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THE ROLE OF TUMOR-ASSOCIATED MACROPHAGES IN TRIPLE-NEGATIVE BREAST CANCER

INVASION AND METASTASIS

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## **DEDICATION**

I would like to dedicate this work to my parents, Gary and Melissa Rabe. Without their tireless work, I would not be where I am today. They instilled in me a curiosity that has led me to a career in science and a desire to help others that brought me to cancer research. Thank you for everything you have done and the encouragement to help get me where I am today.

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## ABBREVIATIONS

Basal-like breast cancer (BLBC)	Immunohistochemistry (IHC)
Bone-marrow derived macrophages (BMDMs)	Macrophage colony stimulating factor (M-CSF also CSF-1)
C-C Motif Chemokine Ligand 5 (CCL5)	Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B)
C-C Motif Chemokine Ligand 7 (CCL7)	Progesterone receptor (PR)
C-C Motif Chemokine Receptor 5 (CCR5)	quantitative real-time polymerase chain reaction (qRT-PCR)
Colony stimulating factor 1 (CSF-1 also M-CSF)	Raf kinase inhibitor protein (RKIP)
Conditioned media (CM)	Toll-like receptor (TLR)
Epidermal growth factor receptor (EGFR)	T regulatory cell (T-reg)
Estrogen receptor (ER)	Tumor necrosis factor receptor 2 (TNFR2)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Tumor-associated macrophages (TAMs)
Granulin or progranulin (GRN)	Tumor educated macrophages (TEMs)
High-mobility group AT-hook 2 (HMGA2)	Triple-negative breast cancer (TNBC)
Human epidermal receptor 2 (HER2)	

## ABSTRACT

Triple-negative breast cancer (TNBC) patients have the highest risk of recurrence and metastasis, cannot be treated with targeted therapies, and many do not respond to chemotherapy, making them a clinically underserved group. While physiological inhibitors of metastasis (metastasis suppressors) play key roles in regulating tumor growth, invasion and metastasis, their role in regulating the tumor microenvironment and immune system is unknown.

Using species-specific RNAseq we determined that expression of the metastasis suppressor Raf kinase inhibitor protein (RKIP) in tumors markedly reduces the number and metastatic potential of infiltrating TAMs. While TAMs isolated from TNBC xenografts drive *in vitro* invasion, RKIP+ derived TAMs did not drive invasion and had decreased secretion of pro-metastatic factors including SLPI, OPN, MMP-12, Galectin-3, VEGF-A, VEGF-D, TNFR2, and PGRN. We determined that RKIP regulates TAM recruitment by blocking HMGA2, which activates CCL5 expression. CCL5 rescued pro-metastatic TAM infiltration as well as tumor intravasation. We additionally showed that factors decreased in RKIP-derived TAMs were restored in CCL5-derived TAMs.

To determine the mechanism of macrophage programming by TNBC cells, we treated bone marrow derived macrophages (BMDMs) with conditioned media (CM) from TNBC cells, CM depleted of exosomes, or tumor exosomes alone. Only exosomes alone showed increased levels of pro-invasive genes. CCL5 expression in tumor cells increased the pro-invasive phenotype of macrophages via tumor exosomes. Additionally, expression of RKIP blocked pro-invasive programming of macrophages through exosomes. Finally, we found that resistance to the CCL5 inhibitor Maraviroc could be transmitted to macrophages through exosomes.

For *in vivo* validation, mice were injected with exosome-depleted tumor cells using shRab27a. While shRab27a in tumors did not change TAM number, it decreased the ability of TAMs to drive TNBC invasion and reduces expression of pro-invasive cytokines including CCL3, CCL7, CCL19, CXCL1, CXCL3, Flt-1, OPN, SLPI, TGF-  $\beta$ 3, TNFSF14, and Thrombospondin. Co-injection of exosome programmed TEMs, which also express these pro-invasive cytokines, resulted in both an increase in number and size of lung metastases. Taken together, these results indicate that exosomes are essential for the pro-metastatic programming of TAMs within the microenvironment as well as transferring therapeutic resistance.

## CHAPTER 1: BACKGROUND AND SIGNIFICANCE

### Significance

One out of every eight women in the United States will develop breast cancer in her lifetime. Of these women, 15-20% will have basal-like or triple-negative breast cancer (TNBC). Triple-negative tumors lack expression of estrogen receptor (ER), progesterone receptor (PR) and amplification of human epidermal receptor 2 (HER2). TNBCs largely (80%) overlap with basal-like breast cancers characterized by overexpression of receptors such as epidermal growth factor receptor (EGFR) and the proto-oncogene Met<sup>1,2</sup>. This disease disproportionately affects African American and low-income women, with rates ~3 times higher than the rest of the population<sup>3</sup>. Because of the poor response to chemotherapy and lack of currently approved targeted therapies for TNBC patients, their 5 year survival rate is only 24%; by contrast, patients who can be targeted with estrogen receptor antagonists or HER2 antibodies have 5 year survival rates of ~98%<sup>4</sup>. *There is clearly a clinical need to further understand the disease biology underlying TNBC progression & find targets for therapy.*

Because of the lack of response to chemotherapy and the lack of targeted therapies, many have sought novel methods for treating TNBC patients, including targeting of the tumor stroma<sup>5</sup>. This study seeks to elucidate the mechanism by which tumor-associated macrophages (TAMs) in the tumor microenvironment drive tumor invasion and metastasis of TNBC. In particular, we sought to elucidate the mechanism by which TAMs are recruited to the tumor microenvironment, the nature of TAMs recruited to TNBC tumors, and the method by which tumors program TAMs once they are recruited to the tumor microenvironment. By elucidating these mechanisms, we

hope that a clear understanding of their biology will help to establish how TAM function and recruitment can be blocked.

### **Triple-negative breast cancer (TNBC)**

Breast cancer is not a single disease, but has many disparate clinical characteristics, disease courses, and responses to treatment<sup>6</sup>. It can be broadly divided into six or more subtypes by molecular profiling, each with different clinical characteristics<sup>7,8</sup>. Of the subtypes of breast cancer, 15-20% are classified as triple-negative breast cancer (TNBC). These tumors lack expression of estrogen receptor (ER), progesterone receptor (PR), and lack amplification of human epidermal receptor 2 (HER2). These tumors often overlap with the basal-like breast cancer (BLBC) subtype; 80% of basal tumors are triple-negative and vice versa<sup>1</sup>. Basal tumors are characterized by their overexpression of the cytokeratins 5, 6, 14, and 17 as well as overexpression of epidermal growth factor receptor (EGFR), the proto-oncogene Met (Met), and the proto-oncogene c-Kit (c-Kit)<sup>2,9</sup>.

While clinical outcomes have improved for many patients with breast cancer, as high as 98% five-year survival rates in patients treated with targeted anti-ER or anti-HER2 therapies, patients with TNBC have five year survival rates closer to 24%<sup>4</sup>. These patients have higher rates of metastasis and more aggressive tumors, leading to heavy disease burden. Currently, the only form of therapy for these patients is surgery and platinum based chemotherapy. However, patient outcome is generally poor on chemotherapy. This disease disproportionately affects African-American women and lower income women, with rates seen approximately three times higher in African-American women compared to the rest of the population<sup>3,10</sup>.

Because of the lack of response to chemotherapy and the lack of targeted therapies, many have sought novel methods for treating TNBC patients, including targeting of the tumor stroma<sup>5</sup>. The stroma consists of extracellular matrix proteins, cancer associated fibroblasts (CAFs), and immune cells, including tumor associated macrophages (TAM). The extracellular proteins provide a scaffold on which cells can travel and invade, while the surrounding stromal cells provide critical signaling to the tumor. These cells do not have the same genomic instability and cellular heterogeneity within each type as tumor cells do, making them more stable targets for therapy.

### **Raf kinase inhibitory protein (RKIP)**

Raf Kinase Inhibitory Protein (RKIP), also known as Phosphatidylethanolamine binding protein1 (PEBP1), is a member of the phosphatidylethanolamine binding protein (PEBP) family. It regulates several key signaling pathways<sup>11</sup>, by binding Raf-1 and inhibiting MAPK signaling<sup>11,12</sup>, suppresses NFκB activation<sup>13</sup>, inhibits GRK2-mediated down-regulation of G protein-coupled receptors<sup>14</sup>, and potentiates the efficacy of chemotherapeutic agents in some cancer cell types<sup>15</sup>. Additionally, RKIP acts as a metastasis suppressor for androgen-independent prostate<sup>16</sup> and TNBC cells<sup>17,18</sup>. RKIP is missing or depleted in a number of tumors including prostate, breast, melanoma, hepatocellular carcinoma, and colorectal<sup>16,19</sup>, suggesting a general suppressor role in solid tumors. It additionally plays a role in chromosomal integrity by preventing MAPK driven inhibition of Aurora B kinase during the spindle checkpoint<sup>20</sup>.

Our lab has also shown that RKIP is able to inhibit TNBC invasion, intravasation, and metastasis in injectable xenograft mouse models dependent on the siRNA let-7 and High-mobility group AT-hook 2 (HMGA2)<sup>17</sup>. Using a bioinformatics analysis of this signaling pathway, the lab

developed an RKIP pathway based metastasis signature (RPMS), that is able to predict those patients that have the worst prognosis among TNBC patients<sup>18</sup>. Studying RKIP regulation of signaling and metastasis has given insight not only into the regulation of several key signaling pathways, but also in the ability to detect patient prognosis based on a combination of signaling pathways<sup>21-24</sup>.

Recent work from our lab has shown that RKIP can also regulate the expression of stromal targets as well as the recruitment of cells into the stromal microenvironment<sup>23</sup>. In collaboration with the Gilad group, we developed a novel high-throughput RNA sequencing (RNAseq) technology that is able to precisely resolve expression of tumor and stroma in a xenograft model based on species differences. This model unambiguously mapped 93.3% of reads with a misassignment rate of 0.01%. This study showed that RKIP not only regulates signaling networks within the tumor cells, but also affect expression of tumor associated targets in the stroma. One particularly interesting result of this study was the effect of macrophage recruitment to the tumor stroma.

### **Tumor-associate macrophages (TAMs)**

Tumor-associated macrophages (TAMs) are generally thought of as alternatively activated or M2. Secretion of growth factors from these alternatively activated or tumor-associated macrophages leads to tumor growth, progression, and metastasis<sup>25,26</sup>. Recent work has shown that the level of M2 macrophages in the tumor stroma (determined through cluster of differentiation 163 (CD163) staining) correlates with outcome in patients, as well as with tumor type. More specifically, M2 macrophage recruitment positively correlates with TNBC tumors

while negatively correlating with ER+ tumors<sup>27</sup>. Therefore, recruitment of alternatively activated M2 macrophages could play a significant role in the outcome of TNBC patients and explain their poor prognosis. Work from Levano et al, 2011 has shown that basal versus luminal subtypes of breast cancer express different repertoires of cytokine receptors allowing them to be differentially affected by M2 macrophages<sup>28</sup>.

The Pollard group first examined the role of TAMs in breast cancer by knocking out macrophage colony stimulating factor-1 (CSF-1) in transgenic mice with the Polyoma Virus middle T antigen (PyMT) oncogene under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). While these mice had no changes in the development of mammary tumors, the tumors had delayed ability to become invasive and metastatic<sup>29</sup>. To study the effect of TAMs on tumor progression specifically, clodronate-tagged liposomes have been utilized to specifically ablate macrophages after tumor development. Macrophage ablation has led to reduced ability of tumor cells to invade and metastasize<sup>25</sup>, as well as increased anti-tumor effect in combination with targeted therapies in liver cancer<sup>30</sup>. IL-4 signaling from CD4+ T cells is able to support the pro-tumorigenic signaling of TAMs, including expression of epidermal growth factor (EGF) that sustains the invasive potential of tumor cells in the MMTV-PyMT model<sup>31</sup>. Work from the Pollard group has more recently shown the importance of CCL2 signaling for macrophage involvement in breast cancer. He has sought to show that blockade of CCL2 signaling could lead to a reduction in the invasive potential of this cancer.

The TAM that are either recruited to the tumor environment or differentiated from monocytes are generally thought to be of an anti-inflammatory, alternatively activated, M2 subtype<sup>32</sup>. However, the exact characteristics of macrophages in the tumor microenvironment

remains unclear. Proteomic analysis of macrophages has shown that differentially derived monocytes do not always show the characteristic dichotomy of markers for classic M1 activation versus alternative M2 activation<sup>33-36</sup>. Rather, some populations display markers of both M1 and M2 origin. It is therefore essential to determine the exact characteristic of the macrophages that infiltrate the tumor microenvironment of TNBC tumors to fully understand the role they can play in driving progression, invasion, and metastasis. However, many of the studies that have been done rest on studying the effect of differentiated THP-1 macrophages (a monocyte cell line) on tumor cells in culture or in the MMTV-PyMT model.

### **M1 vs M2 Macrophages**

The classical description of macrophage function has separated them by dichotomy in promoting tumorigenesis. It has been viewed that macrophages either drive tumor rejection (classified as M1) or tumor progression (classified as M2) depending on the type of macrophage activation<sup>37</sup>. Classic or M1 activated macrophages are stimulated by IFN $\gamma$ , lipopolysaccharide (LPS), or tumor necrosis factor- $\alpha$  (TNF $\alpha$ ). Classical activation of monocytes leads to M1 macrophages that secrete reactive oxygen species (ROS), TNF $\alpha$ , interleukin-1 (IL-1), IL-6, IL-12, IL-23, and cytokines leading to tumor rejection by the immune system. Monocytes activated by IL-4, IL-10, IL-13 and tumor growth factor- $\beta$  (TGF $\beta$ ) lead to alternatively activated M2 macrophages or TAM. These macrophages are known to secrete IL-10, TGF $\beta$ , platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular-endothelial growth factor (VEGF), epidermal growth factor (EGF), and matrix metalloproteinases.

Classical activation of macrophages by IFN $\gamma$ , lipopolysaccharide (LPS), or tumor necrosis factor- $\alpha$  (TNF  $\alpha$ ) polarized, M1 macrophages, secrete inflammatory cytokines important in the body's anti-tumor response as well as in response to bacterial infections. M2 macrophages, activated by factors such as IL4, play an essential role in wound healing. However, secretion of factors from tumor-associated macrophages (TAMs), traditionally classified as M2 activated macrophages, leads to tumor growth, progression, and metastasis<sup>26,38,39</sup> as well drug resistance<sup>40</sup>. However, recent evidence suggests that this division of macrophages into two discrete subtypes incompletely describes the range of macrophage phenotypes present in the tumor microenvironment<sup>41</sup>. Importantly, studies of breast cancer patients show that CD163+ macrophage recruitment positively correlates with TNBC while negatively correlating with ER+ and luminal tumors<sup>27</sup>. Therefore, recruitment of alternatively activated TAMs could play a significant role in the outcome of TNBC patients and explain their poor prognosis.

### **The role of chemokines in TAM recruitment and tumor biology**

TAMs are recruited to mammary tumors through induction of a variety of cytokines and chemokines, where they play essential roles in driving metastasis. Much of the work in the immune environment of breast cancer has been done utilizing the MMTV-PyMT model of breast cancer. This model has established CSF-1 and CCL2 as critical factors for recruiting TAMs to the primary tumor as well as metastatic sites. TAMs recruited by CSF-1 express higher levels of VEGF-A, with increased angiogenesis in the MMTV-PyMT genetically engineered mouse model for breast cancer<sup>42</sup>. Similarly, CCL2 was required for TAM infiltration in primary breast tumors as well as TAM-enabled metastatic colonization of lungs<sup>43</sup>. When GFP monocytes were injected into the

blood stream of mice with tumor cells derived from the MMTV-PyMT model, a CCL2 neutralizing antibody blocked their recruitment to metastatic sites in the lung and subsequently metastatic outgrowth of tumor cells.

Work in other models of breast cancer has suggested that other chemokines, such as CCL5, play a critical role in the recruitment of TAMs to the tumor. Antagonists of CCL5 inhibited TAM recruitment in a syngeneic mouse model<sup>44</sup>. Recent work comparing breast tumors before and after EMT has shown that GM-CSF is able to recruit TAMs to the primary tumor<sup>45,46</sup>. CCL5 has also been shown to play a role in the recruitment of T cells. In particular, work has shown that tumor-derived CCL5 can recruit T-regulatory cells (T-regs) into the tumor, leading to CD8+ T-cell apoptosis. Blockade of CCL5 only lead to decreased tumor growth in immune competent models, suggesting that T-cells are required to reduce tumor bulk. This work additionally demonstrated that TGF-beta signaling downstream of CCL5 was required for its pro-tumor activity in blocking CD8+ T-cell activity<sup>47</sup>. Although factors enabling recruitment of pro- metastatic TAMs to mammary tumors have been identified, the regulation of these pathways by metastasis suppressors and the specific phenotypes of these TAMs are poorly understood. Chemokines clearly play a variety of roles in regulating the microenvironment in addition to simply serving as chemotactic factors for monocytes.

