Despite recent advances in chemotherapies, breast cancer remains a major cause of death among women. Metastasis serves as a marker of cancer progression and dictates patient outcome; it is the ultimate cause of death in a majority of patients. Understanding the progression of breast cancer to metastasis and the ways in which we can alter this development are essential for improving patient outcomes. Macrophages have been identified as the major cell type responsible for metastatic progression in many cancers. Specifically, M2 macrophages exert a pro-tumor phenotype, whereas M1 macrophages are considered to be anti-tumor. Products of M2 macrophages are known to support many steps of the metastatic cascade, from invasion to the promotion of the pre-metastatic niche.

Investigating factors that can alter macrophage phenotypes may reveal novel ways to diminish their pro-metastatic behavior. Recently, thymic stromal lymphopoietin (TSLP) has been identified in allergic disorders as a factor that can exacerbate M2 macrophage phenotypes. While M2 macrophages are part of the typical progression of allergic responses, these M2 products are pro-tumorigenic in cancer settings. TSLP has recently been identified in several human cancers and has been linked to cancer progression. Specifically, it has been identified in human breast cancer as a cytokine that amplifies pro-tumorigenic CD4 T cell responses. The effect of TSLP on macrophages in breast cancer remains understudied, despite data in allergy supporting this interaction. Therefore, we hypothesize that TSLP drives tumor progression to metastasis by amplifying M2-like macrophage responses. We aim to show that tumor-derived TSLP facilitates spontaneous metastasis via an M2 macrophage-dependent mechanism (Aim 1). We feel this is an essential gap in the field of macrophage biology that may expand our understanding of metastasis and lead to novel targets for therapy. Additionally, anti-TSLP therapy has newly been initiated in clinical trials in asthmatic patients. Recent data has suggested that checkpoint inhibitors, which are traditionally ineffective therapies in breast cancer, are efficacious against aggressive breast cancer when combined with therapies that target innate immunity. We aim to show that TSLP blockade enhances cancer immunotherapy efficacy (Aim 2). We plan to evaluate the efficacy of TSLP therapy both as a monotherapy as well as in combination with checkpoint inhibitors. Given the potential importance of TSLP as a therapeutic target, we will also interrogate the prognostic merit of TSLP levels in breast cancer patients. The overall goal of this proposal is to increase our understanding of macrophage biology in breast cancer to expand potential therapeutic options for patients.
SPECIFIC AIMS: Macrophages are now well recognized as key players in tumor progression to metastasis reflecting diverse solid malignancies; most notably, breast cancer which helped form the conceptual foundation for the inverse relationship between macrophage infiltration and prognosis. However, it is also well appreciated that not all macrophages are functionally equivalent. Pivotal studies in macrophage biology have revealed that macrophages actually comprise a continuum of functionally diverse populations, ranging from tumor-protective (‘M1’) to tumor-promoting (‘M2’) phenotypes. Functional heterogeneity is thought to reflect the types and quantities of factors secreted by various malignancies, which, in turn, directly or indirectly influence the resultant macrophage phenotype. Thus, if we can improve our understanding of how macrophage behavior is altered in neoplasia and what tumor-derived factors (TDFs) are responsible for functional diversity we will likely be able to refine current paradigms of patient prognostication and treatment.

TDFs, namely IL-4, IL-13 and IL-10, have been implicated in the ‘polarization’ of macrophages to tumor-promoting phenotypes. Macrophages polarized by such cytokines enhance metastasis, in part, through the production of cytokines that promote angiogenesis or chemokines that enable tumor spread to secondary sites. Moreover, studies elsewhere have now implicated a new mediator of tumor progression, termed thymic stromal lymphopoietin (TSLP), although the mechanisms by which TSLP does so are yet unclear. TSLP has long been known to function in CD4+ Th2 biology; in particular, TSLP has been found to exacerbate Th2 responses in allergic disorders of the lung. However, in contrast to hallmark Th2-polarizing cytokines such as IL-10, TSLP is thought to amplify Th2-mediated activities at a stage subsequent to polarization. This is thought to occur directly or indirectly through myeloid-dependent interactions, including macrophages. As with Th2 biology, TSLP enhances M2 macrophage-mediated activities following polarization by IL-4 or IL-13.

