include Th1 (5 ng/ml of mouse IL-2, 10 ng/ml of mouse IL-12 and 1 µg/ml of anti-IL-4); Th2 (5 ng/ml of mouse IL-2, 10 ng/ml of mouse IL-4, and 1 µg/ml of anti-IFNy); Th17 (20 ng/ml of mouse IL-6, 1 ng/ml of mouse TGF-β1, 1 µg/ml of anti-IFNy, 1 µg/ml of anti-IL-4, and 1 µg/ml of anti-IL-2); and Treg (2 ng/ml of mouse TGF-β1, 1 µg/ml of anti-IFNy, and 1 µg/ml of anti-IL-4). At the end-point, cells were restimulated with 50 ng/ml of PMA and 0.5 µg/ml of ionomycin for 5 hours. Cells were then harvested, stained, and analyzed as described above in the Flow cytometry section.
Peptides

PyMT peptides (MPLTCLVNV, LPSLLSNPTY, YPRTPPELL) with previously established immunogenicity (77) were synthesized by Atlantic Peptides (Lewisburg, PA) with at least 95% purity and used at 10 µg/ml concentration for restimulation and for generating peptide-bound tetramers.

IFNγ ELISPOT assay

ELISpot assays were carried out on Multiscreen HTS IP 0.45µm filter plates (Millipore Sigma). Briefly, the plates were coated with anti-IFNγ capture antibody (clone AN18, BioLegend) overnight at 4°C and blocked with RPMI-1640 supplemented with 20% FBS for 2 hours at 37°C. 500,000 splenocytes were then added, and the plates were incubated in the presence of 10 µg/ml of pooled peptides or 25 ng/ml of PMA and 0.5 µg/ml of ionomycin in positive control wells, or 0.1% DMSO in negative control wells. In addition, for freeze-thawed splenocytes (Figure 7) 50 IU/ml of mouse IL-2 was added to the stimulation medium to enhance T cell survival. After incubating the plate for 44 hours at 37°C, the wells were washed to remove the cells. The wells were then incubated with biotinylated anti-IFNγ detection antibody (clone R4-6A2, BioLegend) for 2 hours at 37°C followed by streptavidin-HRP for 1 hour at room temperature. IFNγ spots were developed using 3-Amino-9-ethylcarbazole chromogen (Sigma) and images were captured by the ELISpot Reader (AID GmbH, Germany). Automatic spot counts were obtained using the AID ELISpot Reader software.

Tetramer assay

The three PyMT peptides folded into MHC-I tetramers with the haplotype H-2Dq with human β2M and conjugated to R-Phycoerythrin (PE) were kindly provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Whole splenocytes were obtained from tumor-bearing mice as described above and flow cytometry staining was performed. Splenocytes were first stained with the fixable viability dye (Ghost Dye UV450, Tonbo Biosciences) for 30 minutes on ice to exclude dead cells. Cells were then stained with the tetramer pool containing all three tetramers (1:100 dilution each) for 30 minutes at room temperature. Mouse FcR blocking was performed by incubating with anti-CD16/CD32 for 10 mins on ice followed by surface marker staining for 30 minutes on ice with a panel of antibodies including CD3ε (APC, clone 145-2C11), CD4
(violetFluor450, clone GK1.5), CD8α (FITC, clone 53-6.7), CD62L (PE-Cy7, clone MEL-14), and CD44 (redFluor710, clone IM7). Additionally, for compensation purposes in the tetramer-PE channel, anti-CD3ε conjugated with PE was used. Data acquisition and analysis were performed as described above (see Flow cytometry section).

Adoptive transfer
For adoptive transfer, T cells isolated from spleens of 6-8 week old naïve WT or Ron SF-/- donors were transferred into the age-matched WT recipients. Briefly, T cells were isolated from whole splenocytes of donor mice using the EasySep Mouse T cell Isolation kit (STEMCELL Technologies) following the manufacture’s recommendation. A total of 2 million T cells were then transferred to the recipient mice via the lateral tail vein, followed by the injection of 500,000 MMTV-PyMT tumor cells via the tail vein 48 hrs later as described previously. Mice were then harvested 3 to 4 weeks after tumor injection; the lungs and spleens were collected for subsequent analysis of metastatic tumor burden and T cell subset phenotyping.