### **Mouse models of TNBC**

Early xenograft studies implanting human breast cancer cell lines in immune deficient mice led to the ability to study human tumor cell growth *in vivo* and examine tumor-stromal interactions, as well as study the effect of adding or blocking certain factors on the growth and

spread of the tumor<sup>48</sup>. Implantable tumors can be grown in a variety of immune deficient mice; athymic-nude mice with reduced T-cell development, mice with the severe combined immune deficiency (SCID) mutation lacking T, B, and mast cell development, the SCID-beige (SCID/BG) mutant that additionally lack functional macrophages and natural killer (NK) cells as well as the X-linked immunodeficiency (XID) mutation that leads to deficient B cell and macrophage development<sup>48</sup>. Currently there are 27 human cell lines that can be classified as TNBC and one of the most highly studied human lines of TNBC is MDA-MB-231<sup>49</sup>, which was derived from the pleural effusion of a breast cancer patient with a poorly differentiated tumor that tended toward papillary configuration. Kang et al, 2003 derived a highly bone-metastatic variant of MDA-MB-231 (termed 1833 or BM1), characterized by high levels of connective tissue growth factor (CTGF), interleukin-11 (IL-11), chemokine (C-X-C motif) receptor-4 (CXCR-4), and matrix metalloproteinase-1 (MMP1) expression<sup>50</sup>.

There are additionally many mouse breast cancer cell lines and genetically engineered mouse (GEM) models have been explored to study breast cancer, each with different characteristics that make it more or less similar to certain human breast cancer subtypes. The 4T1 breast cancer model was originally derived from a BALB/cfC3H mammary tumor and spontaneously forms metastases in distant sites including lymph nodes, lung, brain, and bone<sup>51,52</sup>. It has been widely used as a model for macrophage involvement in breast cancer<sup>53,54</sup>.

Microarray analyses have shown that models employing the inactivation of p53 and/or mutations in BRCA1/2 display characteristics most similar to human TNBC patients based on comparisons of miRNA and mRNA expression<sup>55</sup>. To date, the majority of work done to explore the role of macrophages has focused on using the MMTV-PyMT mouse model or the orthotopic

syngeneic model 4T1. However, the PyMT model falls within the set of luminal tumors and most closely clusters with normal mammary tissue from pregnant mice<sup>56</sup>. The C3(1)Tag model uses the SV-40 T antigen under the control of the 5' flanking, C3(1) region of the rat prostate steroid binding protein (PSBP) promoter to silence activity of p53 and Rb in mammary epithelial cells. In this model, the carcinoma develops without the use of repeated pregnancy or hormone stimulation unlike other mouse mammary tumor models<sup>57,58</sup>. It most resembles human TNBC patient gene expression. The cell lines M6 and M6C, derived from C3(1)Tag FVB/N mice, have both been well characterized in syngeneic models for TNBC<sup>59</sup>. The M6C cell line shows a reduction in HMGA2, OPN, and CCL5 as also seen in the BM1 cell line as described by Yun et al, 2011<sup>18</sup>. Additionally, the E0771 and E0771-LNB (referred to as LNB) mouse tumor cell lines were derived from a C57BL/6 tumor. These lines are triple-negative and have been described as basal-like. The LNB line forms spontaneous metastases in the lungs of C57Bl/6 mice<sup>60</sup>.

## **Exosomes**

Exosomes are small micro-vesicles with a double lipid bilayer, secreted by most cell types, of endocytic origin<sup>61</sup>. They are generally between 40 to 150 nm in diameter, with sizes ranging depending on their origin<sup>62</sup>. The first description of exosomes was published in the early 1980s describing micro-vesicles as 'exfoliated particle' from cells that had a similar ecto-enzyme composition as the cell of origin in culture<sup>63</sup>. Subsequent work in reticulocytes established that exosomes were formed through invagination of the endosome membrane to form multi-vesicular bodies (MVBs)<sup>64,65</sup>. Alternative pathways of secretion occur through pinching off of vesicles at the plasma membrane. However, studies of the origin of exosomes have shown they

differ greatly from other vesicles shed at the membrane, such as apoptotic bodies, and are derived from endosomal origin rather than from the plasma membrane<sup>61,66</sup>. This work demonstrated that proteins found in exosomes often lack expression of surface receptors of high abundance on the cell of origin, as well as proteins found in the Golgi apparatus, mitochondria, and endoplasmic reticulum. Protein components primarily found in exosomes originate from the plasma membrane, endosome, or cytosol<sup>66</sup>. Exosomes, as expected from their origin within the endosome and MVBs, should be enriched for proteins of the endosome compared to the membrane. Membrane proteins are more likely to be found on micro-vesicles formed at the plasma membrane<sup>61</sup>. However, some plasma membrane proteins are enriched on exosomes and help to distinguish them from other micro-vesicles shed from alternative pathways, including CD63 and TSG101<sup>61</sup>. Other cargo in exosomes includes cellular DNA, all types of RNAs, enzymes, and other cellular proteins<sup>61</sup>.

Exosomes form within the late endosome pathway in MVBs, which have a variety of functions including uptake of material targeted for degradation and sorting of material in the endocytic pathway, as well as uptake of material to be secreted<sup>67</sup>. It has been well established that the Rab family of proteins, known for tethering of vesicles prior to fusion with target membranes<sup>68</sup>, is essential for the trafficking of exosomes, their loading, and release<sup>61,68-71</sup>. In particular, knock-down screens of genes involved in exosome secretion identified Rab27a as necessary for full secretion of exosomes from cells. Rab27a is required for docking of MVBs at the plasma membrane<sup>71</sup>. The ESCRT complex additionally assists in MVBs sorting to the membrane before being assisted by Rab proteins to dock and fuse with the plasma membrane via SNARE mediate membrane fusion to release exosomes into the extra-cellular space<sup>72</sup>.

Additional proteins that have been shown to regulate endosomal sorting and exosome secretion include Rab37, involved in endosomal sorting to the membrane<sup>69</sup>. Other work has shown a clear role of Rab27a in regulating degranulation of mast cells, while Rab37 demonstrated a conflicting role<sup>73</sup>. TSG101, a critical component of the ESCRT-1 complex functions in endosomal sorting of exosomes and is found in exosomes from many different cell types<sup>74</sup>. However, inhibition of TSG101 in tumor cells has been shown to decrease growth, migration, and or invasion rates, suggesting it may not be an ideal target to block to study exosome biology *in vivo* during tumorigenesis and metastasis<sup>75</sup>. The most widely used protein to block exosome secretion in cells for *in vivo* studies is Rab27a<sup>61,71</sup>.

The function of exosomes has been somewhat controversial, some suggesting that they are simply a 'cellular garbage bag' as so many cellular components can be found in them. This could be supported by the fact that the process of phagocytic engulfment of material has some resemblance to the processes of exosome formation in MVBs<sup>76</sup>. The ESCRT proteins are essential in the sorting of both exosomes as well as proteins bound for degradation through autophagy. The pathways underlying the sorting of cargo into exosomes need to be further elucidated to better understand how and what material they are shuttling between donor and target cells.

However, several lines of evidence suggest that exosomes differ from these particles. Rab proteins, such as Rab27a, seem to be important for docking at the plasma membrane<sup>71,77</sup>, suggesting that knock-downs of that protein would alter exosome release but not autophagy. Additionally, some confusion arises from contamination of exosomes with apoptotic bodies. Depending on the method of isolation, apoptotic bodies can be isolated along with exosomes. However, these vesicles are formed by blebbing off from the cell membrane, which differs from

the endosomal origin of exosomes. Apoptotic bodies are removed during isolation methods by spinning at 10,000 x g or more commonly by utilizing a 0.22 um syringe filter. Microscopy of isolated vesicles using a nanoSight will also tell you if you have larger vesicles present. Additional validation can be done by staining for markers seen only on exosomes, such as CD63 and TSG101, to determine the purity of isolation.

### **Exosomes in cancer and metastasis**

The role of exosomes has been well established in the creation of a pre-metastatic niche<sup>78,79</sup>. They are essential players in transmitting information from tumors to distant sites throughout the body, altering gene expression. Work from multiple groups has demonstrated that exosomes from tumor cells are preferentially taken up by certain tissue types. One major example is work from David Lyden's group that established exosomes play a critical role in organotropic metastasis<sup>79,80</sup>. Utilizing the bone tropic, lung tropic, and brain tropic derivatives of MDA-MB-231, they established that exosomes from these cell types will preferentially accumulate in the organ corresponding to the metastatic target of the parent tumor cell line. Membrane integrins played a critical role in regulating the cellular target of these exosomes. Blocking exosome uptake with neutralizing antibodies against each integrin or by knocking out the specific integrin on the parental cell blocked the uptake of exosomes at pre-metastatic sites and subsequently blocked the formation of the pre-metastatic niche. Additionally, Integrin levels in human breast cancer patient exosomes were prognostic for outcome<sup>79</sup>. Pancreatic tumor exosomes preferentially target Kuepfer cells (tissue resident macrophages within the liver).

Uptake by these cells helps drive them toward a pro-tumor phenotype establishing a pre-metastatic niche in the liver for pancreatic tumor cells.

Exosomes can also be utilized as biomarkers in biofluids of cancer patients. One example is through differential expression of proteins on the surface of tumor exosomes associated with poor outcome. In breast cancer patients, integrins present on the surface of patient exosomes are predictive of outcome<sup>79</sup>. In TNBC, higher levels of expression of TSG101 have been associated with resistance to paclitaxel treatment in vitro<sup>81</sup>. In addition to RNA, proteins, and enzymes, exosomes also carry double stranded DNA from their parent cell. Sequencing of genomic DNA within exosomes from patient-derived as well as from tumor cell lines has demonstrated that most tumor mutations can be found in the exosomes of patients, and DNA from all chromosomes is present in the exosomes<sup>82,83</sup>. Clinically important mutations in KRAS as well as p53 are seen in pancreatic ductal adenocarcinoma patient exosomes. Additionally, studies in patients with non-small cell lung cancer (NSCLC) that become resistance to EGFR inhibitors has shown that T790M mutations associated with drug resistance can frequently be found in exosome samples, but not necessarily in every tumor biopsies depending on sampling region, and vice-a-versa<sup>84</sup>. This suggests that liquid biopsies may be important in correctly diagnosing patients as well as predicting recurrence in addition to tumor biopsies. These liquid biopsies can come from many sources including the plasma, ascites fluid, or lymphatic fluid of patients with cancer<sup>85</sup>. An additional source of diagnostics has been through the study of RNAs present in tumor patient exosomes<sup>86</sup>. While the molecules found within exosomes can be very powerful diagnostic tools for outcome and recurrence, they are often simply too small a signal among the many exosomes present in the blood stream from immune cells. One method to overcome this issue is the use of

microfluidic devices in conjunction with tagged antibodies specific to tumor cell exosomes. These methodologies are very new and will need to be further refined, but will allow for a much greater ability to specifically study tumor exosomes from patients and more precisely examine their prognostic potential based on *in vitro* studies.

### **The effects of exosomes on immune cells**

One of the most well defined roles of exosomes on immune cells has been the transfer of information between and to immune cells during infection. Roposo *et al* demonstrated that B cells that have become infected with the Epstein-Barr Virus (EBV) shed exosomes with viral antigens. These antigens were bound to MHC class II on the surface of exosomes and induced a T-cell response<sup>87</sup>. This research demonstrated that tumor signals can be sent distantly in the body not only to trigger pro-tumor effects, such as the formation of the pre-metastatic niche, but also to present antigens from infected cells to distant cells, triggering an immune response. This suggests a broader role of exosomes in the transfer of information between cells regardless of the distance between them. Another example of infected cells transmitting signals to regulate the activity of immune cells are those infected with Ebola virus. Virally infected cells transmit VP40 protein in exosomes to immune cells, making them less able to respond to viral infection<sup>88</sup>. Just as cells can trigger an immune response by the transfer of viral antigens via MHC on the surface of exosomes distantly to other immune cells, antigen presenting cells can utilize exosomes for distant stimulation of T cells. Work from Zitvogel *et al* demonstrated that when dendritic cells (DCs) were pulsed with tumor antigens, exosomes from these DCs could activate T cell mediated tumor control<sup>89</sup>.

Tumor cells can also modulate the activity of immune cells directly. Gene expression can be drastically modulated in T cells that are exposed to tumor exosomes, thereby altering their function in the tumor environment<sup>90</sup>. Tumor cell exosomes can also be the source of antigens taken up by antigen presenting cells, leading to cross-priming of T cells and subsequent tumor control and rejection<sup>91</sup>. However, this work demonstrated that stimulation by tumor exosomes could not lead to DC maturation. Numerous studies have demonstrated that stimulation of macrophage cell lines with tumor exosomes can stimulate secretion of pro-tumor cytokines dependent on TLR signaling<sup>92-94</sup>. However, the necessity of exosomes in programming naïve macrophages to a pro-tumor phenotype has not been established.

## **Summary**

Women with triple-negative breast cancer (TNBC), the most aggressive subtype of breast cancer, have the shortest survival times and the highest rate of relapse. While strides have been made in treating patients with ER+ breast cancer with estrogen antagonists and HER2+ breast cancer with HER2 neutralizing antibodies, TNBC patients have no currently available forms of targeted therapy<sup>1</sup>, and most (~77%) succumb to metastases within 5 years. Additionally, the nature of how metastasis relies on signaling from the tumor microenvironment is not well understood, particularly in TNBC patients. The ability to understand these interactions could both inform clinicians about risk factors leading to TNBC as well as lead to potential therapeutic targets.

In particular, macrophages have also been linked to human breast cancer invasion and metastasis as well as drug resistance, and recently have been implicated in TNBC<sup>27,37,95</sup>. In

general, the M1-like pro-inflammatory macrophages are thought to suppress TNBC metastasis, whereas the tumor-associated macrophages (TAMs) are characterized as M2-like pro-metastatic macrophages. However, recent proteomic and RNA studies indicate that macrophage populations are composed of more phenotypic subtypes than previously recognized<sup>96</sup>.

The phenotype of TNBC TAMs and the mechanisms by which they are recruited and interact with TNBCs tumor cells are not well understood and require further characterization. Some studies have implicated tumor secreted exosomes in modulating the nature of TAMs *in vitro*, but no studies to date have explored whether exosomes are required for TAM programming *in vivo*. Exosomes have been shown to play integral roles in transferring information to and between immune cells. Antigen presenting cells are known to take up tumor material, antigens, or exosomes that can alter their function and signal other immune cells to alter their effect on tumor growth and invasion. While macrophages in particular are known to take up tumor exosomes that trigger changes in function, the necessity for exosome transfer of information from tumor cells to TAMs is not known.

Therefore, this work sought to understand the mechanism regulating the recruitment of TAMs into the core of highly metastatic TNBC tumors in addition to TNBC-TAM crosstalk directing polarization of macrophages to a pro-metastatic, TAM phenotype. Chapter 3 describes our work to understand how the metastasis suppressor, RKIP, regulates the microenvironment. We sought to elucidate how RKIP can regulate the recruitment of TAMs, as well as the phenotype of recruited TAMs. We sought to establish that the chemokine CCL5 was not only recruiting TAMs, but also regulating their phenotype. Utilizing patient expression data, we examined whether genes highly expressed in TAMs in TNBC patients were prognostic for survival. Chapter 4 sought

to establish the mechanism by which TAMs are programmed or polarized by TNBC cells. Exosomes play a critical role in transmitting signals from tumor cells to macrophages to program them. We additionally sought to examine the role CCL5 and RKIP could play in regulating this programming, as their expression is critical for TAM recruitment *in vivo*. Our work sought to show that phenotypes observed in tumor-derived TAMs were recapitulated by exosome programming of macrophages *in vitro*. Additionally, we sought to examine whether tumor exosomes were essential for the recruitment and programming of TAMs.

## CHAPTER 2: MATERIALS AND METHODS\*

### Cell Culture

MDA-MB-436, MDA-MB-231, 293T, L929, and 4T1.2 were obtained from ATCC. MDA-MB-231 1833 (referred to as BM1) cells were obtained from Andy Minn and E0771-LNB cells were obtained from Robin Anderson<sup>60</sup>. Numerous vials were frozen upon original receipt of the cells, and all work was done within 15 passages of the initially received lines. Late passage cells were sent to Idexx for cell line authentication using STR analysis. BM1, MDA-MD-436, 4T1.2, and LNB cell lines were cultured in DMEM media supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin and L929 grown in RPMI 1640 with the same supplementation as above. Cells were transduced with lentiviral vectors for shRNA knockdown or overexpression from GE/Dharmacon. Cells were selected for 14 days using 3 µg/ml of puromycin or 10 µg/ml of blasticidin after lentiviral transduction before use.

### Lenti-viral Transductions

All lenti-viral work was done according to institutional biosafety rules, utilizing BSL3 practices and performed in a BSL2 hood. One million 293T cells were plated in a T-25 flask the evening prior. The following day, lentiviral vectors were incubated with 3<sup>rd</sup> generation viral packaging vectors and Transit LT-1 for 30 mins as described by the manufacturers protocol. DNA/LT-1 mixtures were then used to transfect 293T cells for viral production. Transfected cells

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\* Sections of this chapter have been reprinted with additional resources in part from the supplemental methods of Frankenberger et al<sup>120</sup> under the fair use policy of Cancer Research

were grown for 24 – 48 hours prior to viral harvesting. After incubation, viral containing media was removed, centrifuged at 2,000 x g to removed dead cells and debris, then filtered through a 0.45 um PES syringe filter. Polybrene was added to media for a final concentration of 8 ng/ml. Media was then added to target cells. Following a 24-hour transduction period, cells were washed, trypsinized and plated. All viral waste was decontaminated with 10% bleach prior to disposal. Transduced cells were then selected under high antibiotic concentrations for 10 days to ensure only transduced cells remained alive. Antibiotics were not used for subsequent cell culture.

### **CCL5 Knock-Out**

Lentiviral all-in-one plasmids containing Cas9 as well as guide RNAs were purchased from Applied Biological Materials. Virus was produced as described above. BM1 cells were then infected, then selected for 14 days using 3 ug/ml of puromycin. Transduced cells were assayed for CCL5 expression using a CCL5 ELISA from Ray Biotech. Samples were concentrated 10x to ensure even low levels in KO cells could be measured.