Altogether, the finding that TSLP can exacerbate M2 macrophage phenotypes (in allergic models) and that TSLP is involved in tumor progression raises the novel hypothesis that TSLP drives tumor progression to metastasis by amplifying M2-like macrophage responses. We further hypothesize that tumor-derived TSLP modifies macrophage function either within the primary tumor microenvironment or the metastatic ‘niche’. To test these hypotheses, we will make use of the well-characterized 4T1 mammary tumor model and its isogenic non-metastatic cell line variants, 67NR and 168FARN. Thus far, our preliminary data are promising and show that: 1) tumor-derived TSLP levels correlate with metastatic phenotype; 2) silencing TSLP expression in TSLP-producing 4T1 tumor cells reduces spontaneous lung metastasis even in face of high primary tumor burden; 3) treatment of M2-polarized macrophages with TSLP results in significantly higher levels of the TSLP-dependent chemokine, CCL17, compared to the vehicle-treated controls; and 4) tumor-associated CCR4 expression, the cognate receptor for CCL17, is also related to metastatic phenotype. These preliminary data, along with the well-documented role of TSLP in type-2 immune responses, provide the rationale for the following aims:

1. To test the hypothesis that tumor-derived TSLP facilitates spontaneous metastasis via an M2 macrophage-dependent mechanism. We suggest a novel mechanism by which TSLP acts in neoplasia. Here, we will test whether tumor-derived TSLP drives lung metastasis by directly engaging tumor-infiltrating macrophages either within the primary tumor microenvironment or the metastatic niche of the lung. First, with regard to macrophages residing within the primary tumor microenvironment, we will investigate whether TSLP alters their production of MMP9 and VEGF, key mediators of angiogenesis and ultimately metastasis. Secondly, with regard to macrophages residing within the metastatic niche, we will investigate whether TSLP alters their production of CCL17, a chemokine capable of aiding metastasis of CCR4-expressing tumor cells. Altogether, we expect to define new axes by which macrophages potentiate metastasis.

2. To test the hypothesis that TSLP blockade enhances cancer immunotherapy efficacy. We will investigate the impact of TSLP blockade, as a monotherapy or in combination with an immunotherapy, using the 4T1 tumor model. Based on data elsewhere, immune checkpoint inhibitors, such as anti-CTLA-4 or anti-PD-1 have shown some therapeutic value in the 4T1 model, affirming feasibility. Here, we will combine this ‘proof-of-principle’ immunotherapy with TSLP blockade using neutralizing antibody. We posit that this combinatorial approach will disrupt two distinct mechanisms of immune suppression to enhance anti-metastatic activity. The finding that TSLP plays an unrecognized role in macrophage-tumor biology also raises the notion that TSLP can serve as a novel prognostic marker. Using a bank of retrospective breast cancer patient materials at Roswell Park, we will quantify sera TSLP levels, followed by immunohistochemical analysis of TSLP expression in matched tumor samples and correlate these results with clinical outcome. We expect that patients with higher TSLP levels will have a poorer prognosis compared to those with lower TSLP values.

Overall, in TSLP-expressing tumor systems, this TDF may bear previously unrecognized pro-tumorigenic properties, impairing macrophage-mediated mechanisms of tumor control. New knowledge gained from this study has the potential to advance not only our basic understanding of macrophage-tumor biology, but also lead to the discovery of novel blood or micro-environmental biomarkers to track tumor status in vivo.
Our rationale to focus on TSLP reflects several reasons. First, TSLP has recently been identified in several human and murine cancers, namely mammary carcinoma, although its role in these neoplastic settings still remains elusive. Secondly, in models of allergic disorders, TSLP has been shown to enhance CD4+ Th2, as well as M2-like macrophage responses. Interestingly, TSLP does not seem to behave as a classical polarizing cytokine, but rather as a regulatory cytokine that acts at a later stage, one that modulates functional behavior after commitment to a polarized phenotype. For example, in the case of the macrophage response in allergy, TSLP has been shown to increase the production of the chemokines CCL17 or CCL22. Of further interest, these same chemokines have been shown to facilitate tumor migration to lung microenvironments. Altogether, these findings support the possibility for a novel TSLP-macrophage axis in at least certain cancers; that is, those that produce TSLP. We posit that TSLP is a previously unrecognized TDF that augments the pro-metastatic activities of macrophages either at primary or metastatic sites, and will be the focus of Aim 1. Immunotherapy is on the forefront of cancer research and the use of immune checkpoint inhibitors, such as anti-CTLA-4 or anti-PD-1 antibodies, has shown great promise in at least subsets of patients. Strategies to maximize the efficacy of checkpoint inhibition in solid tumors, especially breast cancer, are highly sought after. Until recently, it was thought that breast cancer may be refractory to immunotherapies. However, preclinical studies are now showing that checkpoint inhibitors may be effective in mammary cancer in concert with approaches that eliminate suppressive influences within the tumor microenvironment. Additionally, clinical trials are now underway in asthmatic patients using anti-TSLP therapy. Thus, TSLP blockade in concert with immune checkpoint inhibitors may offer a unique way to improve therapeutic efficacy in mammary cancer, which will be the focus of Aim 2. Given the potential importance of TSLP as a therapeutic target, Aim 2 will also interrogate the prognostic merit of serum TSLP levels in breast cancer patients.