Data availability
scRNA-Seq data from CD45+ sorted tumor-bearing lung samples are available on NCBI Gene Expression Omnibus (GEO) database under the accession number GSE155011.

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Figure legends.

Figure 1. Specific loss of host SF-Ron significantly protects mice from breast cancer metastasis: A. Quantification of mean fluorescence intensity (MFI) for FL-Ron protein on peritoneal macrophages by flow cytometry (n=3 mice/group). B. Quantification of MFI by flow cytometry for PD-L1, CD80, and CD86 upon treatment of peritoneal macrophages from each group with or without MSP for 18 hours (n=3 mice/group). C. Survival plot depicting significant delay in the primary tumors reaching 1cm in diameter in Ron TK-/- and Ron SF-/- mice. D. Representative images of the fixed lungs from the spontaneous metastasis experiment (left), and quantification of the percent lung area occupied by metastasis by ImageJ (right; n=13-14 mice/group). E. Representative images of orthotopic primary tumor (top panel) and spontaneous lung metastasis (bottom panel) tissue sections stained with H&E. F. Representative images of the fixed lungs from all three genotypes of mice (left), and quantification of percent lung area occupied by metastasis at the end point by ImageJ (right; n=16-23 mice/group). G. Histological sections showing micro-metastases across lungs of WT, Ron TK-/-, and Ron SF-/- mice. Sections were taken at 14 days after tumor cell injection. All scale bars correspond to 800µm. Log-rank (Mantel-Cox) test was used for Kaplan-Meier analysis and One-way ANOVA with Tukey’s correction was performed for statistical analysis of the MFI and lung tumor burden quantification, error bars represent SEM. (ns) p > 0.05, (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p ≤ 0.0001.

Figure 2. Lack of host SF-Ron promotes anti-tumor immune responses: A-G. Flow cytometry analysis of immune cell populations from the spleens of tumor bearing WT and Ron SF-/- mice. Bar graphs show frequency of: Ly6G+ CD11b+ myeloid cells (A), CD4+ CD25+ FOXP3+ splenic regulatory T cells (B), F4/80+ CD11b+ macrophages (C), CD4+T cells (D), CD8+ T cells (E), and IFNγ producing cells within the CD4+ and CD8+ compartment (F,G) (n=16-23 mice/group). H-J. Quantification of metastatic tumor-infiltrating T cells. K. Representative immunohistochemistry images showing lung metastatic nodules and T cell infiltration into the metastatic nodules. All scale bars...
correspond to 100μm. Unpaired t-test was performed for statistical analysis, error bars represent SEM. (ns) p > 0.05, (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p ≤ 0.0001.

Figure 3. Single cell transcriptomic analysis reveals robust immune cell infiltration in the TME of Ron SF-/− mice. A. Workflow of scRNAseq of immune cells isolated from tumor-bearing lungs of WT and Ron SF-/− mice. CD45+ cells from at least 8 mice per group were pooled together as a single sample, and equal number of cells were processed for 10X scRNAseq. B. Number of immune cells of major cell populations present in the TME that were identified by typical markers as shown in Figure S5b. C. tSNE plot of scRNAseq data showing 16 distinct cell clusters identified by CIPR tool. D. tSNE plots showing selected marker gene expression of infiltrating immune cells. E. tSNE plot depicting leukocytes in WT and Ron SF-/− mice, emphasizing the enrichment of T cells and alveolar macrophages in Ron SF-/− mice. Mac: macrophage, Mono: monocyte, Neut: neutrophil, CD8T: CD8+ T-cells, CD4T: CD4+ T cells, eff: effector, Strom: Stromal cells, B: B cells, NK: Natural killer cells, pMaclike: peritoneal macrophage-like cells, Progen: Progenitors, IntMac: Interstitial macrophages, DC: Dendritic cells, pDC: Plasmacytoid DCs, AlvMac: Alveolar macrophages.