### **qRT-PCR Primers**

Hs GAPDH-F: TGCACCACCACCTGCTTAGC

Hs GAPDH-R: GGCATGGACTGTGGTCATGAG

Mm Hmga2-F: GAGCCCTCTCCTAAGAGACCC

Mm Hmga2-R: TTGGCCGTTTTTCTCCAATGG

Hs CCL5-F: CCAGCAGTCGTCTTTGTCAC

Hs CCL5-R: CTCTGGGTTGGCACACACTT  
Mm Ccl5-F: TTTGCCTACCTCTCCCTCG  
Mm Ccl5-R: CGACTGCAAGATTGGAGCACT  
Mm Slpi-F: GGCCTTTTACCTTTACGGTG  
Mm Slpi-R: TACGGCATTGTGGCTTCTCAA  
Mm Mmp12-F: CTGCTCCCATGAATGACAGTG  
Mm Mmp12-R: AGTTGCTTCTAGCCCAAAGAAC  
Mm Ccl7-F: GCTGCTTTCAGCATCCAAGTG  
Mm Ccl7-R: CCAGGGACACCGACTACTG  
Mm Tnfr2-F: ACACCCTACAAACCGGAACC  
Mm Tnfr2-R: AGCCTTCCTGTCATAGTATTCCT  
Mm Grn-F: ATGTGGGTCCTGATGAGCTG  
Mm Grn-R: GCTCGTTATTCTAGGCCATGTG  
Hs Grn F: ATCTTTACCGTCTCAGGGACTT  
Hs Grn R: CCATCGACCATAACACAGCAC  
Hs TNFR2 F: CGGGCCAACATGCAAAAGTC  
Hs TNFR2 R: CAGATGCGGTTCTGTTCCC  
Hs CCL5 Sequencing F: TTAGGGGATGCCCTCAACT  
Hs CCL5 Sequencing R: CTGAGACTCACAGACTGCTG  
Hs Rab27a-F: GCTTTGGGAGACTCTGGT  
Hs Rab27a-R: TCAATGCCCACTGTTGTGATAAA  
Mm Rab27a-F: TCGGATGGAGATTACGATTACCT

Mm Rab27a-R: TTTTCCCTGAAATCAATGCCCA

### **Antibodies, Cytokine Arrays, ELISAs**

RKIP (derived in lab from serum of rabbits exposed to an RKIP peptide)

CCL5 antibody (AF-278-NA, R&D Systems)

CCL5 ELISA (ELH-RANTES-1, Ray Biotech)

Mouse Cytokine Array (L308, Ray Biotech)

Rab27a (AF7245, R&D Systems)

### **Invasion Assays**

As previously described,  $2 \times 10^4$  BM1 cells or  $1 \times 10^5$  E0771-LNB cells were plated in 24-well trans-well inserts with 8  $\mu\text{m}$  pores (Corning) coated with growth factor depleted basement membrane extract (Trevigen)<sup>97,98</sup>. After incubating at 37 C for 24 hrs, inserts were transferred to an empty well and stained with 4 ng/ $\mu\text{l}$  of Calcein AM (Corning) for one hour. Stained cells were gently wiped with Q-tips to remove cells on the top layer of the insert, then placed in non-enzymatic dissociation solution (Trevigen) using gentle shaking for one hour at 37 C and 150 RMP. Fluorescence was measured using a Victor X3 fluorescent plate reader with excitation at 465 nm and emission at 535 nm.

### **Tumor educated macrophage programming**

Bone marrow was isolated from the femur and tibia of 6-10 week old C57Bl/6 mice (Charles Rivers). Red cell lysis buffer (Santa Cruz) was used to removed red blood cells. Remaining

bone marrow was counted and 1 million cells were plated per well in a 6-well plate and cultured in RPMI 1640 supplemented with 10% FBS (Corning) and 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen) and 30% L929 Conditioned Media. Media was replenished at days two and six during culture. On day seven, bone-marrow derived macrophages (BMDMs) were washed 2-3 times with PBS (Corning) and then treated with tumor cell conditioned media or serum free media containing isolated tumor exosomes supplemented with 20 ng/ml of mouse M-CSF. BMDM negative controls were grown only in serum free DMEM with 20 ng/ml of M-CSF. Exosome size and numbers were determined after isolation using a nanoSight microscope.  $1 \times 10^8$  exosomes/mL were used for programming with BM1 tumor exosomes or  $1 \times 10^9$  exosomes/mL for LNB exosomes.

For TEMs, after programming, cells were washed with PBS 2-3 times. Then 1 mL of serum free DMEM was added. After incubation at 37 C for 24 hours, media was removed and cells and cell debris were removed by centrifugation at 2,000 x g for 10 mins.

For co-injection, TEMs were injected into the fat pad of C57Bl/6 mice mixed with E0771-LNB cells at a ratio of 1 TEM: 1 Tumor cell. Lung mets were assayed at the end of the study by fixing in formalin and sectioning. Six 5 µm sections were quantified for number and size of metastases. Sections were 100 µm apart, and number of metastases counted were added to give number of metastases per lung.

### **Tumor Associated Macrophage Isolations**

As previously described<sup>98</sup>, tumors were grown to approximately 0.75 g before being harvested. Tumors were dissociated both physically with scissors to 1-2 mm pieces and using C-

tubes and a gentleMACS dissociator (Miltenyi Biotech) as well as enzymatically using the human tumor dissociation kit (Miltenyi Biotech). Cells were filtered through a 70 um mesh filter. Mononuclear cells were isolated using Ficoll-Paque PREMIUM (GE Healthcare) gradient centrifugation at 420 RPM for 45 minutes. Macrophages were then obtained using CD11b positive selection beads (Miltenyi Biotech). Flow cytometry with CD11b, F4/80, CD45, CD11c, CD205, and CCR5 was performed to determine the purity and heterogeneity of isolated TAMs.

For tumor derived macrophages,  $1 \times 10^6$  TAMs were plated in one well of a 6-well plate. After 30 minutes, cells were washed with PBS to ensure only viable macrophages attached to the plate remained. Cells were incubated for 24 hours to obtain conditioned media in serum free DMEM. Cells and cell debris were removed by centrifugation at 2,000 x g for 10 mins prior to use in subsequent assays.

For co-injection studies, isolated TAMs were immediately injected into the fat pad of nude mice mixed with MDA-MB-231 tumor cells at a ratio of 1 TAM: 2 Tumor cells.

### **Conditioned Media**

For THP-1 conditioned media,  $5 \times 10^6$  THP-1 cells were plated in a T-75 flask with 5 mL of 10% serum containing DMEM. Media was collected after 24 hours and cells and cell debris were removed by centrifugation.

For tumor derived macrophages,  $5 \times 10^5$  TAMs were plated in one well of a 6-well plate. After 30 minutes, cells were washed with PBS to ensure only viable macrophages attached to the plate remained. Cells were incubated for 24 hours to obtain conditioned media in serum free DMEM. Cells and cell debris were removed by centrifugation.

## Statistical Analysis

Statistical analysis for patient data sets was done using R and is described in those sections of Materials and Methods. Please refer there for further detail on tests used.

Otherwise, all graphics were made and all statistical analysis was done using Graph Pad Prism. Unless otherwise noted, bar graphs represent the mean ( $\pm$  standard error of the mean (SEM))

For comparing statistical differences between means, a T-test was used. For samples where there was no significant difference in variance between samples and samples were normally distributed or where sample size was too small to determine distribution type, a Student's T-test was used. If sample variance differed significantly between groups, Welch's correction was taken when the T-test was performed (does not assume equal variance between groups during test). If the samples were not normally distributed, a Mann-Whitney T test was used (does not assume normal distribution of samples).

The following was used to denote p-value unless state otherwise in figure legends: \*  
 $0.05 > p \geq 0.01$ ; \*\* $0.01 > p \geq 0.001$ ; \*\*\*  $0.001 > p \geq 0.0001$ ; \*\*\*\*  $0.0001 > p$

## Mice

All mice were housed and handled according to the University of Chicago Institutional Animal Care and Use Committee guidelines. Athymic nude, Balb/c mice, and C57Bl/6 were purchased from Charles Rivers. Mice were injected with  $2 \times 10^6$  human or  $5 \times 10^5$  mouse tumor

cells. Tumor volumes were measured twice per week and calculated as  $\text{volume} = (\pi/6) \times \text{width}^2 \times \text{length}$ . Tumors were grown to  $\sim 500 \text{ cm}^3$  and removed for analysis.

C57BL/6J-*Wnt1-Hmga2*<sup>-/-</sup> and C57BL/6J-*Wnt1-Hmga2*<sup>+/+</sup> mice were provided by the Chada lab and have been described previously. PCR-based genotyping was performed for the *Hmga2* and *Wnt* locus.

Maraviroc Treatments: Tumors were allowed to engraft for 3 days prior to treatment. After 3 days, mice were treated every 12 hours for 21 days by oral gavage and tumors measured twice weekly. Control mice received 0.1 mL of water with an equivalent amount of DMSO used to dissolve Maraviroc. Treated mice received 8 mg/kg of Maraviroc every 12 hours. Statistical differences in F4/80 staining were determined by using a Mann-Whitney T-test between treated and untreated groups, while difference in tumor weight utilized an unpaired T-test with Welch's correction. Statistical differences in tumor growth were determined using a paired T-test. A Pearson correlation was used to compare the correlation between tumor weight and %F4/80+ cells.

## **Immunohistochemistry**

All immunohistochemistry was performed at the University of Chicago Human Tissue Resource Center. Tissue sections were deparaffinized and rehydrated through xylenes and serial dilutions of EtOH to distilled water, and then incubated in antigen retrieval buffer at 97°C for 20 minutes. Primary and secondary antibody incubations were carried out in a humidity chamber at room temperature, and detected using an Elite kit (PK-6100, Vector Laboratories) and DAB (DAKO, K3468) system according to the manufacturers' protocols. Following staining, tissue

sections were briefly immersed in hematoxylin for counterstaining and were covered with cover glasses. Stained tissue sections were scanned at 20X magnification and analyzed using Aperio Imagescope ePathology® software. To quantify infiltrating macrophages, the number of pixels positive for staining were normalized to total number of pixels inside the tumor stroma border. Alternatively number of F4/80 positive cells per area were measured.

### **RNAseq**

For RNAseq experiments, we injected one rear mammary fat pad from each of five athymic nude mice with MDA-MB-231-derived BM1 breast carcinoma cells suspended in PBS and allowed tumors to develop for four weeks. We then sacrificed the mice and extracted both the tumor-bearing and uninjected fat pads, bisected each fat pad, and separately isolated RNA from each tissue section. We then sequenced these RNA samples using the Illumina HISEQ2000 platform and separated the mouse and human reads to generate tumor-specific and stroma-specific gene expression estimates. We compared these estimates with similarly-derived expression estimates from fat pads taken from mice injected with BM1 cells stably expressing the RKIP metastasis suppressor at physiological levels, and from mice that were sham injected and do not contain developing tumors.

Tumor samples were preserved in 2ml RNAlater (Ambion) prior to analysis. RNA was extracted using the RNEasy Mini kit (Qiagen) according to the manufacturer's protocol under RNase-free conditions and performing the optional 5-minute on-column treatment with DNase I to remove contaminating genomic DNA from the RNA sample. Total RNA was eluted in RNase free water (Qiagen), and samples were aliquoted for analysis and stored at -80°C until further

use. After ensuring that the extracted RNA samples were of high quality using an Agilent Bioanalyzer (RIN  $\geq$  8.5), we used the RNA to generate sequencing libraries using the Illumina Truseq RNA kit according to the manufacturer's protocol, and further size-selected each sample via polyacrylamide gel extraction using the MinElute Gel Extraction Mini Kit (Qiagen). The libraries were subsequently analyzed a second time using an Agilent Bioanalyzer to quantitate the library concentration and to verify that fragment sizes were consistent across samples prior to sequencing.

We sequenced all samples in multiplex (8 samples per lane) using an Illumina HISEQ2000 to generate 108bp single-end reads. We chose to generate long reads rather than paired-end reads because they contain more sequence information and are consequently easier to separate by species origin. A total of three flow cells were sequenced for the mouse samples with the lane assignments permuted. Technical replicates were never sequenced on the same lane.

Next, we separated sequencing reads in each sample by species origin. Our approach is conceptually similar to previously published methods, but modified slightly to improve recovery of annotated transcripts. First, we used bwa (Li and Durbin, 2009) to align the raw \*.fastq file to four sequence assemblies: the full set of human Refseq mRNA transcripts (hg19), the full set of mouse Refseq mRNA transcripts (mm9), the human genome (hg19), and the mouse genome (mm9). This resulted in four separate \*.sai files for each sample, which we then converted to \*.sam files using samtools (Li et al., 2009). We then jointly analyzed the \*.sam files to determine to which of the four assemblies each read could be aligned.

Reads that aligned uniquely to the transcriptome assembly in one species but did not align to any of the other assemblies, were considered transcriptome-specific and added to the

transcriptome-specific \*.sam file for the appropriate species. Reads that could be aligned to any combination of assemblies from both species, or which could be aligned to multiple genes or genomic regions within a species were considered ambiguous and removed from further analysis. We used the transcriptome-specific \*.sam files to estimate expression levels for each gene by counting the number of reads that were unambiguously aligned to each gene. In our analysis, regions within a given transcript may be functionally unalignable because of sequence similarity between species, and so both the overall length of each transcript and the expected distribution of reads across it is unknown. This prevents us from meaningfully estimating individual isoforms or splice variants, and we consequently chose to perform a gene-wise analysis by combining reads mapped to all known isoforms for a given gene into a single expression estimate. Specifically, we generated gene expression estimates as the number of reads that could only be aligned to transcript assemblies annotated to a single gene, even if the read could be aligned to multiple places within a transcript, or to multiple isoforms that were annotated to the same gene.

### **Categorical Analysis of RNAseq mRNA expression**

To perform ontological enrichment analyses we compared the specified set of significant genes to the total set of successfully-modeled genes in the corresponding dataset. We identified enriched categories with the GOrse function implemented in GOrse using the default settings (Wallenius approximation), and corrected p-values for multiple testing using the Benjamini and Hochberg approach.

We used GOrse to determine the ontological categories enriched within sets of genes whose boundaries may be clearly defined (e.g., differential expression at an FDR of 0.01). For

statistics that are continuous and in which appropriate cutoff values are less clear, such as correlation coefficients, we used GSEA with the statistics considered as a ranked list using the default settings.

### **Enrichment of differential expression in cell type-specific gene sets**

To determine whether differences in gene expression observed in our data were consistent with infiltration of specific cell types into the tumor microenvironment, we used the RefDIC database of mouse immune cell expression data (23) to identify genes whose expression was highly specific to a particular immune cell type, and then determined whether there was disproportionate evidence that these genes were differentially expressed within the set of P-values quantifying the evidence for altered expression levels between metastatic and non-metastatic stroma tissue. To do so, we used the RefDIC Specific Gene Finder tool using the default settings to extract the set of genes whose expression is specifically upregulated in each of 13 specific immune cell types. Each cell type was analyzed using all P-values from genes specific to the cell type in question, with all of the P-values used as the background set.

We determined whether each set of genes specifically upregulated within each cell type was disproportionately differentially expressed within our data using an approach combining bootstrapping with standard visualization using a quantile-quantile plot (Q-Q plot). In a standard Q-Q plot an observed distribution of n values (typically P-values) is compared to the theoretical distribution by ordering the observed values and plotting them against n ordered random draws from the theoretical distribution as the respective independent and dependent variables in a 2d plot. In this visualization strategy, deviations above the line  $x=y$  correspond to an enrichment of

values more extreme than are expected to result from random draws from the theoretical distribution.

We used a bootstrapping strategy to compare the P-values observed for the genes specific to each immune cell type to the overall distribution of P-values summarizing the evidence for differential expression between metastatic and non-metastatic stroma. For each gene set containing  $n$  genes whose expression is specific to a particular cell type, we randomly sampled 100 sets of  $n$  P-values from the full distribution using the weights predicted by the PWF generated above, rank-ordered them, and then took the mean and standard deviation of the P-values at each rank. We then used these values as the dependent variables in the Q-Q plot, and determined divergence from  $x=y$  in excess of the standard deviation by inspection. The entire set of Q-Q plots for this analysis is included as Supplementary Figure S5.

### **Patient Data Sets**

Four datasets were RMA pre-processed, median centered by sample, and z-score transformed: BrCa871 ( $n = 871$ ; consisting of GSE1456, GSE2990, GSE3494, GSE7390, and GSE11121), BrCa443 ( $n = 443$ ; consisting of GSE5327, GSE2034, and GSE2603), BrCa341 ( $n = 341$ ; consisting of GSE6532, GSE12093, GSE31519), and BrCa295 (NKI-295) as done previously (9)

### **Patient Data Classification**

Patients were classified into TNBC and non-TNBC categories using an algorithm previously described (30). Briefly, mRNA expression of estrogen, progesterone and her2/neu receptor genes was modeled as bimodal distribution of two Gaussian peaks then binned into positive or

negative expressing patients. Those patients classified as absent all three receptors were categorized triple negative.

### **Patient Data Correlation**

mRNA expression levels of genes were compared between TNBC and non-TNBC patients using a Student's T-Test to predict if a significant difference exists in the mean gene expression of the two patient populations.