INNOVATION: We believe this work is conceptually innovative for the following reasons:

1. It is currently unknown if TSLP can influence macrophage function in cancer, although recent studies in allergic models suggest that TSLP can significantly alter M2-macrophage behavior. We aim to identify TSLP as an underexplored influence of tumor progression via macrophage-dependent mechanisms. Although TSLP has recently been identified in mouse mammary tumor models, these models have focused mainly on the effects of TSLP on primary tumor growth, and the potential role of the Th2 response during that particular stage of disease. Therefore, little is known about the effects of TSLP on metastasis and the role played by macrophages at that stage of disease given the well-documented involvement of macrophages in the etiology of solid tumor progression. We already have in place mouse models of mammary cancer with which we can study/modulate the effects of TSLP on tumor progression to metastasis and macrophage biology.

2. Combination therapy with checkpoint inhibitors is a newly expanding field; the limited, but remarkable success of checkpoint inhibitors in at least some patients, has led to a push to increase the efficacy of these modalities. We suggest that our novel approach of combining anti-TSLP therapy, which has recently been initiated in asthmatic patients, has strong rationale. This combinatorial approach may have direct implications for cancer clinical trials.

3. TSLP has yet to be identified as a prognostic factor in human cancer patients, despite rationale in both breast and other cancer types to explore this avenue. We are uniquely poised to answer this question due to our location in a comprehensive cancer center and the availability of all resources needed to assess the relationship between TSLP levels and prognosis in primary breast tumor tissues.
A new model for the role of tumor-derived TSLP in macrophage biology and cancer therapy. As previously reported\(^2\), the tumor microenvironment produces factors, such as IL-10, which promote the differentiation of naïve T cells to a Th2 phenotype. Polarized Th2 cells release cytokines such as IL-4 and IL-13 that promote macrophage differentiation to an M2-phenotype\(^2\). These M2-macrophages have been demonstrated\(^2\) to further advance tumor progression by facilitating tumor angiogenesis, leading to metastasis. Tumor-derived TSLP has been shown to amplify Th2 responses subsequent to subset polarization\(^2\)\(^3\). Recent findings led us to hypothesize that tumor-derived TSLP augments metastasis by enhancing the pro-metastatic activity of M2-like macrophages, and that this may be a useful therapeutic target in mammary cancer. We postulate that TSLP may be acting directly on macrophages in the primary tumor microenvironment and/or metastatic ‘niche’, in concert with polarizing type-2 cytokines. One potential mechanism involves a novel chemokine-chemokine receptor axis, such as CCL17-CCR4, whereby TSLP enhances the release of CCL17 from macrophages in the lung, instigating tumor metastasis of CCR4\(^*\) tumor cells. Another mechanism we propose is an increased production of pro-invasive factors by tumor-infiltrating macrophages within the primary tumor microenvironment, such as MMP9 or VEGF. We further posit that alleviation of the M2-macrophage response by TSLP blockade in combination with the immune checkpoint inhibitors, anti-PD-1 or anti-CTLA-4, may be a useful therapeutic approach. We will therefore focus in Aim 1 on the mechanism of TSLP-mediated metastasis via macrophages at the primary or metastatic sites. Aim 2 will focus on the utility of TSLP as a therapeutic target and the prognostic merit of TSLP in breast cancer patients. This TSLP-macrophage axis will be a novel advance to our understanding of macrophage-tumor biology, and its influence on metastasis, and may lead to new or alternative therapies in cancer systems where TSLP may be relevant.