Figure 4. Loss of SF-Ron alters the composition of tumor-infiltrating T cells A. tSNE plot showing 13 T cell clusters extracted from the combined dataset. B. Heat map showing the average expression of selected genes in each cluster that are linked to memory, self-renewal, activation, and inhibitory T cell functions. C. tSNE plots showing the Tcf7, Ccr7, and Lef1 expression in tumor-infiltrating T cells of WT and Ron SF-/− lungs. D. Flow cytometry quantification of lung CD4 T cells presenting naïve-like phenotype. E-F. Quantification of metastatic tumor-infiltrating TCF1 expressing CD4 (E) or CD8 T cells (F). G. Representative images of PyMT lung metastases from WT and Ron SF-/− mice subjected to a 5-marker multiplexed immunofluorescence stain to demonstrate the TCF1 expressing CD4 or CD8 T cells infiltration into tumor. All scale bars correspond to 20μm. Statistical analysis was performed by two-tailed unpaired student t-test, and error bars represents the SEM. (ns) p > 0.05, (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p ≤ 0.0001. nai: naïve, scm: stem cell memory, em: effector memory, CTL: cytotoxic T lymphocyte, exh: exhausted, dn: double-negative.
Figure 5. Recruitment of CD4 T-cells from lymph nodes is required for control of metastasis in Ron SF−/− mice. A. Representative images of the fixed lungs from WT and Ron SF−/− mice treated with either isotype control or anti-CD4 antibody (left), and bar graphs representing the percent lung area occupied by metastasis at the endpoint after indicated treatment (right; n=8 mice/group). B. Quantification of percentage lung area occupied by metastases in WT and Ron SF−/− mice treated with FTY720 or vehicle control at the end-point of the experiment (n=5 mice/group). C. Intracellular staining of master transcription factors denoting Th1 (Tbet), Th2 (Gata3), Th17 (RORγt), and Treg (Foxp3) on isolated CD4+ T cells from spleens of WT and Ron SF−/− mice at the endpoint. D. Box plot analysis of each Th subtype in the CD4+ T-cell pool in spleens of WT and Ron SF−/− mice. The box presents the 25th and 75th percentiles of the variables, and the horizontal bar corresponds to the 50th percentile. Individual values from each sample were plotted as points. E. Flow plots demonstrating differentiation of different Th subtypes from CD4+ T cells isolated from lungs of tumor-bearing WT and Ron SF−/− mice. Purified CD4+ T cells were labelled with CTD and incubated with Th differentiation media for 96 hours. CTD versus major markers indicating Th1 subsets (Th1: Tbet, Th2: Gata3, Th17: IL17A, and iTreg: Foxp3) are shown. Data are representative of two individual experiments in which each sample represents a pool of cells from 5-10 mice per group. Statistical analysis was performed by One-way ANOVA with Tukey’s correction and error bars represents the SEM. (ns) p > 0.05, (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p ≤ 0.0001.

Figure 6. Lack of host SF-Ron promotes tumor specific immune responses and depletion of CD8+ T-cells rescues metastasis in Ron SF−/− mice: A: Representative images of the anti-IFNY ELISPOT assay performed by stimulating splenocytes from tumor bearing mice in vitro with tumor specific MHC-I PyMT peptides. B. Quantification of anti-IFNY responses from the ELISPOT assay C. Flow cytometric quantification of frequency of tumor specific CD8+ T cells after staining splenocytes with PyMT-D9 tetramer pool. D. Frequency of tumor specific effector memory T cells within the CD8+ T-cell population quantified by flow cytometry. E. Representative images of the fixed lungs from WT and Ron SF−/− mice treated with either isotype control or anti-CD8 antibody. F. Quantification of the percent lung area occupied by metastasis at the endpoint after indicated treatment (n=7-8 mice/group). G. H&E and PyMT immunohistochemistry stain of mammary tissue with or without tumor collected from
tumor rechallenged site. Black arrow indicates a residual tumor cell cluster that is
detected in one Ron SF/- mouse. H. Numerical comparison of the presence of
mammary tumor in WT and Ron SF/- mice two weeks after tumor re-challenge. I.
Quantification of PyMT peptides-specific IFNγ spots per million splenocytes isolated
from WT or Ron SF/- mice 2 weeks after tumor rechallenge. Statistical analysis of
Figure B-D, and H were performed by two-tailed unpaired student t-test. One-way
ANOVA with Tukey’s correction was performed for statistical analysis of Figure F. Error
bars represents the SEM. (ns) p > 0.05, (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p
≤ 0.0001.