### **Patient Data Survival**

We combined RKIP, HMGA2, CCL5 genes with a macrophage metagene to produce a simple classifier representing activation of a signaling pathway defined by the experimental results represented in this manuscript. The four components were designated independently as high or low as to whether they are above (in the case of HMGA2, CCL5 and the macrophage metagene) or below (in the case of RKIP) median expression across each data set for the level of mRNA in each component in each patient. For patients that have 3 of the 4 components designated, we declare the pathway to be "active" and stratify in to two groups based on this.

We tested the significance of the association to metastasis free survival in the framework of the model using a log rank test.

## **CHAPTER 3: Metastasis suppressors regulate the tumor microenvironment by blocking recruitment of pro-metastatic tumor-associated macrophages<sup>98, †</sup>**

### **Abstract**

Triple-negative breast cancer (TNBC) patients have the highest risk of recurrence and metastasis. Because they cannot be treated with targeted therapies, and many do not respond to chemotherapy, they represent a clinically underserved group. TNBC is characterized by reduced expression of metastasis suppressors such as Raf Kinase Inhibitory Protein (RKIP), which inhibits tumor invasiveness. Mechanisms by which metastasis suppressors alter tumor cells are well characterized; however, their ability to regulate the tumor microenvironment, and the importance of such regulation to metastasis suppression is incompletely understood.

Here we use species-specific RNA sequencing to show that RKIP expression in tumors markedly reduces the number and metastatic potential of infiltrating TAMs. TAMs isolated from non-metastatic RKIP+ tumors, relative to metastatic RKIP- tumors, exhibit a reduced ability to drive tumor cell invasion and decreased secretion of pro-metastatic factors including PRGN and shed TNFR2. RKIP regulates TAM recruitment by blocking HMGA2, resulting in reduced expression of numerous macrophage chemotactic factors, including CCL5. CCL5 overexpression in RKIP+ tumors restores recruitment of pro-metastatic TAMs and intravasation, while treatment with the CCL5 receptor antagonist Maraviroc reduces TAM infiltration. These results highlight the importance of RKIP as a regulator of TAM recruitment through chemokines such as CCL5. The

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clinical significance of these interactions is underscored by our demonstration that a signature comprised of RKIP signaling and pro-metastatic TAM factors strikingly separates TNBC patients based on survival outcome. Collectively, our findings identify TAMs as a previously unsuspected mechanism by which the metastasis suppressor RKIP regulates tumor invasiveness, and further suggest that TNBC patients with decreased RKIP activity and increased TAM infiltration may respond to macrophage-based therapeutics.

## **Introduction**

Of the approximately 40,000 women diagnosed with breast cancer each year, 15-20% will have triple-negative breast cancer (TNBC). The most aggressive subset of breast cancer, TNBCs lack expression of the estrogen, progesterone and HER2/neu receptors. While clinical outcomes have improved for many patients with breast cancer, TNBC patients have higher rates of metastasis, more aggressive tumors, higher disease burden, and early recurrence<sup>99</sup>. Additionally, this disease disproportionately affects African-American women, with rates approximately three times higher in African-American women<sup>3,10</sup>. Moreover, only 30% of TNBC patients are responsive to platinum based chemotherapy<sup>100</sup>. Therefore, there is interest in novel approaches for treating TNBC patients, including targeting of the tumor stroma<sup>5</sup>.

One possible strategy is to mimic the action of physiological tumor metastasis suppressors such as Raf Kinase Inhibitory Protein (RKIP). RKIP, a member of the evolutionarily conserved phosphatidylethanolamine family, has been implicated as a metastasis suppressor for prostate, breast and other solid tumors<sup>16,97,101</sup>. RKIP inhibits key signaling pathways including Raf/MAP kinase, GRK2-regulated  $\beta$ -adrenergic receptor, and NF- $\kappa$ B activation<sup>101</sup>. Previously, RKIP was

shown to suppress the expression of many pro-metastatic genes in TNBC cells by inhibiting transcriptional regulators such as HMGA2<sup>13,18,97</sup>. Because previous studies have focused on the effects of metastasis suppressors in tumor cells, their role in regulating the tumor microenvironment is unknown.

Multiple lines of evidence have shown that the microenvironment regulates both tumor progression and metastasis. In particular, macrophages have been shown to play a dual role in tumor growth, either driving tumor rejection or tumor progression depending on the type of macrophage activation<sup>37</sup>. Classical activation of macrophages by IFN $\gamma$ , lipopolysaccharide (LPS), or tumor necrosis factor-  $\alpha$  (TNF  $\alpha$ ) leads to polarization of M1 macrophages that secrete inflammatory cytokines important in the body's anti-tumor response. M2 macrophages, activated by factors such as IL4, play an essential role in wound healing. Secretion of factors from tumor-associated macrophages (TAMs), thought to be M2, leads to tumor growth, progression, and metastasis<sup>26,38,39</sup> as well drug resistance<sup>40</sup>. However, recent evidence suggests that this division of macrophages into two discrete subtypes incompletely describes the range of macrophage phenotypes present in the tumor microenvironment<sup>41</sup>. Importantly, studies of breast cancer patients show that CD163+ macrophage recruitment positively correlates with TNBC while negatively correlating with ER+ and luminal tumors<sup>27</sup>. Therefore, recruitment of alternatively activated TAMs could play a significant role in the outcome of TNBC patients and explain their poor prognosis.

TAMs are recruited to mammary tumors through induction of a variety of cytokines and chemokines, where they play essential roles in driving metastasis. For example, TAMs recruited by CSF-1 express higher levels of VEGF-A, with increased angiogenesis in the polyoma middle T

genetically engineered mouse model for breast cancer<sup>42</sup>. Similarly, CCL2 was required for TAM infiltration in primary breast tumors as well as TAM-enabled metastatic colonization of lungs<sup>43</sup>. Antagonists of the CCL5 receptor (CCR5) inhibited TAM recruitment in a syngeneic mouse model<sup>44</sup>. Finally, recent work comparing breast tumors before and after EMT has shown that GM-CSF is able to recruit TAMs to the primary tumor<sup>45,46</sup>. Although factors enabling recruitment of pro- metastatic TAMs to mammary tumors have been identified, the regulation of these pathways by metastasis suppressors and the specific phenotypes of these TAMs are poorly understood.

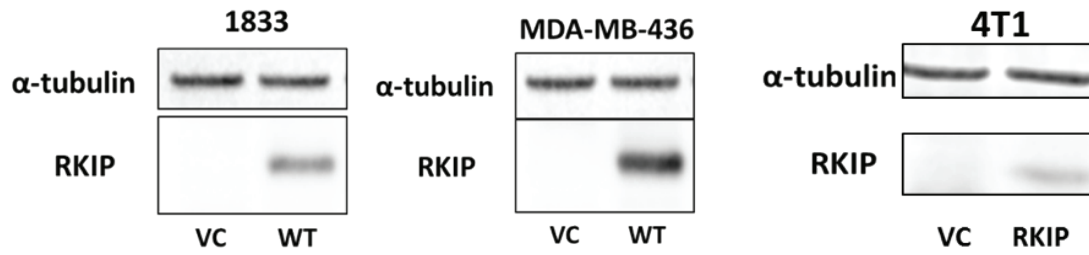
Here we combine species-specific RNA sequencing, protein secretion profiling, functional assays, and gene knockdown studies in xenograft and syngeneic breast cancer models to characterize the effects of the metastasis suppressor RKIP on TAMs and to identify the molecular mechanisms that mediate these effects. Our findings demonstrate that RKIP blocks a subset of TAMs that secrete pro-metastatic factors and are enriched in human TNBC patients. These results suggest that one mechanism by which metastasis suppressors alter tumor invasiveness is by regulating TAMs.

## **Results**

### **Non-metastatic RKIP+ tumors contain fewer macrophages**

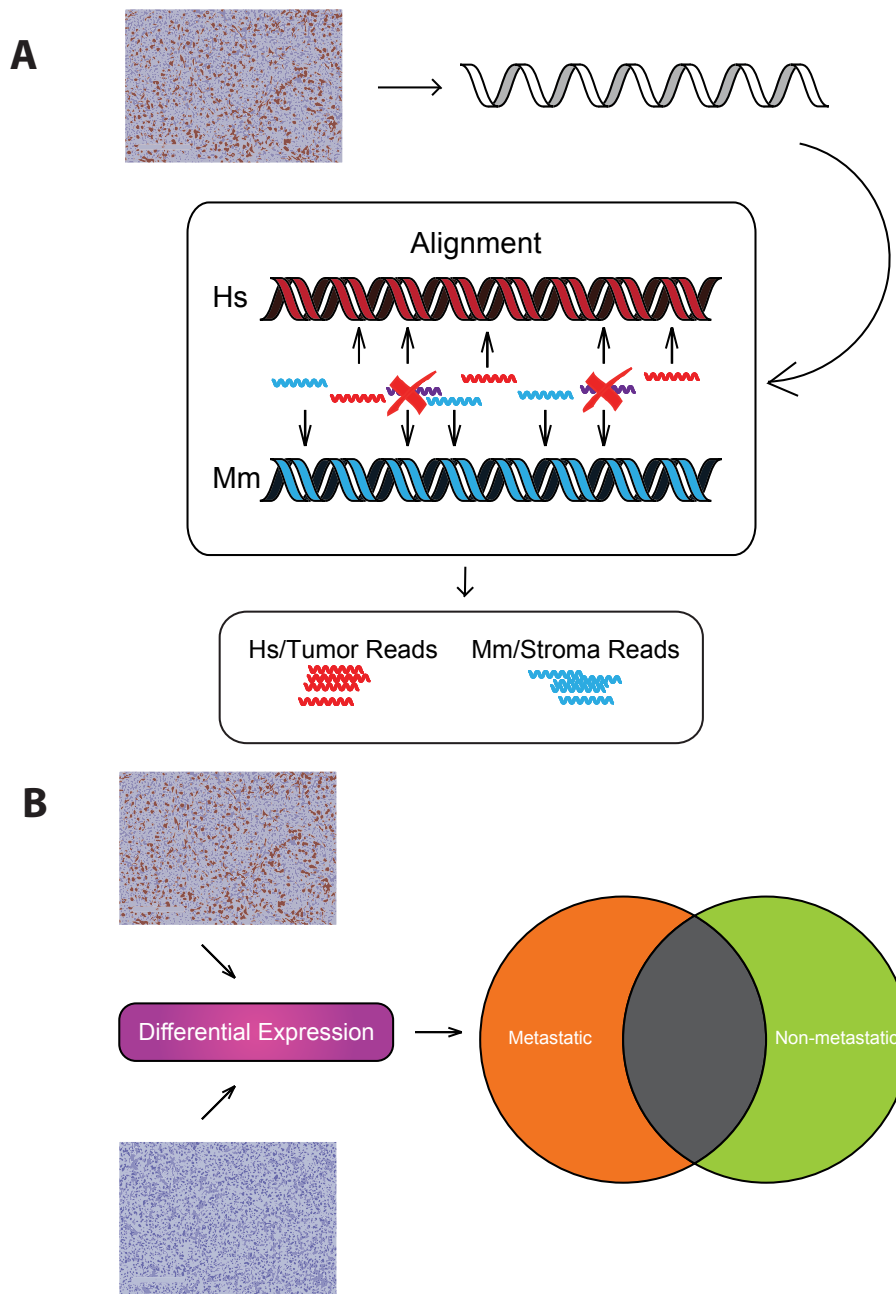
To compare metastatic and non-metastatic tumors that were isogenic, we used highly metastatic BM1 derivatives of the MDA-MB-231 human TNBC cell line stably expressing either the metastasis suppressor Raf Kinase Inhibitory Protein (RKIP+) or a vector control (control) (Fig 1)<sup>18,97</sup>. Tumor cells were injected orthotopically into athymic nude mice, and RNA from the

tumors was then isolated and sequenced. To overcome the challenge of distinguishing between tumor-specific and stroma-specific gene expression, we used next-generation RNA sequencing (RNAseq) in this xenograft mouse model to separate sequencing reads based on their species of origin (Fig 2).



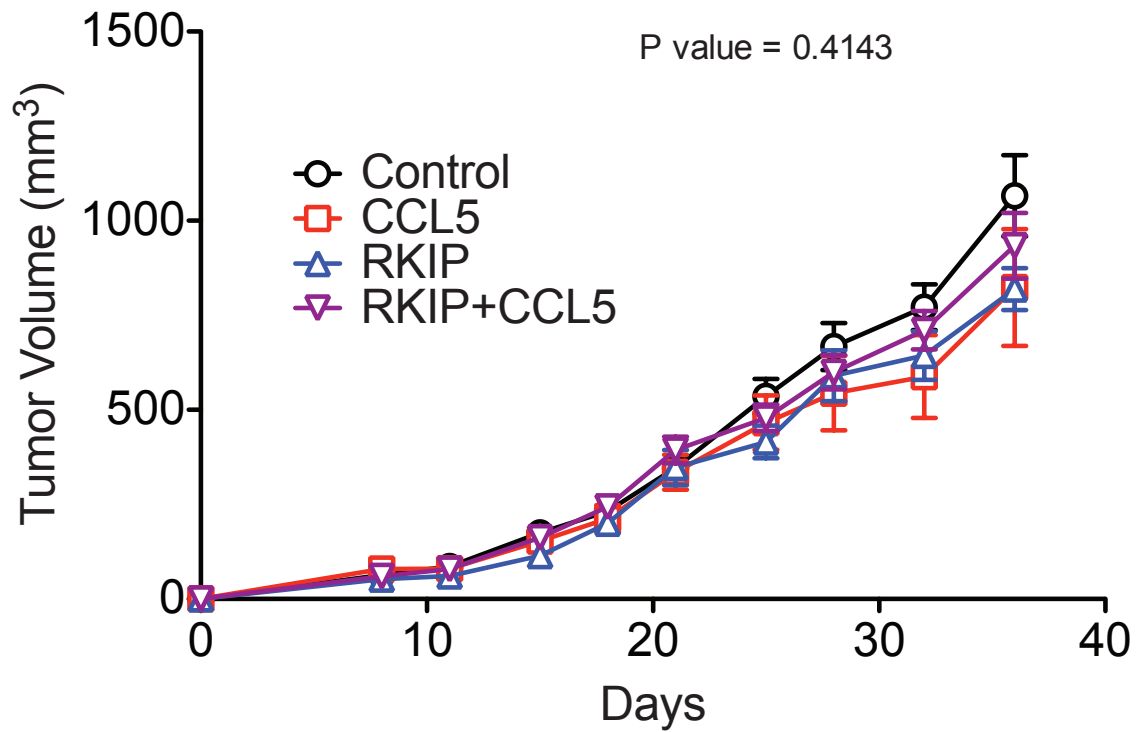
**Figure 1: Validation of RKIP expression in cell lines.**

Protein lysate from human bonetropic metastatic MDA-MB-231 derived BM1 (1833), MDA-MB-436 and mouse 4T1.2 triple negative breast cancer expressing pCDH1 vector or wild-type RKIP were immunoblotted with RKIP and tubulin antibodies



**Figure 2: Schematic of tumor and stroma RNAseq**

A) Scheme depicting comparison between tumor and stroma-derived mRNA expression levels from isogenic TNBC xenograft models in which B) the metastatic phenotype is suppressed by exogenous expression of RKIP.



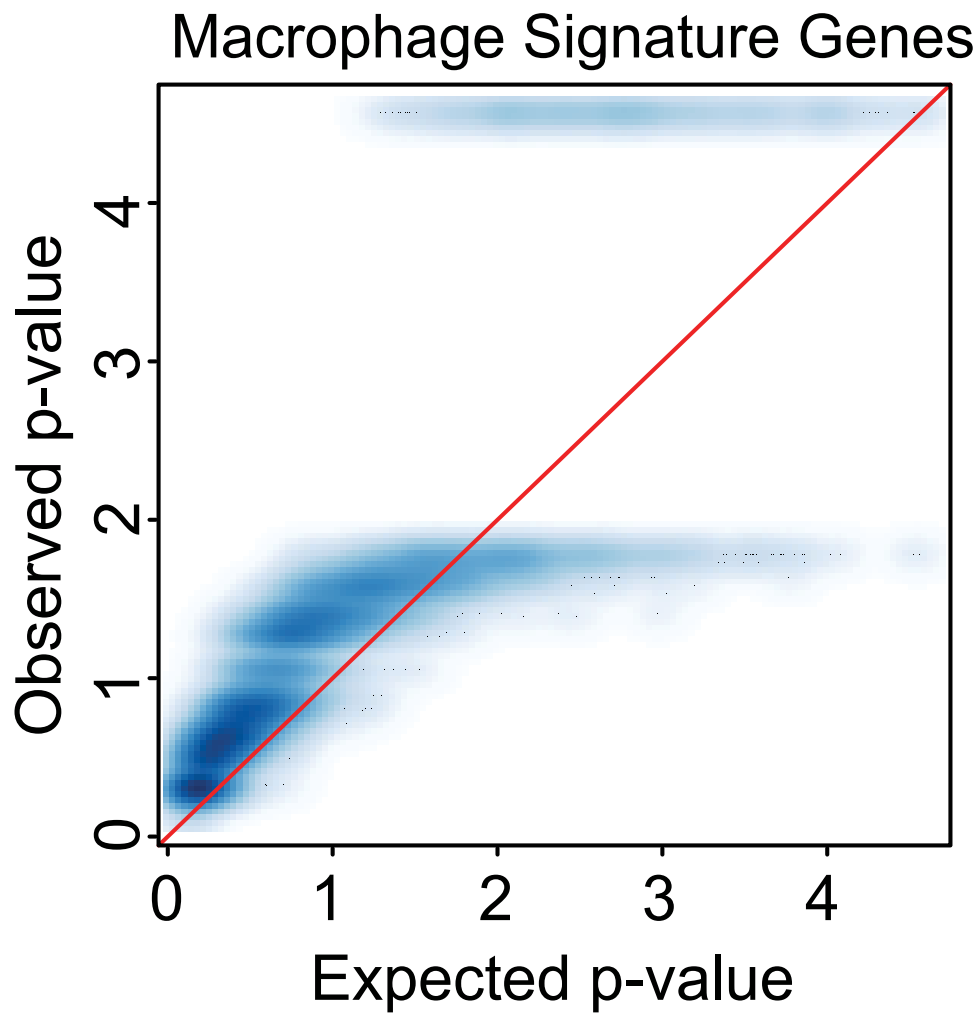
**Figure 3: RKIP and CCL5 do not change tumor growth rates.**

Tumor growth was measured twice per week using calipers. Tumor volume was calculated as  $(\pi/6) \times \text{width}^2 \times \text{length}$ . P-value was obtained using a 2-way ANOVA comparing the different tumor groups over time.