IV. APPROACH

Preliminary Studies: TSLP is highly expressed by the aggressive 4T1 mouse mammary tumor model and plays an unrecognized role in tumor progression to metastasis. We first developed a model of TSLP underexpression, and utilized shRNA silencing of TSLP in the 4T1 mammary tumor cell line. We successfully silenced TSLP expression in 4T1 cells (i.e., 4T1-KD; henceforth, 4T1-TSLP\(^{26}\)) based on mRNA levels (not shown) or secreted protein levels (Fig. 2A) compared to parental 4T1 cells or 4T1 cells transfected with the scrambled control vector. We showed that TSLP expression is not significantly different between parental 4T1 and 4T1-VC cells (henceforth, 4T1-TSLP\(^{26}\)), suggesting no overt off-target effect of RNA interference. We have also shown that the non-metastatic 4T1 isogenic cell line variants 67NR and 168FARN\(^{26}\) do not express significant levels of TSLP (Fig. 2B). We have demonstrated that 4T1-TSLP\(^{26}\) cells grow significantly slower in vivo compared to 4T1-TSLP\(^{26}\) cells (Fig. 3A), which translated to a significant survival advantage (Fig. 3B). In contrast, no differences in cell proliferation were observed in vitro (not shown). More importantly, we have shown, for the first time, that knockdown of TSLP results in decreased lung metastasis at similar endpoint primary tumor volumes (Fig. 3C). Here, lungs (Fig. 3C) were histologically analyzed from groups of mice with similar endpoint tumor volumes (Fig. 3A). We have also demonstrated that TSLP results in increased CCL17 production, a known TSLP-regulated chemokine, in bone marrow-derived M2-polarized macrophages (Fig. 4A). While neither TSLP nor IL-4 alone was able to induce CCL17 expression, the combination of IL-4 and TSLP resulted in significantly increased CCL17 expression. We have shown that the metastatic ability of 4T1 and its variants, 168FARN and
67NR, directly correlates to levels of CCR4, the cognate CCL17 receptor (Fig. 4B). 4T1 spontaneously metastasizes to the lung and expresses the highest levels of CCR4. This supports our hypothesis that TSLP facilitates metastasis to sites such as the lung via a ‘macrophage chemokine-tumor chemokine receptor axis’.

**APPROACH**

**Aim 1: To test the hypothesis that tumor-derived TSLP facilitates spontaneous metastasis via an M2 macrophage-dependent mechanism. Rationale:** TSLP influences primary tumor growth through a CD4+ Th2-dependent mechanism. However, when CD4+ T cells are depleted in settings of high tumor TSLP levels, primary tumor growth is abrogated without impairing metastatic activity. This suggests that there is a distinct cell type responsible for TSLP-mediated metastasis. It is known that M2-macrophages are a key player for metastasis to secondary sites. Here, we postulate that tumor-derived TSLP acts to promote metastasis by potentiating M2-macrophage activity within the primary tumor or metastatic microenvironment. In the primary tumor, we hypothesize that TSLP enhances macrophage MMP9 and/or VEGF expression, which are known mediators of tumor angiogenesis and invasion. In the metastatic lung microenvironment, we hypothesize that TSLP enhances macrophage CCL17 production, recruiting CCR4-expressing tumor cells. While we recognize that macrophages may drive metastasis via multiple mechanisms, including their ability to suppress immune responses, Aim 1 of this proposal will focus on a novel TSLP-macrophage-tumor axis.

**Research Plan:** Sub-aim 1A: Do alterations in tumor-derived TSLP levels affect metastatic outcome through mobilization of macrophages? Our preliminary data show high levels of macrophages within 4T1 primary tumor tissue (~30%, n=5), suggesting that macrophage infiltration carries biologic relevance. Moreover, studies elsewhere have shown that macrophage depletion in a range of solid tumor models leads to a significant reduction in primary tumor growth. Thus, these data indicate that macrophages in the 4T1 model are protumorigenic. First, we will extend these experiments and deplete macrophages using two approaches, pharmacologic and genetic. Using the experimental design in Fig. 3A, we will test the effects of macrophage depletion on orthotopic primary tumor growth, followed by its effects on metastasis at experimental/humane endpoints. Pharmacologic depletion will be achieved by clodronate liposomes, as previously described. Clodronate treatment will be initiated (100 mg/kg, i.p.) after tumors first become measurable, followed by injections (50 mg/kg) biweekly to maintain depletion until endpoint. To confirm these findings or if macrophage depletion by clodronate liposomes is ineffective, we will seek to deplete macrophages using Trabectedin, a chemotherapeutic agent recently shown to cause a selective and profound depletion of monocytes/macrophages both in the periphery and tumor microenvironment. Briefly, Trabectedin will be injected (0.15 mg/kg, i.v.) once weekly after tumors first become measurable, and continue throughout the duration of the experiment. For either drug, depletion efficiency will be determined by flow analysis of spleen/tumor tissues. Genetic depletion will be achieved by the use of the DTR-mCherry mouse (Jackson Labs), which encodes for a Cherry-DTR fusion protein under the CSF-1 receptor promoter, which is flanked by lox P sites. Monocyttes/macrophages will be selectively depleted following administration of diphtheria toxin. Depletion will be verified, as described above, by flow cytometry based on the expression of mCherry.