Figure 7. SF-Ron/- T cells are sufficient to protect from metastasis and
pharmaceutical inhibition of Ron suppresses metastatic progression by
promoting tumor-specific immune responses. A. Representative images of the fixed
lungs from WT mice that are adoptively transferred with T cells either from WT mice or
Ron SF/- mice (left) and quantification of the percentage lung area occupied by
metastasis at the end-point of the experiment (right; n=10 mice/group). B-C. Flow
cytometric quantification of effector memory CD4 T cells (B) and the frequency of Th1
(characterized as IFNγ producing CD4 T cells after 5 hours restimulation with PMA and
ionomycin, (C) in whole splenocytes isolated from adoptively transferred WT mice. D.
Representative images of the fixed lungs from WT mice treated with either Ron inhibitor
(Roni) or vehicle control (Veh) (left), and the bar graph shows the quantification of the
percentage lung area occupied by metastasis at the end-point of the experiment (right;
n=8 mice/group). E. Representative images of the anti-IFNγ ELISPOT assay performed
by stimulating thawed splenocytes from tumor bearing mice in vitro with tumor-specific
MHC-I PyMT peptides. Data represents mean ± SEM (n= 5 per group). F. Flow
cytometric quantification of frequency of tumor specific CD8+ T cells after staining
thawed splenocytes from Ron inhibitor- or vehicle-treated mice with PyMT-Dq tetramer
pool. Statistical analysis were performed by two-tailed unpaired student t-test, error bars
represents the SEM. (ns) p > 0.05, (*) p ≤ 0.05, (**) p ≤ 0.01, (***) p ≤ 0.001, (****) p ≤ 0.0001.
Figure 4.

A. tSNE plots showing differences in CD4 and CD8 T cells between WT and Ron SF-/- mice.

B. Heatmap showing expression levels of various genes in different immune cell populations.

C. tSNE plots comparing naive-like CD4 T cells (CD62L+CD4+) in WT and Ron SF-/- mice.

D. Bar graph showing significantly higher percentage of naive-like CD4 T cells in Ron SF-/- compared to WT mice (p < 0.01).

E. Bar graph showing significantly lower percentage of tumor-infiltrating TCF1+ CD4 T cells in Ron SF-/- compared to WT mice (p < 0.05).

F. Bar graph showing no significant difference in tumor-infiltrating TCF1+ CD8 T cells between WT and Ron SF-/- mice (ns).

G. Immunofluorescence images showing CD4 and CD8 expression in WT and Ron SF-/- mice.
Figure 6.

A. WT
Ron SF-/-

B. # IFNγ spots / 10^6 Splenocytes

C. % Tet+CD8+ splenic T cell

D. % Tet+ EM CD8+ splenic T cell

E. Isotype
Anti-CD8

F. % Lung area covered by metastases

G. H&E
PyMT

H. | Number of mice with detectable tumor | WT | Ron SF-/- | Chi-square value | P value |
--- | --- | --- | --- | --- |
| 9 | 0 | 15.2 | < 0.0001 (****) |
Figure 7.

A. WT Transfer  Ron SF-/- Transfer

B. % Splenic CD4+ effector memory cells (CD62L-CD44+)

C. % IFN\(\gamma\)+ CD4+ splenocytes

D. Veh  Roni

E. 

F. % Tet+ CD8+ splenocytes

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Blocking short-form Ron eliminates breast cancer metastases through accumulation of stem-like CD4+ T cells that subvert immunosuppression


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