<b>GOid</b>	<b>Category</b>	<b>Qvalue</b>
GO:0032103	positive regulation of response to external stimulus	1.3E-05
GO:0006953	acute-phase response	1.55E-05
GO:0010759	positive regulation of macrophage chemotaxis	2.98E-05
GO:0010758	regulation of macrophage chemotaxis	3.94E-05
GO:0006955	immune response	4.1E-05
GO:0071622	regulation of granulocyte chemotaxis	4.6E-05
GO:0050921	positive regulation of chemotaxis	4.91E-05
GO:0048520	positive regulation of behavior	5.5E-05
GO:0071675	regulation of mononuclear cell migration	5.71E-05
GO:0030169	low-density lipoprotein particle binding	7.67E-05
GO:0071674	mononuclear cell migration	7.69E-05
GO:0071621	granulocyte chemotaxis	8.47E-05
GO:0071345	cellular response to cytokine stimulus	0.000113246
GO:0050920	regulation of chemotaxis	0.000124252
GO:0002526	acute inflammatory response	0.000131065
GO:0071813	lipoprotein particle binding	0.00021975
GO:0071814	protein-lipid complex binding	0.00021975
GO:0048246	macrophage chemotaxis	0.00022022
GO:0050795	regulation of behavior	0.000222061
GO:0045087	innate immune response	0.000225704
GO:0070098	chemokine-mediated signaling pathway	0.000230038
GO:0034097	response to cytokine stimulus	0.000294786
GO:0030246	carbohydrate binding	0.000440636

**Figure 4: Ontological categories changed by RKIP in the tumor**

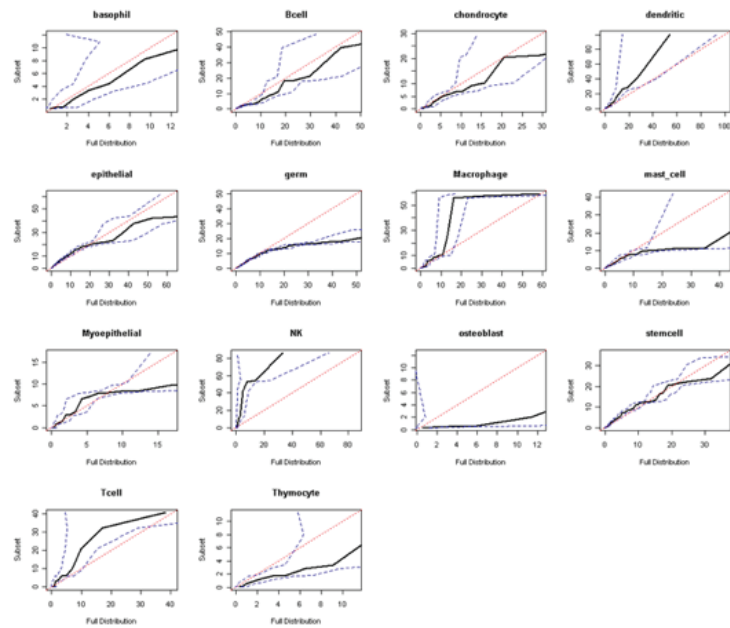
Ontological categories enriched among genes whose expression level is significantly decreased by RKIP relative in TNBC xenograft tumors. Highlighted in red are categories involving macrophages and macrophage chemotaxis. (FDR = 0.01 for all comparisons).



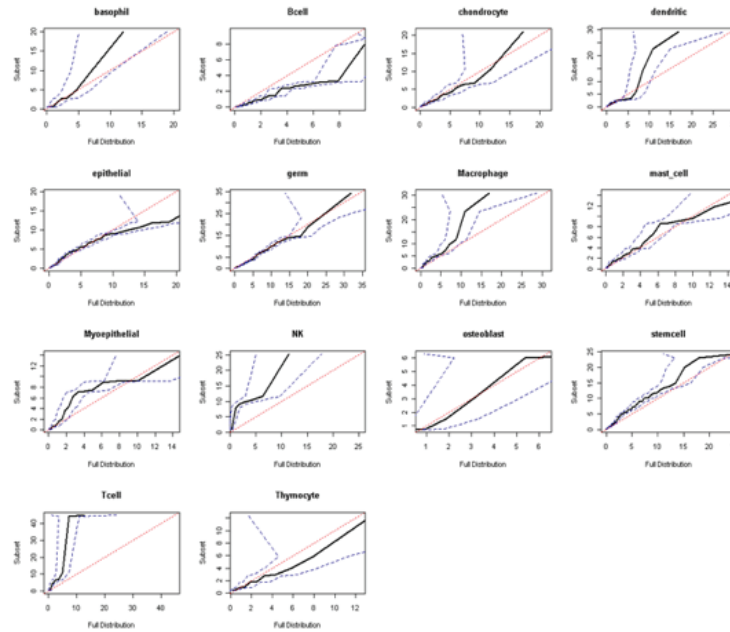
**Figure 5: Quantile-Quantile plot of macrophage specific gene expression**

Quantile-quantile (qq)-plot showing ranked  $-\log_{10}$  transformed p-values among macrophage-specific genes (y-axis) relative to a similar bootstrapped distribution in blue (x-axis). Distortion of the density above  $x = y$  (red line) indicates that the measured p-values are systematically lower than expected by chance.

Metastatic



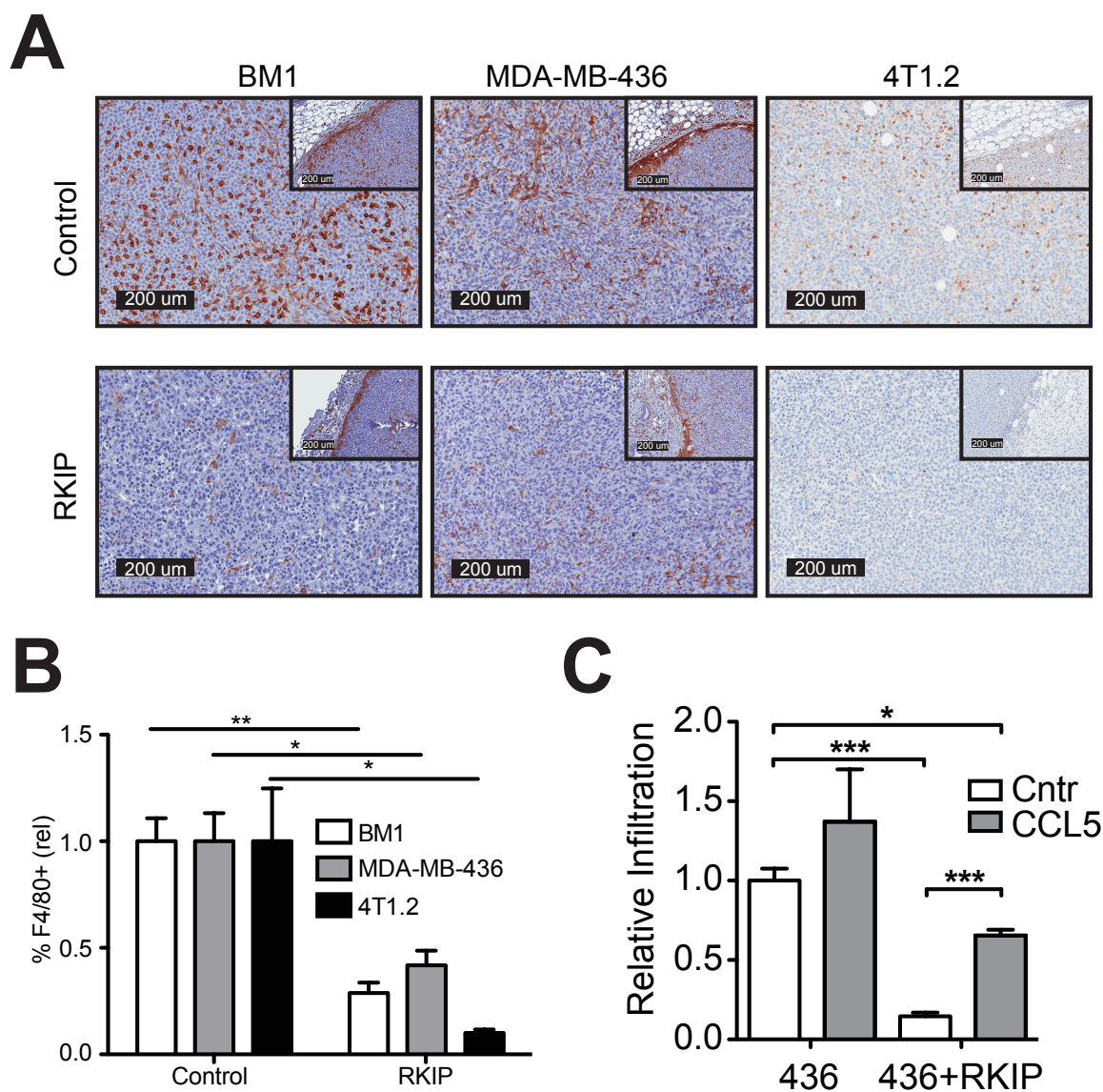
Non-metastatic



**Figure 6: Quantile-quantile plot of immune cell specific genes**

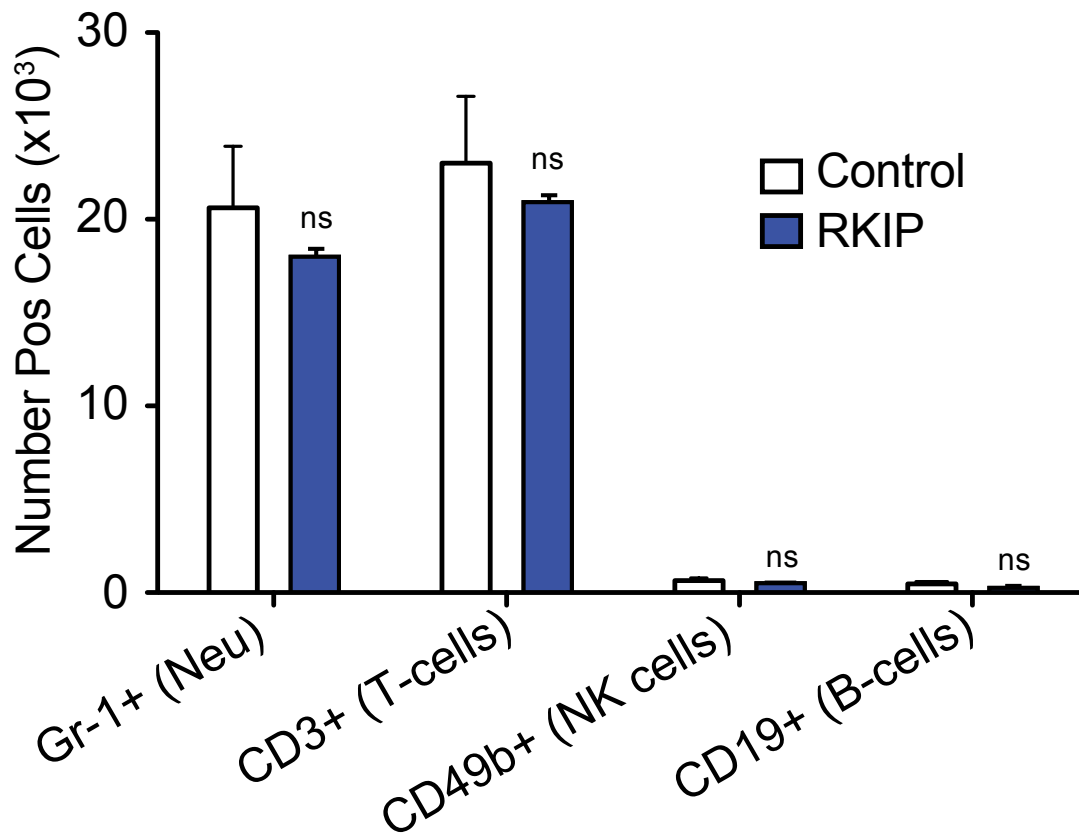
Quantile-quantile plots of immune cell specific genes compared between metastatic BM1 tumors and non-metastatic RKIP+ tumors.

We observed dramatic changes in stromal gene expression profiles between RKIP+ and control tumors even though the tumors did not significantly differ in size (Figure 3). Analysis of gene expression changes between RKIP+ and control tumor cells using GOseq revealed that the most significant difference was the immune response and, specifically, macrophage chemotaxis (Q-value =  $2.2 \times 10^{-4}$ , Figure 4). Using mouse gene sets characteristic of common immune cell types<sup>102</sup>, we observed a clear depletion of gene expression associated with macrophages in the RKIP+ tumor microenvironment (Figure 5), and this was the most robust change observed (Figure 6). Immunohistochemical staining confirmed a marked reduction in the number of tumor-associated macrophages (TAMs) at the primary tumor site in RKIP+ tumors relative to controls, both in xenograft (BM1, MDA-MB-436) and syngeneic (4T1.2) tumor models (Figure 7). Moreover, the effect of RKIP on TAMs was quite specific, as we did not observe significant differences in the number of B cells (CD19+), T cells (CD3+), NK cells (CD49b+), or neutrophils (Gr-1+) when analyzed by flow cytometry (Figure 8).



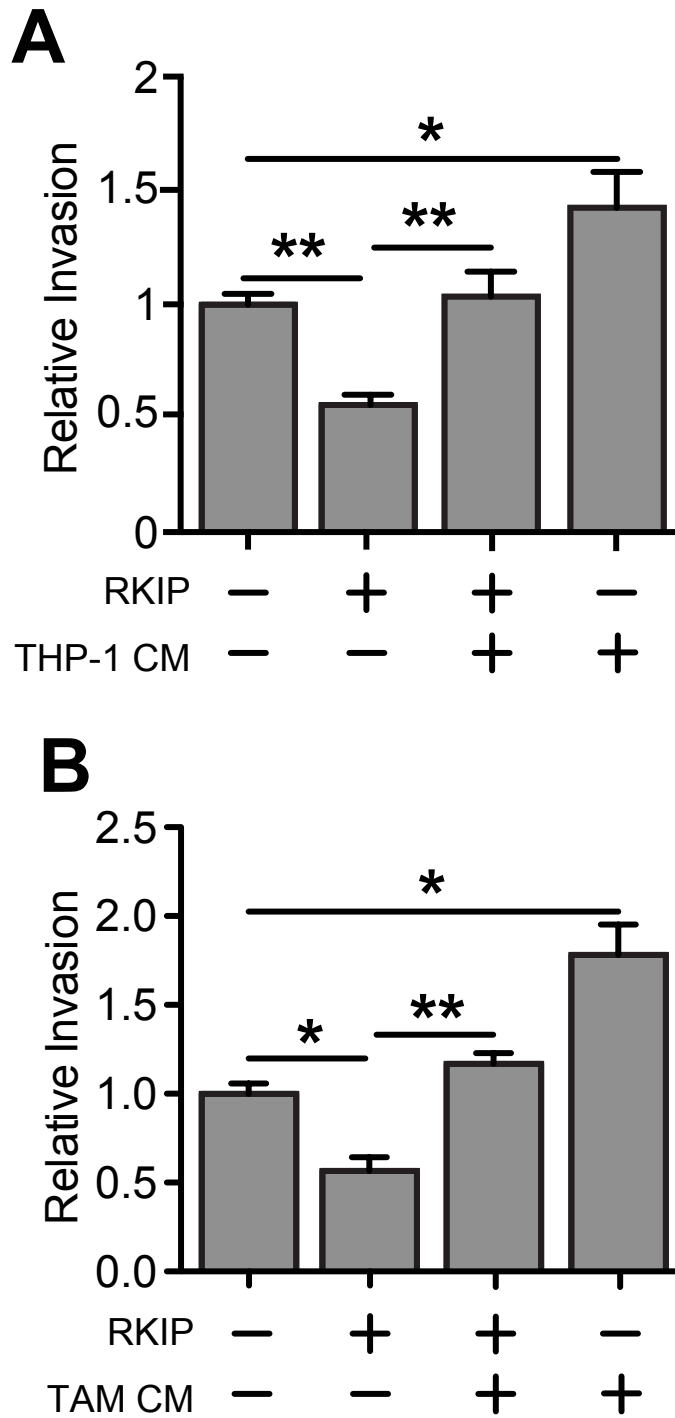
**Figure 7: Non-metastatic RKIP+ tumors contain fewer macrophages**

A) Representative images of relative macrophage presence in xenograft (BM1 and MDA-MB-436) and syngeneic (4T1.2) tumor models with and without RKIP expression were sectioned and immunostained for F4/80. B-C) Relative macrophage infiltration was quantified as the proportion of inner tumor mass positively stained with F4/80 via immunohistochemistry.