To determine whether the pro-metastatic activity of macrophages is influenced by tumor-derived TSLP levels, mice will be implanted with 4T1 expressing or lacking TSLP expression (as in Fig. 2A). First, we will implant mice with 4T1-TSLP\(^{hi}\) or 4T1-TSLP\(^{lo}\) cells; macrophages (CD45\(^{hi}\)CD11b\(^{hi}\)F4/80\(^{hi}\)Ly6G\(^{hi}\)) from either primary tumor or lung will be flow-purified (at varying tumor sizes) and analyzed for pro-metastatic features in Fig. 1 (i.e., MMP9, VEGF, CCL17). This will be important for studies in Sub-aim 1B (primary site) or 1C (metastatic site). Secondly, to show that the effect of macrophage depletion on metastasis is TSLP-dependent, we will perform depletion experiments outlined above in mice bearing 4T1-TSLP\(^{hi}\) or 4T1-TSLP\(^{lo}\) cells. If tumor-derived TSLP potentiates pro-metastatic infiltrating macrophages, depletion in mice bearing 4T1-TSLP\(^{hi}\) cells will reduce metastasis to a level seen in mice bearing 4T1-TSLP\(^{lo}\) cells. Thirdly, we will establish a TSLP gain-of-function approach. We will transfect 4T1 variants, 67NR and 168FARN, with a full-length TSLP expression vector. We posit that the (over)expression of TSLP in 67NR or 168FARN will increase the pro-metastatic ability of macrophages, based on assays above. While we recognize that metastatic potential has many influences, this experiment allows us to determine how tumor-derived TSLP levels impact metastasis.

**Sub-aim 1B: Do TSLP-conditioned macrophages potentiate metastasis by their actions within the primary tumor microenvironment?** To determine if TSLP plays a role in promoting metastasis by affecting macrophages...
at the primary tumor site, we will utilize a ‘macrophage-tumor co-implantation’ approach, which has been extensively utilized by our laboratory\textsuperscript{34} and elsewhere. Since the variable in this experiment is whether TSLP modulates the behavior of macrophages, which in turn influence metastasis by their actions in the primary tumor microenvironment, macrophages will be recovered from 4T1-TSLP\textsuperscript{hi} or 4T1-TSLP\textsuperscript{lo} tumor tissue, as in Sub-aim 1A. Macrophages from 4T1-TSLP\textsuperscript{hi} or 4T1-TSLP\textsuperscript{lo} tumors will then be co-mixed ex vivo with 4T1-TSLP\textsuperscript{lo} cells, and the admixture implanted into new groups of female BALB/c mice. 4T1-TSLP\textsuperscript{lo} cells will be used for this part of the experiment to minimize the impact of additional tumor-derived TSLP in the host. Tumor growth and metastasis will be measured as in Fig. 3. Controls will include mice receiving 4T1-TSLP\textsuperscript{hi} tumors to determine the extent of maximal metastatic activity. In short, this approach will allow us to determine if macrophages previously exposed to TSLP in vivo can re-instate/boost metastatic ability of 4T1-TSLP\textsuperscript{lo} cells.

To minimize recruitment of endogenous macrophages, additional experiments will be performed using antibody-based methods that impede trafficking. The CCL2-CCR2 axis has been shown to play critical roles in mobilizing monocytes to sites of inflammation or pathologic conditions, such as cancer, and targeting this interaction in vivo has been shown to significantly block macrophage accumulation. Briefly, anti-CCL2 or isotype-matched Abs (from BioXcell; clone 2H5 or hamster IgG) will be injected (20 mg/kg, i.p.) after tumors first become measurable and continue thereafter twice a week, as described\textsuperscript{35}, until endpoints are reached. In separate cohorts of mice, tumor tissues will be collected at various time points and analyzed by flow cytometry for changes in macrophage frequencies as a way to quantify the efficacy of this blockade strategy.

To determine whether macrophage-mediated effects on metastasis involve VEGF- or MMP9-dependent mechanisms, experiments will be performed using genetically deficient mouse models. In the case of VEGF, a VEGF-A\textsuperscript{flo/llo} mouse model is publicly available\textsuperscript{36}, while the Lyz2-cre mouse model (which targets macrophage populations) is currently available in our laboratory (from Jackson Labs). Since the VEGF mouse is on a B6 background, it will be backcrossed (at least 8 generations) onto a BALB/c background which will then be bred to the Lyz2-cre mouse (on a BALB/c background) to generate experimental progeny. To that end, wild-type (fl/fl) and VEGF-A conditional knockout progeny will be implanted with 4T1-TSLP\textsuperscript{hi} or 4T1-TSLP\textsuperscript{lo} cells, and the impact of VEGF-A loss in macrophages on metastasis analyzed as above. Macrophage infiltration into primary tumor tissue will be verified by flow analysis, as above. In regard to the MMP9 studies, we will make use of a global MMP9 null mouse model (from Jackson Labs), since a conditional knockout strain to the best of our knowledge is not available. As with the VEGF studies, the MMP9 mouse, which is on a B6 background, will be backcrossed (at least 8 generations) onto a BALB/c strain to generate appropriate experimental progeny. Since the impact of MMP9-loss could reflect multiple cell types, macrophages will be recovered from tumor tissue of (4T1-TSLP\textsuperscript{hi}) wild-type or MMP9\textsuperscript{-/-} mice for subsequent use in the co-implantation experiments.