**Figure 8: RKIP does not affect infiltration of neutrophils, T-cells, NK cells, or B-cells in BM1 xenograft tumors**

Cells isolated from digested tumors following a Ficoll-PAQUE gradient isolation were stained for Gr-1, CD3, CD48b, and CD19. For each sample, number of positively staining cells was determined by flow cytometry. Comparisons were made between 3 samples per group and statistical difference was tested using a Student's two-tailed T-test. No statistical differences were seen between control BM1 tumors and RKIP over-expressing tumors.



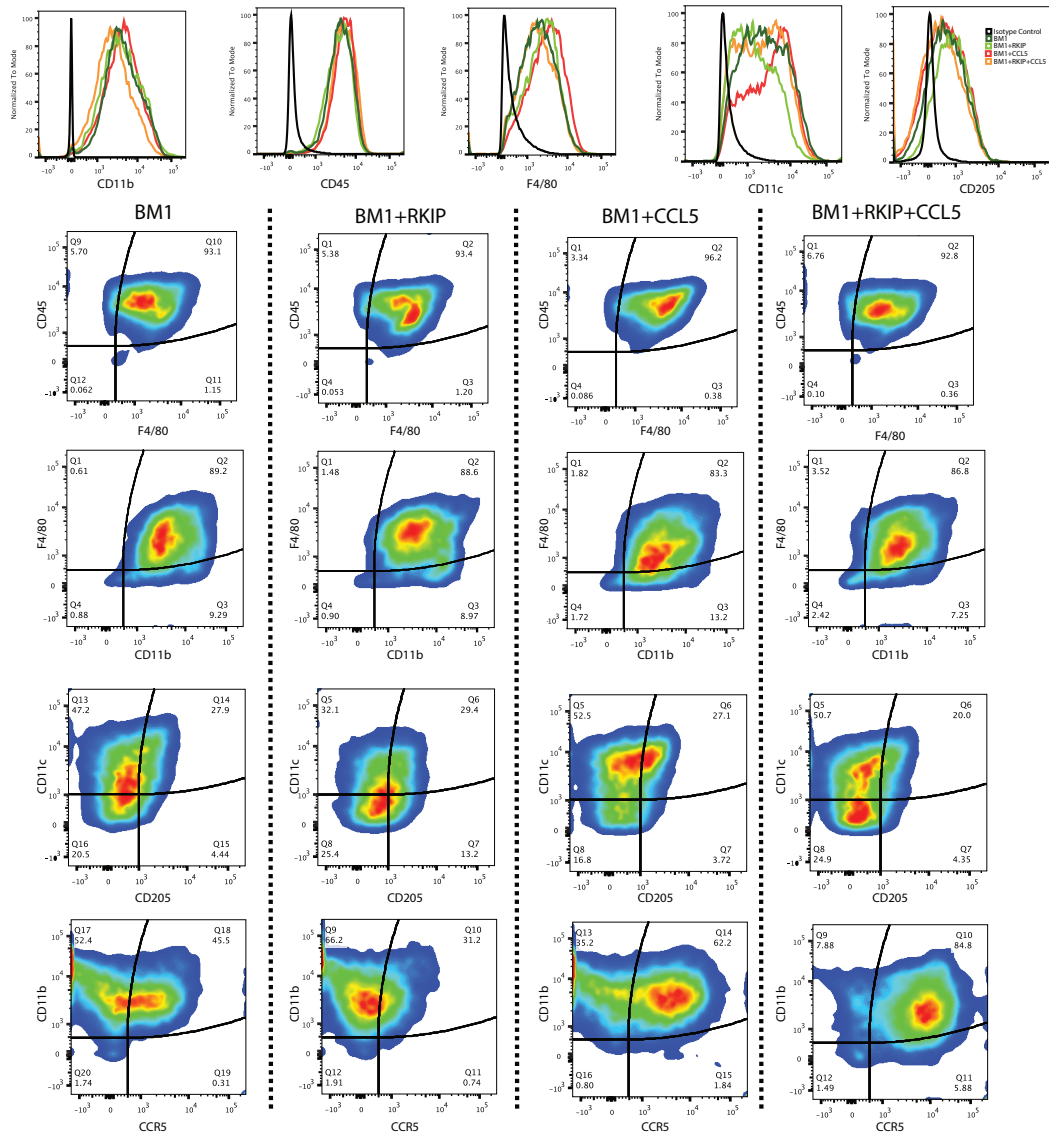
**Figure 9: THP-1 and TAM CM rescue RKIP blocked invasion**

A-B) BM1 or BM1+RKIP tumor cells were pretreated with conditioned media collected from various types of macrophages for 24h; A) THP1 human monocytic cell line (n=8 per group), B) TAMs isolated from BM1 tumors (n=5 per group)

### **RKIP suppresses recruitment of a distinct TAM population that potentiates tumor cell invasion**

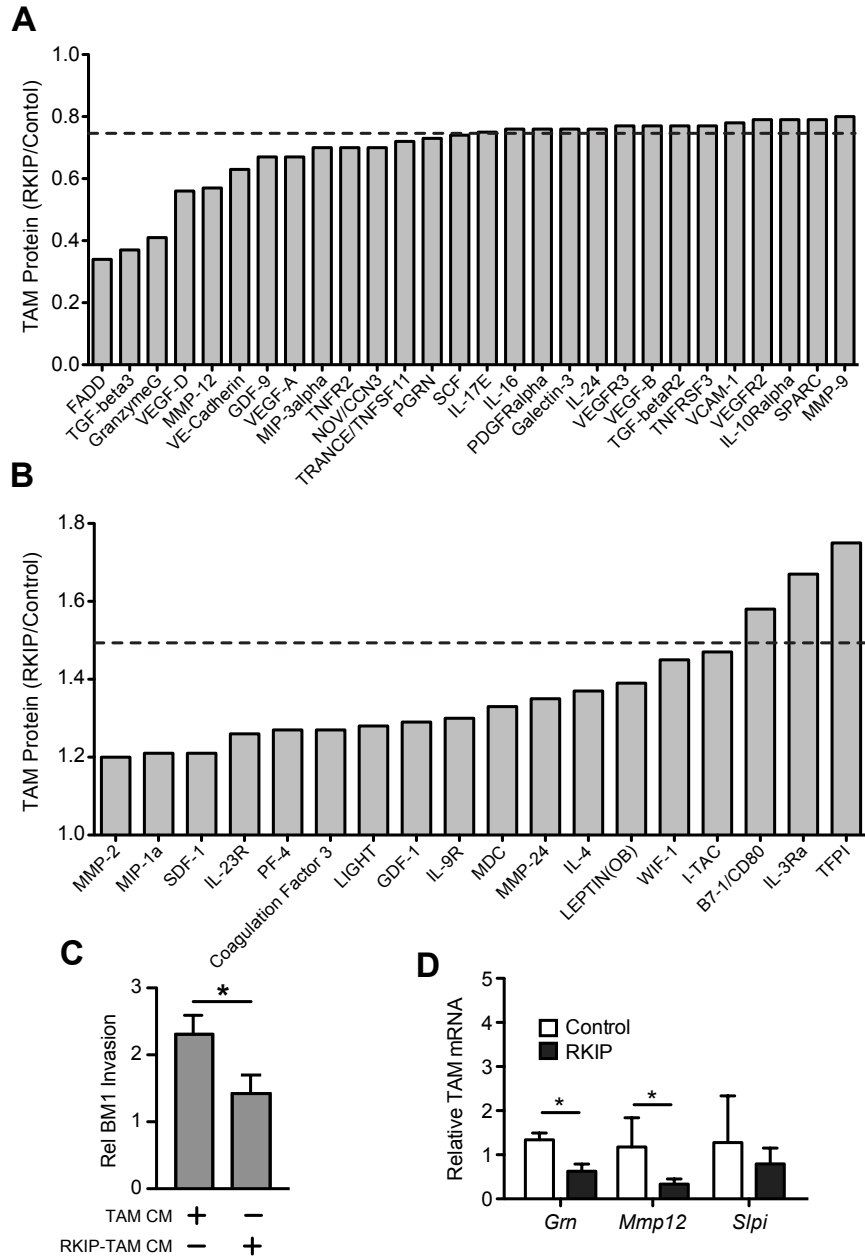
Since RKIP regulates the number of macrophages in tumors (Figures 5-7) and TAMs are known to play a significant role in tumor biology<sup>37</sup>, we hypothesized that changes in TAMs may in part explain suppression of intravasation by RKIP. In support of this hypothesis, treating RKIP+ BM1 tumor cells with conditioned media (CM) from a human monocytic cell line (THP1) restored tumor cell invasion relative to levels observed in control BM1 tumor cells (Figure 9A). Similar results were observed with the CM of TAMs purified from control BM1 tumors (Figure 9B). These findings demonstrate that TAMs from metastatic tumors can overcome blockade of tumor cell invasion by RKIP.

It is well established that, depending on environmental conditions, TAMs can adopt phenotypes with pro-tumor (“M2-like”) or anti-tumor (“M1-like”) properties<sup>38</sup>. We therefore explored the possibility that, in addition to reducing the number of macrophages in tumors, RKIP might also alter their functional properties to suppress metastasis. To test this hypothesis, we purified TAMs from BM1 tumors (metastatic) and RKIP+ BM1 tumors (non-metastatic), which were uniformly CD45+, CD11b+, F4/80+, and CD205- (Figure 10), and we assessed their functional phenotype using two interrelated approaches.



**Figure 10: Purity and heterogeneity of isolated TAMs**

Control BM1 tumors (green), BM1 tumors over-expressing RKIP (light green), over-expressing CCL5 (red), and over-expressing RKIP and CCL5 (orange) were digested using the Miltenyi Biotech human tumor dissociation kit using C tubes. Immune cells were isolated from the single cell suspension using a Ficoll-Paque gradient. Macrophages were isolated from other immune cells using positive CD11b selection using CD11b magnetically labeled beads using the Miltenyi Biotech AutoMACS separator. Purity was determined by flow cytometry of CD45, CD11b, CD11c, CD205, and F4/80. Purity of isolated cells was determined by comparing the number of CD11b+, CD45+ cells. To determine the heterogeneity of macrophages we examined the number of CD11b+, F4/80+ cells.



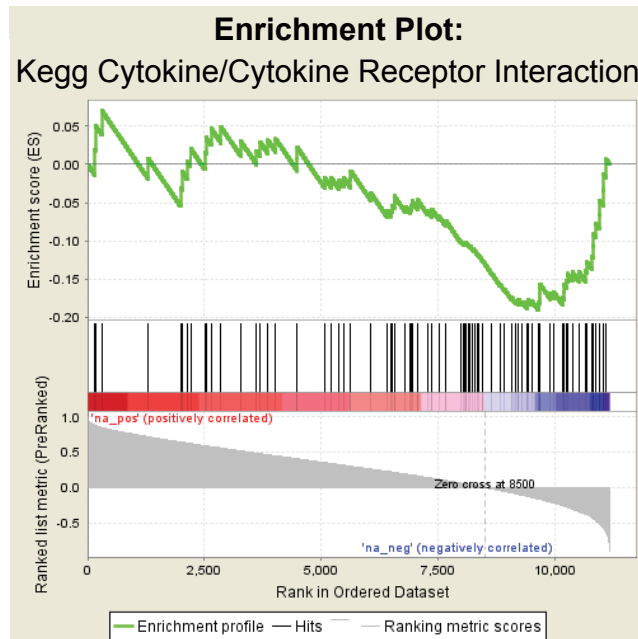
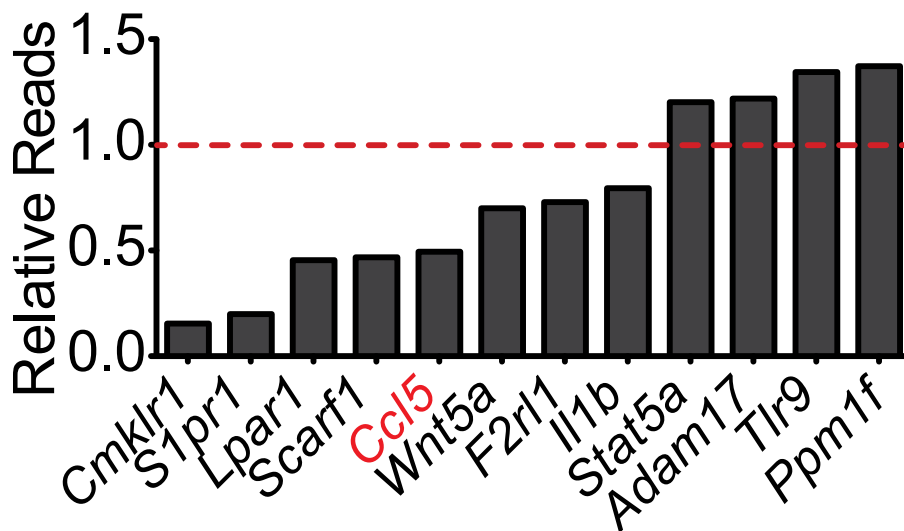
**Figure 11: RKIP suppresses recruitment of a distinct TAM population that potentiates tumor cell invasion**

A-B) TAM conditioned media from four independent tumors was analyzed for protein levels using RayBiotech L308 Mouse Cytokine Arrays. Protein abundance for RKIP derived TAMs were normalized to control tumor TAMs. A) Proteins with 0.8 or lower relative abundance, B) Proteins with >1.2 relative abundance. C) TAMs isolated from BM1 or BM1+RKIP tumors (n=6 per group). Relative invasion is against BM1 grown in a media control. P-values were calculated using an unpaired T-test with Welch's correction. D) Relative mRNA was measured from three independent TAM samples per group. Relative mRNA was calculated as compared to control TAMs, with *Gapdh* as the reference gene.

First, we determined the effect of TAM conditioned media on tumor cell invasion *in vitro*. Pretreating BM1 tumor cells with the conditioned media (CM) of TAMs isolated from control BM1 tumors, like THP1 cells, potentiated invasion (Figure 11A). In sharp contrast, factors secreted by TAMs from RKIP+ BM1 tumors had no significant effect on tumor cell invasiveness (Figure 11A). These results indicate that the TAMs from metastatic and non-metastatic (RKIP+) tumors have distinct phenotypes.

Second, we quantified the relative abundance of 400 proteins including inflammatory and tumorigenic factors (e.g. cytokines, growth factors) in the TAM conditioned media using the RayBiotech L308 mouse cytokine array. TAMs purified from RKIP+ BM1 tumors relative to control BM1 tumors were distinguished by reduced abundance of a number of pro-metastatic factors<sup>37</sup> including TGF- $\beta$ 3, VEGF-D, MMP-12, GDF-9, VEGF, sTNFR2, and GRN (Figure 11B). We also observed induction of secreted factors in the CM of RKIP+ BM1 tumors including CD80 and TFPI, two potential anti-tumor proteins<sup>103–105</sup>. We confirmed differential regulation of *Mmp12* and *Grn* (granulin) transcripts in TAMs isolated from BM1 versus RKIP+ BM1 tumors by qRT-PCR (Figure 11C).

Taken together, the direct functional evidence and protein expression analysis suggest that RKIP suppresses recruitment of a TAM population that secretes a set of pro-invasive and pro-metastatic proteins.

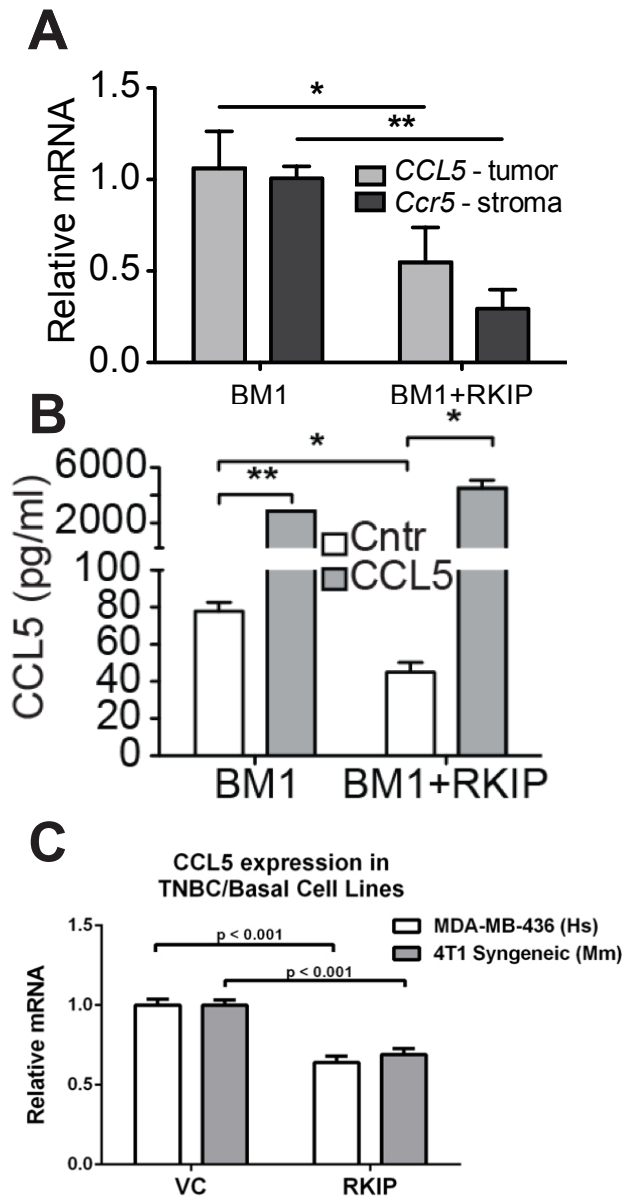
**A****B**

**Figure 12: RKIP regulates cytokine signaling and CCL5 expression**

A) GSEA identifies enrichment of genes involved in cytokine-cytokine receptor interactions (black lines) among genes with anticorrelated expression levels in tumors and surrounding stroma. B) Tumor genes differentially expressed in RKIP tumors relative to control ( $p < 0.05$ ) are shown from the external stimulus (GO) category from our RNAseq data.