Sub-aim 1C: Do TSLP-conditioned macrophages potentiate metastasis by their actions within the metastatic microenvironment? To determine whether macrophages conditioned by tumor-derived TSLP impact metastasis by acting in the lung microenvironment, we will modify the co-implantation experimental design. Here, mice will be implanted with 4T1-TSLP\textsuperscript{lo} tumors, followed by surgical resection (of a primary tumor mass ~7x7mm). This will allow us to study metastasis without any ongoing influence from the primary tumor microenvironment. Thus, in lieu of the co-implantation step, macrophages will be recovered from the metastatic lung microenvironment from a separate cohort of 4T1-TSLP\textsuperscript{hi} or 4T1-TSLP\textsuperscript{lo} tumor bearing mice and then adoptively transferred i.v., as described\textsuperscript{37}. Briefly, flow-purified macrophages (3x10\textsuperscript{6} cells/mouse) will be injected via tail vein weekly until ethical/animal endpoints are reached. Experiments will be performed in CD45.1 congenic recipients to assess the efficiency of donor macrophage (CD45.2) trafficking to the lung. Controls will include mice receiving 4T1-TSLP\textsuperscript{hi} tumors to determine maximal metastatic activity in this post-surgery setting.

We are hypothesizing macrophages which inhabit the metastatic niche influence metastasis via a novel chemokine-chemokine receptor pathway, CCL17-CCR4. This is based on our preliminary data (Fig. 4A), as well as published data which demonstrate that TSLP can enhance production of CCL17 by M2-polarized macrophages\textsuperscript{5}. We further hypothesize that the metastatic circuit is then achieved by the recruitment of CCR4-expressing cells, the cognate receptor for CCL17. Additional preliminary data not only confirm that 4T1 cells express high levels of CCR4, but that CCR4 expression correlates with metastatic phenotype (Fig. 4B). To test this hypothesis, we will utilize both CCR4 loss- and gain-of-function methods. First, we will silence CCR4 in 4T1-TSLP\textsuperscript{lo} cells and analyze their ability to metastasize (relative to vector control). Secondly, the impact of CCR4 expression on metastasis will be tested by adoptive transfer, wherein the CCR4\textsuperscript{lo} and CCR4\textsuperscript{lo} 4T1 variants will be implanted and resected, followed by adoptive transfer of TSLP-conditioned lung macrophages.

Next, we will transfect 67NR or 168FARN cells with a full-length expression vector encoding murine CCR4 and then analyze the ability of these transfectants to metastasize using the same adoptive transfer paradigm. Again, while we recognize that metastatic potential is influenced by a variety of factors, this proof-of-principle experiment will allow us to determine whether differential expression of tumor-derived CCR4 levels also impact the lung metastasis. Thirdly, in a reciprocal fashion to inhibiting the expression of the relevant receptor, we will inhibit the production CCL17 using a genetically deficient mouse model. As with the VEGF
and MMP9 models, a CCL17-deficient mouse model is publically available, but on non-BALB/c strain. Accordingly, mice will be backcrossed to provide a source of macrophages for the adoptive transfer studies.

**Expected Outcomes:** We expect that depletion of macrophages, either pharmacologically or genetically, will result in decreased metastasis in mice bearing 4T1-TSLP tumors, comparable to that seen in mice bearing 4T1-TSLP cells (with or without macrophage depletion). We expect that macrophages conditioned by TSLP in vivo will enhance metastasis compared to macrophages exposed to lower levels of TSLP. We also expect that macrophages exposed to 4T1-TSLP tumors will exhibit greater production of MMP9, VEGF, and/or CCL17. We anticipate that deficiency of one or more of these products from macrophages will decrease their pro-metastatic ability. Knockdown of CCR4 (in 4T1-TSLP cells) or loss of CCL17 expression (by macrophages) will decrease TSLP-mediated metastasis. Overall, these studies have the potential to unveil for the first time that tumor-derived TSLP enhances macrophage-mediated mechanisms of metastasis by modulating their ability to produce angiogenic factors, such as VEGF or MMP9, or pro-metastatic chemokines, such as CCL17.