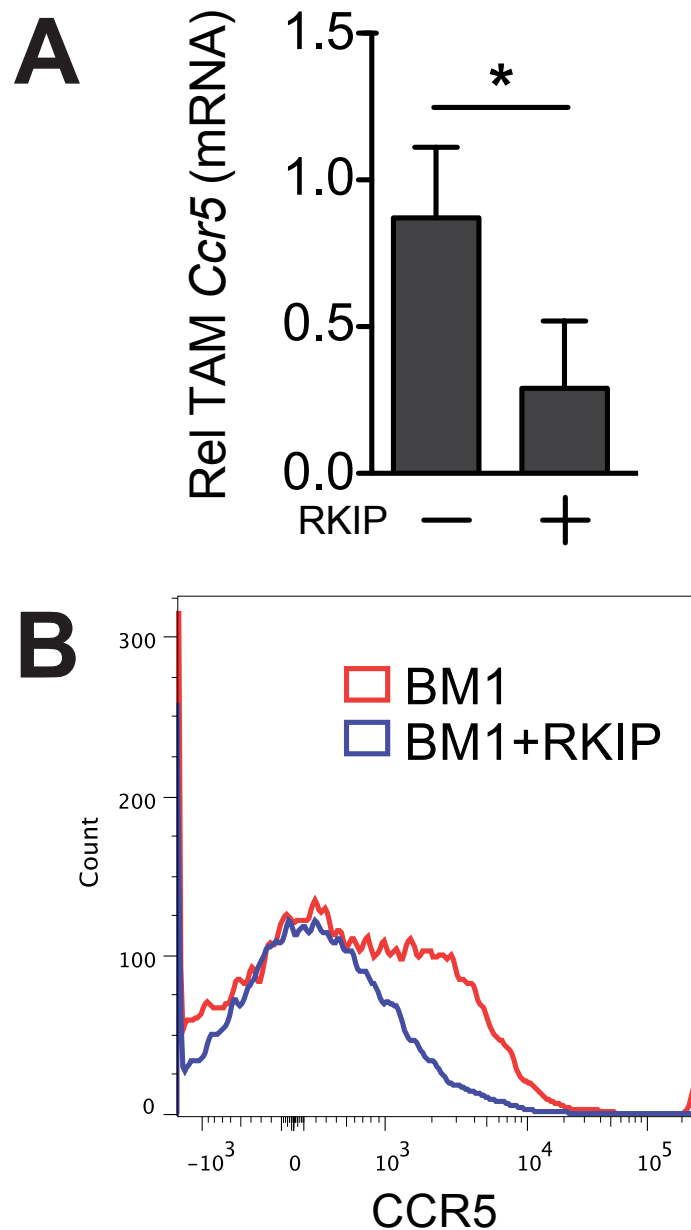
### **Overexpression of CCL5 restores TAM recruitment in RKIP+ tumors.**

To determine the mechanism by which RKIP regulates TAM number and function, we examined our RNAseq data comparing BM1 and RKIP+ BM1 tumors. RKIP+ tumors suppressed numerous genes involved in cytokine/cytokine receptor interactions, particularly in relation to external stimulus and macrophage chemotaxis (Figures 12; 4) (FDR =  $3.9 \times 10^{-2}$ ). The foremost of the chemokine factors was CCL5 (Figure 12)<sup>44,106</sup>. We therefore analyzed CCL5 expression by species-specific qRT-PCR (Figure 13) and by ELISA (Figure 13), and confirmed its down-regulation in RKIP+ tumors. Similar decreases in CCL5 transcripts were observed following RKIP expression in human MDA-MB-436 and mouse 4T1.2 tumor cell lines (Figure S9). Thus, RKIP suppresses CCL5 expression in multiple human and murine tumor cell lines.



**Figure 13: RKIP regulates CCL5 expression in multiple cell lines**

A) qRT-PCR was performed on mRNA purified from xenograft tumors (BM1 & BM1+RKIP). Species-specific primers were used to detect relative mRNA of CCL5 (Hs) and Ccr5 (Mm) in the tumor and stroma, respectively. Relative mRNA was normalized to GAPDH (Hs) or Rpl4 (Mm). B) CCL5 levels were measured from media of cells cultured for 24 hours in SF media using a RayBiotech CCL5 ELISA. A standard curve was used to calculate amounts of protein from three independent experiments. C) qRT-PCR was performed on mRNA purified from human (MDA-MB-436) and mouse (4T1.2) TNBC cell lines that do (RKIP) and do not (VC) express exogenous RKIP. Species specific primers were used to detect relative mRNA abundance of CCL5 (Hs) or Ccl5 (Mm) calculated using  $2^{-\Delta\Delta CT}$  normalized to GAPDH (Hs) or *Gapdh* (Mm).



**Figure 14: RKIP regulates CCR5 expression in isolated TAMs**

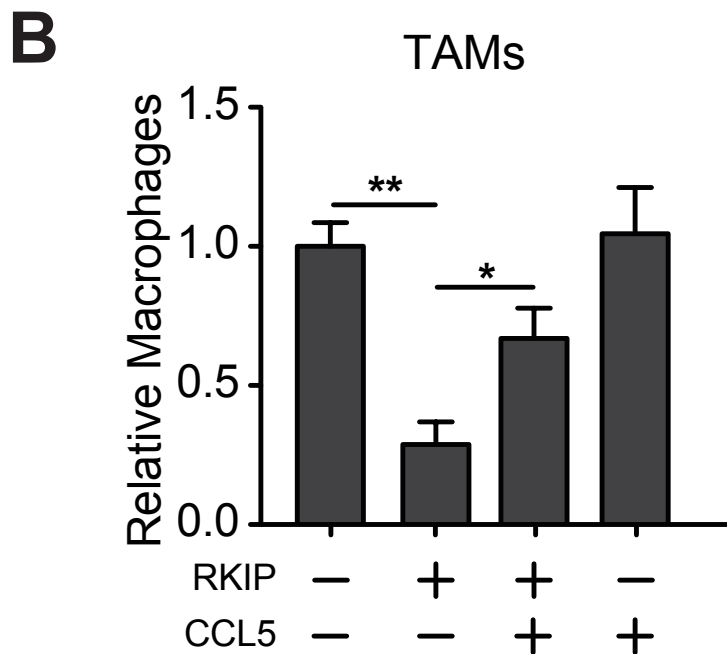
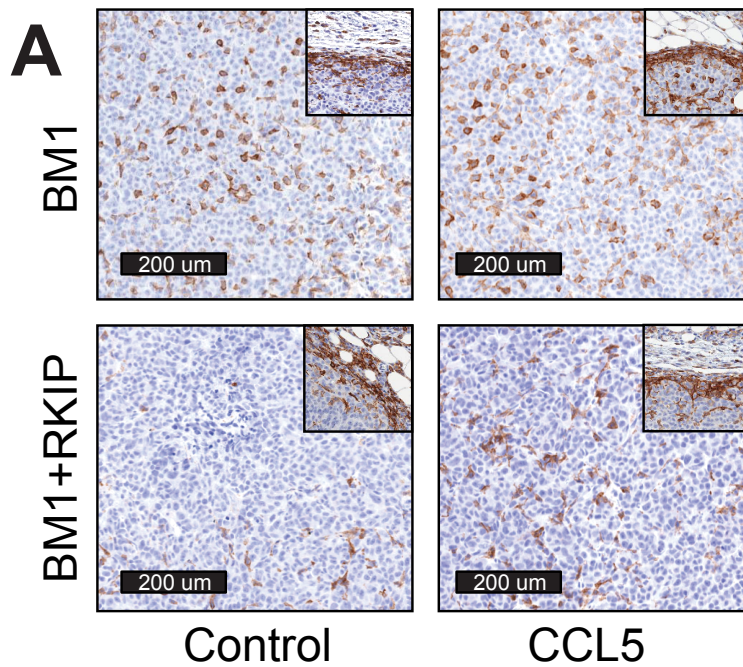
A) Relative mRNA was calculated using qRT-PCR relative to BM1 TAMs with Gapdh as the reference gene. B) Flow cytometry of BM1 (red) and BM1+RKIP (blue) isolated TAMs are also shown for CCR5 of equal numbers of TAMs isolated from tumors using CD11b+ selection following Ficoll-PAQUE gradient centrifugation

We used three approaches to investigate whether suppression of CCL5 by RKIP plays an important role in regulating macrophage accumulation into tumors *in vivo*. First, since CCL5 recruits macrophages via interaction with its receptor CCR5<sup>44,107</sup>, we measured *Ccr5* expression in the stroma. We found that *Ccr5* levels were significantly reduced in RKIP+ tumor stroma by qRT-PCR using species specific primers (Figure 13). In addition, when comparing the same number of TAMs, we observed significant decreases in the mean *Ccr5* expression by qRT-PCR (Figure 14). Using flow cytometry, we determined that the decrease in mean *Ccr5* was due to a reduction in the number of CCR5+ TAMs in RKIP tumors (Figure 14).

Second, we transfected CCL5 into BM1 cells stably expressing RKIP or control vector (Figure 13B) and observed rescue of TAM infiltration in tumors expressing RKIP and CCL5 (RKIP+CCL5) compared to those just expressing RKIP alone (RKIP+) (Figure 15). There was a corresponding increase in the number of CCR5+ macrophages in CCL5-rescued tumors (Figure 10), consistent with CCL5 recruitment of TAMs.

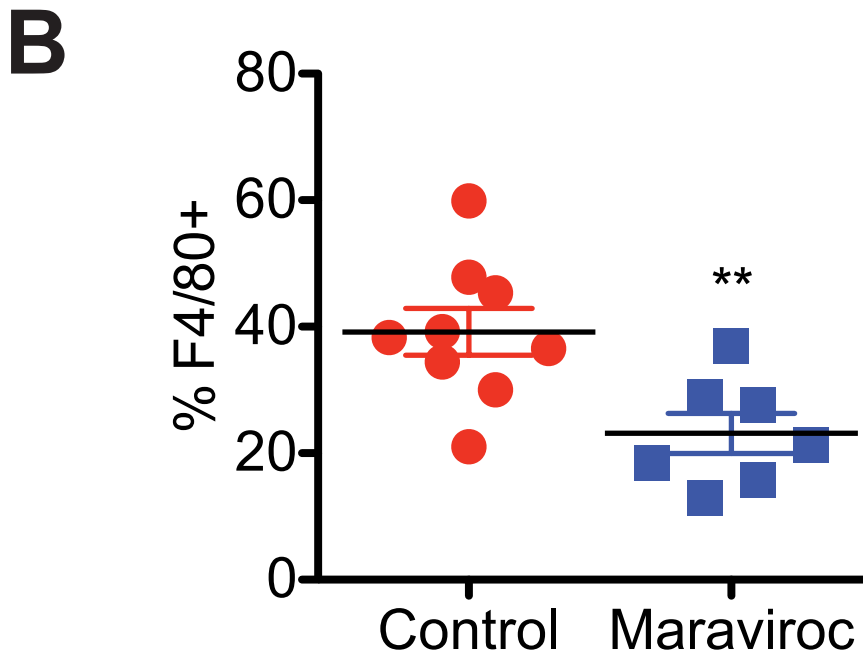
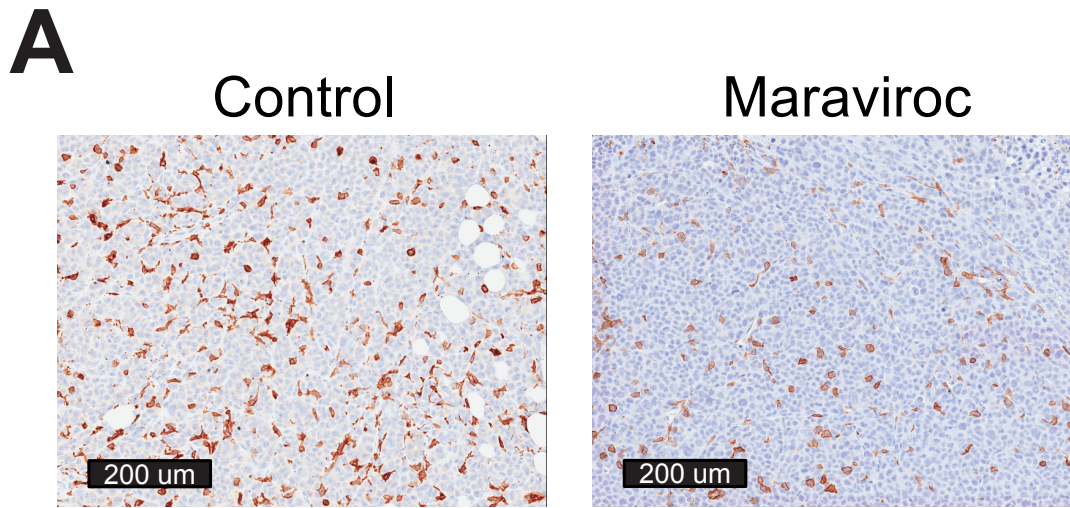
Third, we determined if a reduction in CCL5 signaling alone was necessary for TAM infiltration and tumor growth. We treated mice with the orally bioavailable CCR5 inhibitor Maraviroc twice daily by oral gavage and found that antagonizing the CCL5 receptor significantly lowered the number of TAMs recruited into BM1 control tumors as well as decreased tumor growth (Figure 16). To determine if the number of TAMs infiltrating into the tumor was simply due to a difference in tumor size, we performed a Pearson correlation between the tumor weight and the percent of F4/80+ cells in the tumor. We found that, whether we examined the total population of tumors or the control and Maraviroc-treated tumors individually, there was no correlation between the size of the tumor and the percent of F4/80+ cells in the tumor (Figure

17). Together, these findings suggest that modulation of CCL5 expression is one important mechanism by which RKIP controls tumor macrophage recruitment.



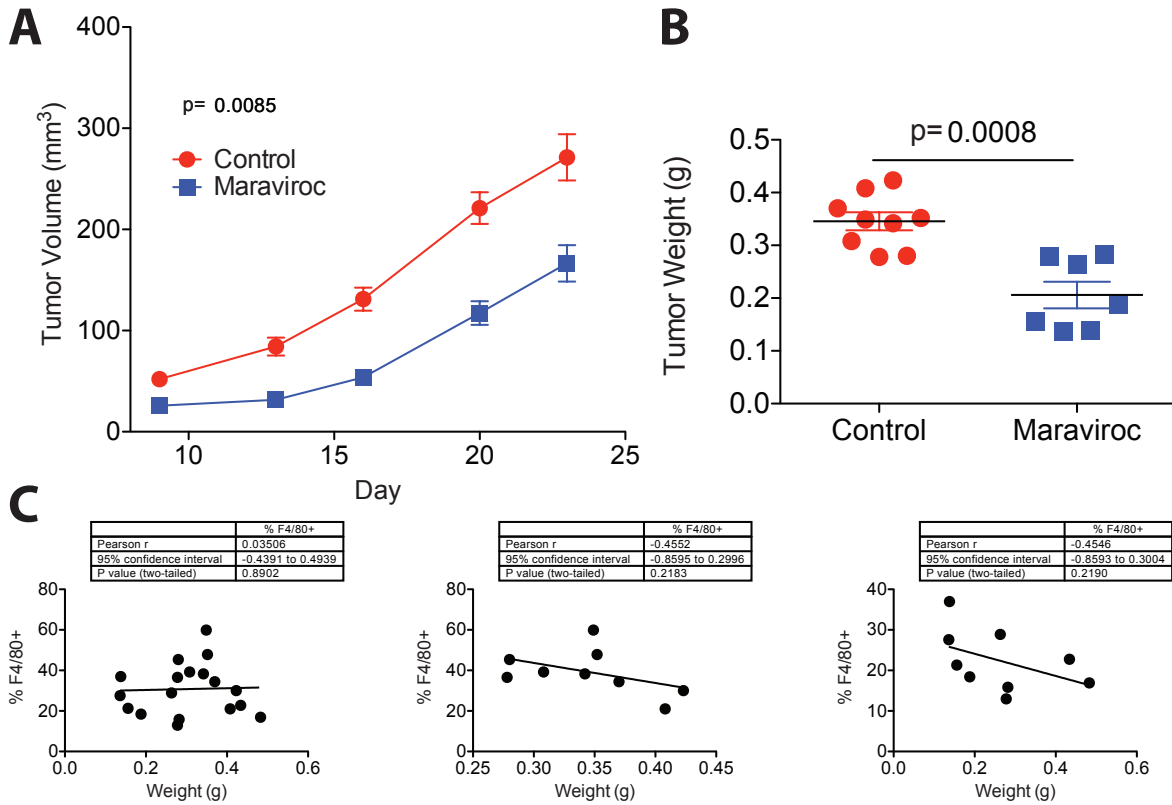
**Figure 15: CCL5 over-expression restores recruitment of macrophages to the tumor core**

E) Representative images of macrophage presence in BM1 tumors with and without RKIP and CCL5 expression. F) Relative macrophages in BM1 and BM1+RKIP tumors with or without exogenous CCL5 expression in tumor cells. Infiltration was quantified as the proportion of total tumor area positively stained with F4/80 (n=3 per group).