**Potential Problems and Alternative Strategies:** If our macrophage depletion strategies are ineffective, we will investigate alternative models, such as the CD11b-DTR or MAFIA mouse models (Jackson Labs). In the event flow purification of macrophages is problematic, TSLP-conditioned M2-polarized macrophages will be generated in vitro as in Fig. 4A. If we do not observe a significant decrease in metastasis despite effective macrophage depletion/add-back, we will explore the role of other myeloid populations, such as dendritic cells, myeloid-derived suppressor cells, or CD4+ Treg cells using pharmacologic (antibody depletion) or genetic-based approaches. It is important to note that TSLP may potentiate M2-macrophage activity via CD4+ Th2 responses. To address this issue, we will perform experiments in T cell-deficient hosts, such as SCID or RAG2-/- mice, and/or antibody deplete CD4+ T cells in normal mice in vivo. If our VEGF, MMP9, or CCL17 loss-of-function approaches are unable to show a significant role for these macrophage products, we will analyze the expression of additional metastasis-related gene products, such as fibroblast growth factor, TGF-β, CCL22, or CXCL12. Additionally, endogenous TSLP may interfere with the interpretation of co-implantation or adoptive transfer experiments. In this case, we will deplete TSLP using commercially available neutralizing antibody.

**Aim 2: To test the hypothesis that TSLP blockade enhances cancer immunotherapy efficacy.** Rationale: The notion that tumor-derived TSLP plays unrecognized roles in macrophage-mediated metastasis implies that TSLP has therapeutic or prognostic merit. To that end, the first part of Aim 2 will investigate if TSLP blockade improves responses to immunotherapy in the 4T1 model, while the second part of Aim 2 will investigate if TSLP levels in sera or tumor tissue of breast cancer patients carry prognostic value. The preclinical studies will utilize commercially available anti-TSLP antibody. The translational utility of this approach is strengthened by recent clinical trials initiated in humans to treat asthma. The immunotherapy component will make use of immune checkpoint blockade targeting CTLA-4 or PD-1, which are now FDA-approved strategies for solid cancers such as metastatic melanoma. While we recognize that immune-based therapy is not novel, it carries translational relevance. Overall, the novel concept concerns ‘TSLP blockade in cancer’, which we posit (Aim 1) disrupts a negative regulatory axis in macrophage-tumor biology to improve a clinically relevant therapy.

**Sub-aim 2A: Can the combination of both treatment factors promote anti-metastatic activity more effectively than either one alone?** Initial studies will make use of anti-CTLA-4 antibody, based on our earlier work showing that CTLA-4 blockade has a partial, but significant antitumor effect. Once primary tumors become measurable, mice will be injected i.p. with anti-CTLA-4 antibody (clone 9H10; BioXcell), anti-TSLP antibody (clone 152614; R&D Systems) or both. Treatment with anti-CTLA-4 antibody will be at 3-day intervals for a total of 3 injections (100µg/mouse), as described, whereas treatment with anti-TSLP antibody (100µg/mouse) will be maintained at 5-day intervals throughout the course of the experiment, as described. Efficacy will be determined by inhibition of primary tumor growth and lung metastasis. Subsequent studies will explore this combinatorial regimen in a post-surgery setting (as in Sub-aim 1C). Treatment will be initiated within a week post-surgery to ensure that mice fully recover from the procedure. Any mice showing evidence of primary tumor regrowth will be technically excluded from analysis. If we observe complete tumor growth inhibition, mice will be re-challenged with 4T1 cells orthotopically (≥8 weeks post-initial implant) to determine the extent of tumor immunity. Resistance to tumor re-challenge will be used as another assessment of potential benefit.

**Sub-aim 2B: What are mechanisms by which the combination regimen mediates antitumor activity?** Our hypothesis is that TSLP blockade will alleviate the pro-metastatic phenotype of intratumoral macrophages and enhance therapeutic efficacy of CTLA-4-based immunotherapy via a T cell-dependent mechanism. We will therefore examine the effect of the combination treatment, both the macrophage and T cell responses. For the former, macrophages will be flow-purified from primary tumor tissue or the metastatic microenvironment (at varying primary tumor sizes) and analyzed for changes in pro-metastatic phenotype (i.e., MMP9, VEGF and
CCL17 levels). For the latter, T cells will be examined by flow analysis of primary/metastatic tumor tissues for changes in percentages/numbers of CD4+ and/or CD8+ T cells. The role of T cells will be assessed functionally, based on in vivo depletion of CD8+, CD4+ cells, or both, prior to treatment.

**Sub-aim 2C: To determine the prognostic relevance of TSLP levels in human breast cancer.** The notion that tumor-derived TSLP is relevant to metastasis and may be a therapeutic target provides the impetus for exploring TSLP levels in breast cancer patients as a unique biomarker. As with perhaps all cancers, breast cancer is a heterogeneous disease with multiple subgroups reflecting differences in histology, stage, grade, differentiation, and hormone receptor (HR) status, among other characteristics. Here, we will focus our exploratory study on patients with late-stage invasive ductal carcinoma (IDC), since it is a common histologic subtype. We will investigate differences in TSLP levels between healthy and disease state. Insights gained about a potential TSLP-breast tumor relationship would be most informative between healthy and advanced disease states (e.g., stages III/IV), which is currently unknown. TSLP levels will be quantified by ELISA. This initial analysis will include a total of 30 – 50 patients, as well as 30 age/gender/race-matched healthy donors. If the data reveal significant differences between these two cohorts, this analysis will be extended to patients with earlier stage disease (n =30 – 50). (Please refer to statistical section below).

It is important to emphasize that Roswell Park has an outstanding resource of patient materials (with well-annotated demographic and clinical information), and our laboratory has already successfully made use of breast cancer patient materials. Our laboratory also has active IRB-approved, de-identified nonhuman subject research (NHSR) protocol to conduct these studies. Criteria for selecting patient materials for study will reflect: 1) samples procured at time of diagnosis; and 2) patients with no known prior (anticancer) therapy, and no known prior cancer diagnosis of any kind. Patient sera TSLP levels will then be stratified according to two broad cohorts, high or low, based on median cutoff. The data will then be plotted in relation to overall survival and progression-free survival. To determine whether neoplastic tissue is a source of TSLP, we will perform immunohistochemical (IHC) analysis of primary tumor tissue (i.e., formalin-fixed paraffin-embedded blocks) for TSLP expression from a selected match of patients reflecting low/high serum TSLP (n=10 from each cohort).

**Statistical Analyses:** Statistical analysis will be performed in consultation with Dr. Austin Miller, a senior biostatistician at Roswell Park. Data will be reported as mean ± SEM of multiple biologic experiments. Significance will be determined by unpaired t-tests or Mann-Whitney. Differences in tumor growth by treatment will be based on non-linear mixed effects modeling. TSLP levels will be compared between patients with disease to healthy donors, as well as pairwise between each group by Wilcoxon Rank Sum tests (80% power to detect >3FC). P < 0.05 will be considered significant. For both mouse and human studies, survival outcomes will be illustrated by Kaplan-Meier plots, and inferred by log-rank tests (70% power to detect HR=5).

**Expected Outcomes:** These studies will extend our preliminary data, demonstrating that CTLA-4 blockade in combination with TSLP blockade will significantly inhibit tumor metastasis compared to either treatment alone. We also expect that the mechanism of CTLA-4 based therapy will require CD8+ and/or CD4+ T cells, and that the efficacy of the combination treatment will be accompanied by diminution of the pro-metastatic phenotype of the infiltrating macrophages. We expect that breast cancer patients will express higher levels of circulating TSLP, compared to matched controls demonstrating that TSLP presence is disease-dependent. We also expect that TSLP levels will be variable among patients with IDC, but will correlate with measurements of clinical outcome. Thus, patients with higher levels of TSLP are likely to correlate with a poorer prognosis.

**Potential Problems & Alternative Strategies:** If enhanced antitumor or anti-metastatic responses are not observed, we will modify the dose/frequency/schedule of each antibody. If this fails to show additional impact, we will include anti-PD-1 antibody. While we are initially proposing to analyze the indicated number of human samples, we will strive to examine as many as possible during this award period to boost clinical significance. If we do not observe significant differences between TSLP levels in patient sera or tumor tissue by IHC, we will compare TSLP levels to other clinical characteristics, such as TNM classification and HR status. We will analyze fresh primary tumor tissue for TSLP expression by qPCR. Recently, we have received approval of a de-identified IRB protocol (BDR 030312) to obtain fresh primary tumors from treatment-naïve patients with IDC at time of surgery. Samples are enzyme treated to produce single cell suspensions and cryopreserved. This renewable 3-year protocol allows for analyses of 150 tumor samples. So far, we have procured 47 samples.

**Significance:** Overall, this work has the potential to expand our understanding of macrophage-tumor biology and the impact of a newly defined TDF (TSLP) on regulating the interplay of that interaction. Moreover, the identification of TSLP as a relevant TDF may bear significant prognostic or therapeutic implications.