**Figure 16: Maraviroc reduces the number of macrophages in the tumor core**

Effect of Maraviroc on BM1 tumor macrophage numbers was assessed by immunostaining for F4/80. A) Representative images of F4/80 staining of tumors. B) Data are displayed as the %F4/80+ cells in the core of the tumor.

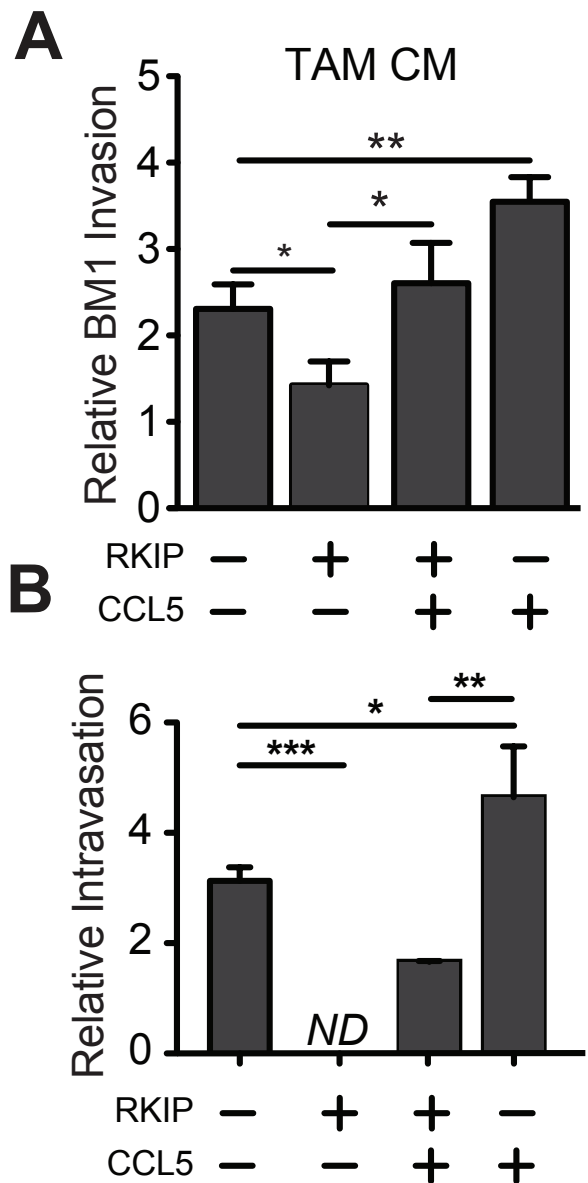


**Figure 17: Maraviroc treatment reduces size of tumors**

A) Tumor growth was measured twice per week using calipers. Tumor volume was calculated as  $(\pi/6) \times \text{width}^2 \times \text{length}$ . P-value was obtained for tumor growth using a paired T-test. Differences in tumor weight were determined using a Student's T-test. B) Correlations and p-values between TAM infiltration and tumor size were determined using a Pearson correlation.

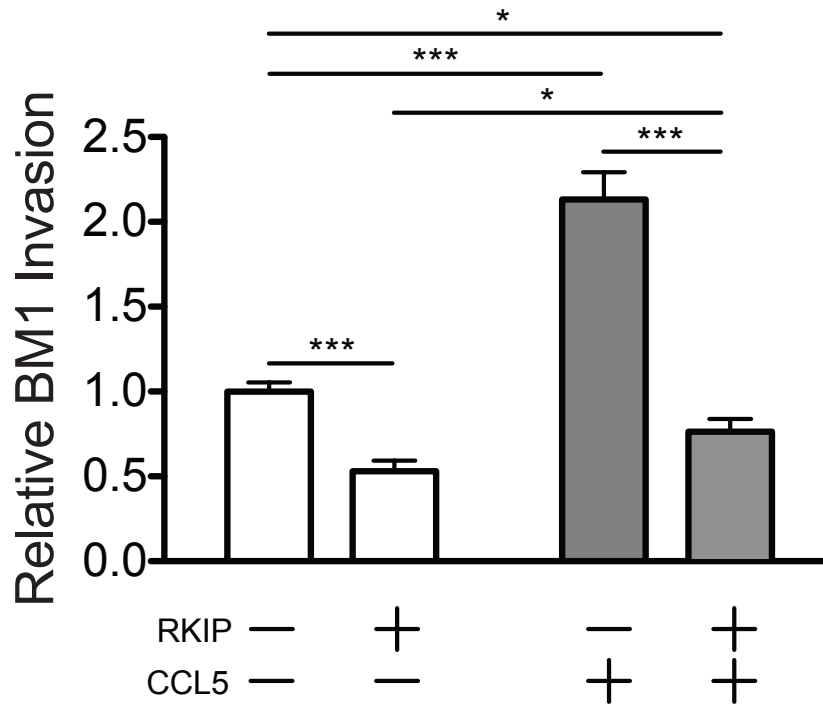
**Overexpression of CCL5 restores a prometastatic TAM phenotype and overcomes metastasis suppression in RKIP+ tumors.**

To determine if CCL5 overexpression in RKIP+ BM1 tumors could also restore a TAM phenotype that promotes tumor invasion, we first conducted functional assays. Whereas TAMs isolated from BM1 RKIP+ tumors had no effect on BM1 invasion; TAMs isolated from RKIP+ BM1 tumors overexpressing CCL5 induced tumor cell invasion with similar efficiency as TAMs isolated from metastatic BM1 tumors (Figure 18A). Since invasion enables tumor cell entry into vessels, we investigated whether overexpression of CCL5 in RKIP+ BM1 tumor cells could overcome the inhibitory effect of RKIP on intravasation. Consistent with RKIP's ability to suppress metastasis, RKIP expression in BM1 tumor cells potently inhibited intravasation into blood vessels (Figure 18B)<sup>18</sup>. Importantly, elevating CCL5 expression in RKIP+ BM1 cells produced a partial but significant recovery of tumor cell invasion (Figure 19) and intravasation into blood vessels (Figure 18B). CCL5 overexpression also potentiated both invasion (Figure 19) and intravasation in control metastatic tumor cells (Figure 18B).



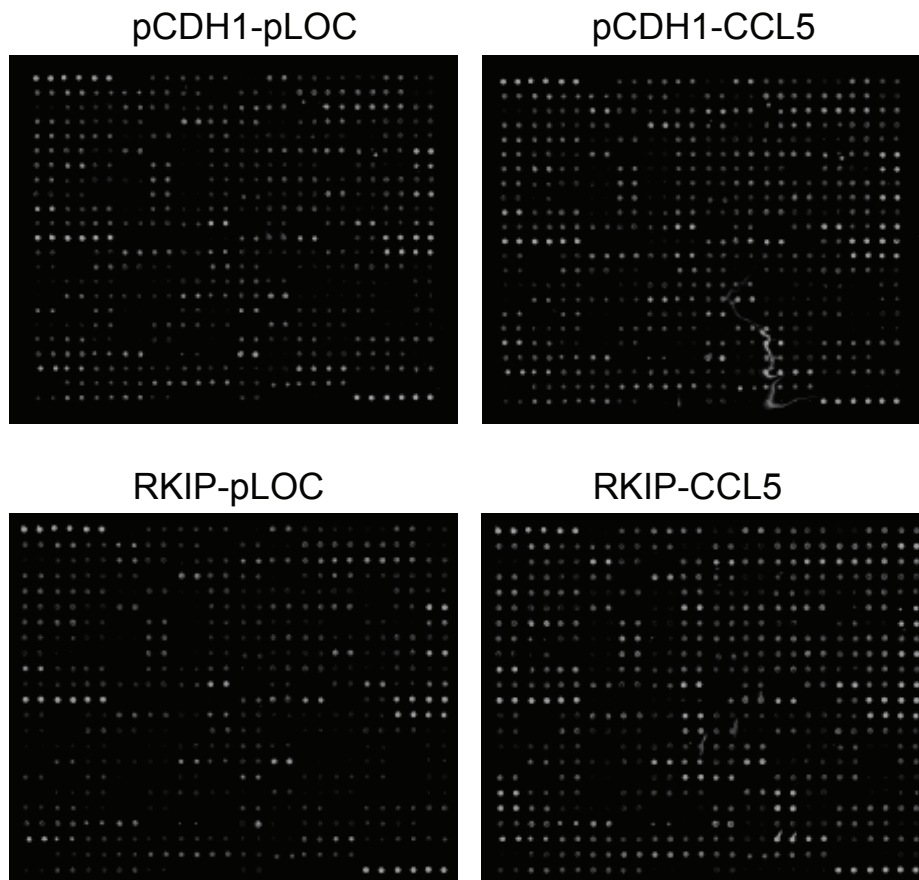
**Figure 18: CCL5 restores both TAM pro-invasive phenotype as well as tumor cell intravasation blocked by RKIP**

A) BM1 cells were pretreated with TAM conditioned media (BM1, BM1+RKIP, BM1+RKIP+CCL5, or BM1+CCL5 TAMs) for 24 hours prior to invasion assays, using TAMs from four independent tumors each. P-values were obtained using an unpaired T-test with Welch's correction, n=6. B) Relative intravasation of tumor cells into blood 4 weeks following injection was estimated by quantifying the ratio of human *GAPDH* (tumor) to mouse *Gapdh* by qRT-PCR (n=4 per group)



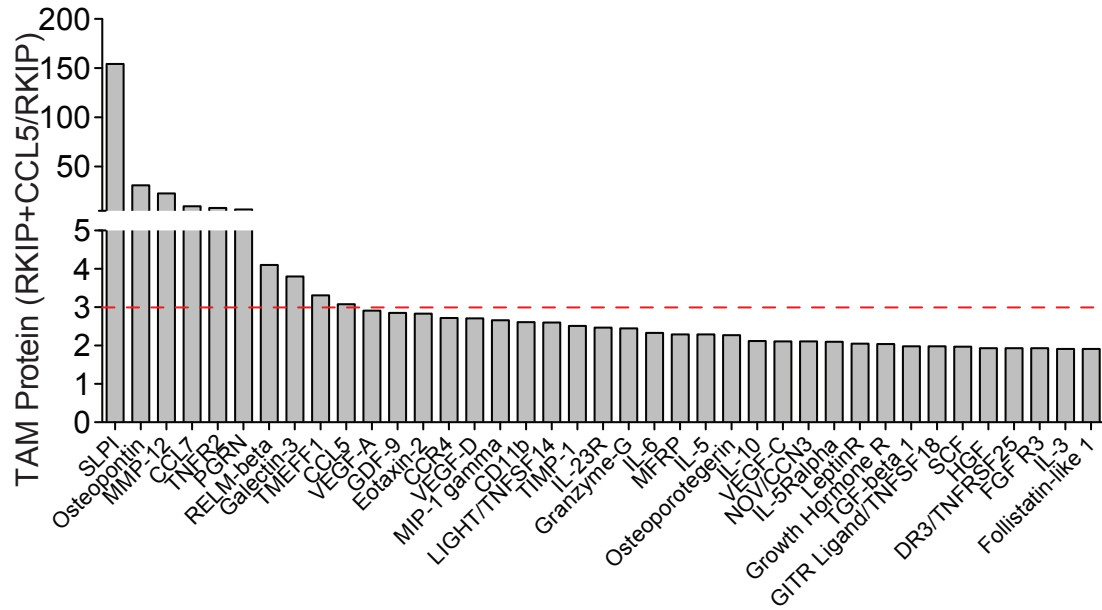
**Figure 19: CCL5 partially rescues tumor cell autonomous invasion blocked by RKIP**

20,000 BM1 cells (control, RKIP, CCL5, or RKIP+CCL5 transduced) were plated in transwell inserts coated with matrigel, and invaded cells were stained with Calcein and measured after 24 hours. Relative invasion is calculated by the amount of Calcein uorescence normalized to control cell invasion. P-values were calculated using an unpaired T-test with Welch's correction, N = 8 per group. \* 0.05 > p ≥ 0.01 \*\* 0.01 p ≥ 0.001 \*\*\* 0.001 > p



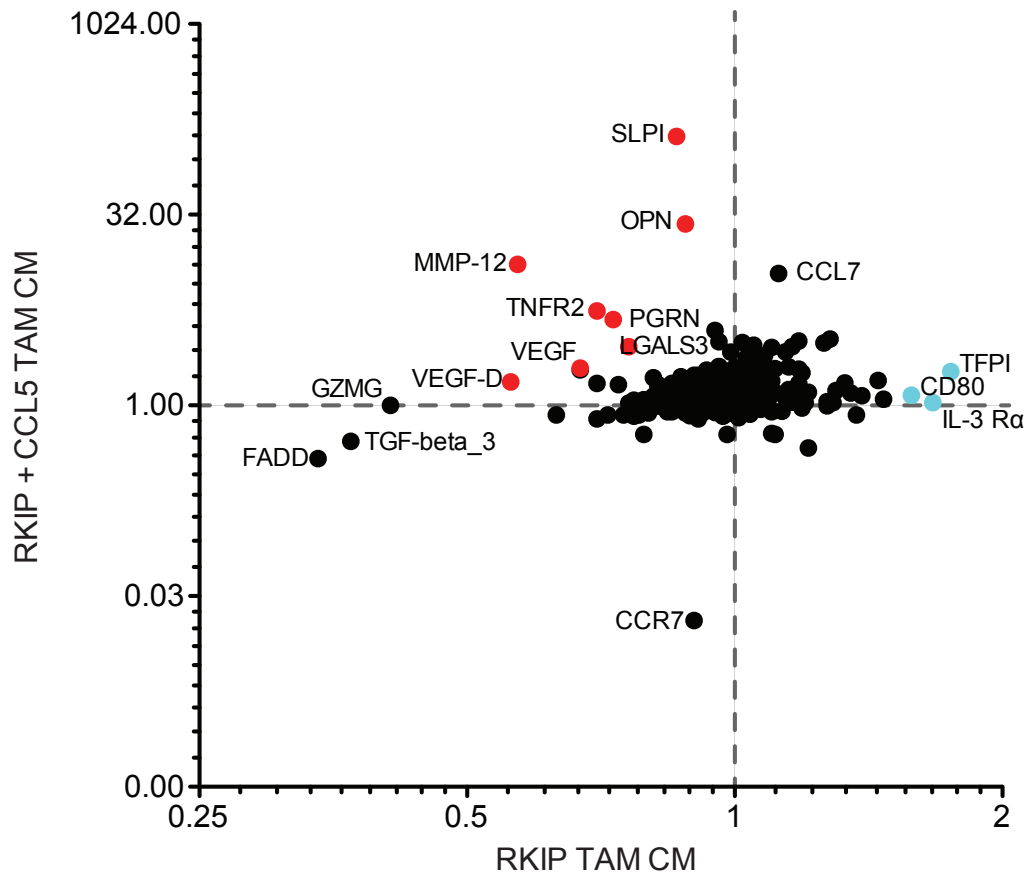
**Figure 20: L308 cytokine arrays or TAM CM**

Images shown of RayBiotech L308 Mouse Cytokine Arrays analysis of TAM conditioned media from four independent tumors



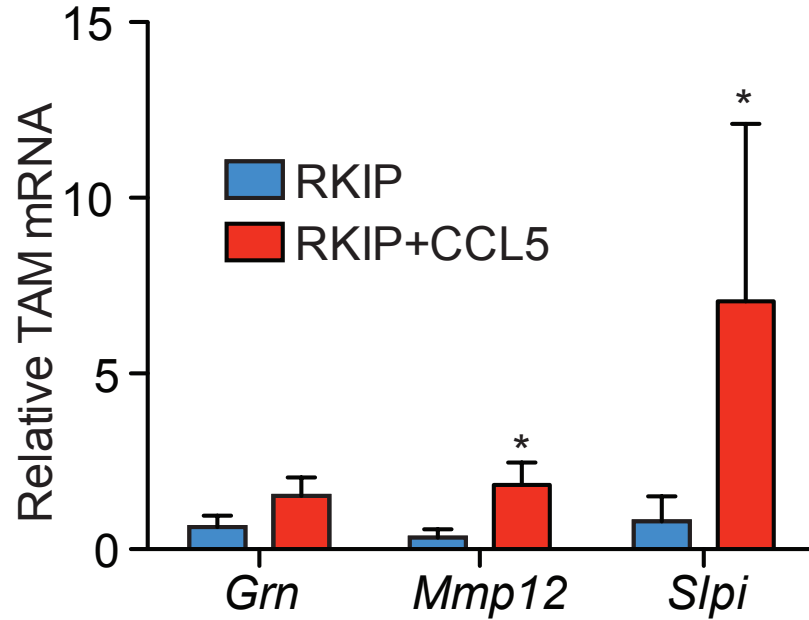
**Figure 21: Proteins most highly secreted in CCL5-recruited TAMs compared to RKIP**

TAM conditioned media from four independent tumors were combined analyzed for protein levels using RayBiotech L308 Mouse Cytokine Arrays. RKIP+CCL5 derived TAMs were normalized to RKIP derived TAMs with a cutoff set at greater than 3-fold expression to examine which proteins were over-expressed in CCL5-recruited tumors compared to those reduced by RKIP.



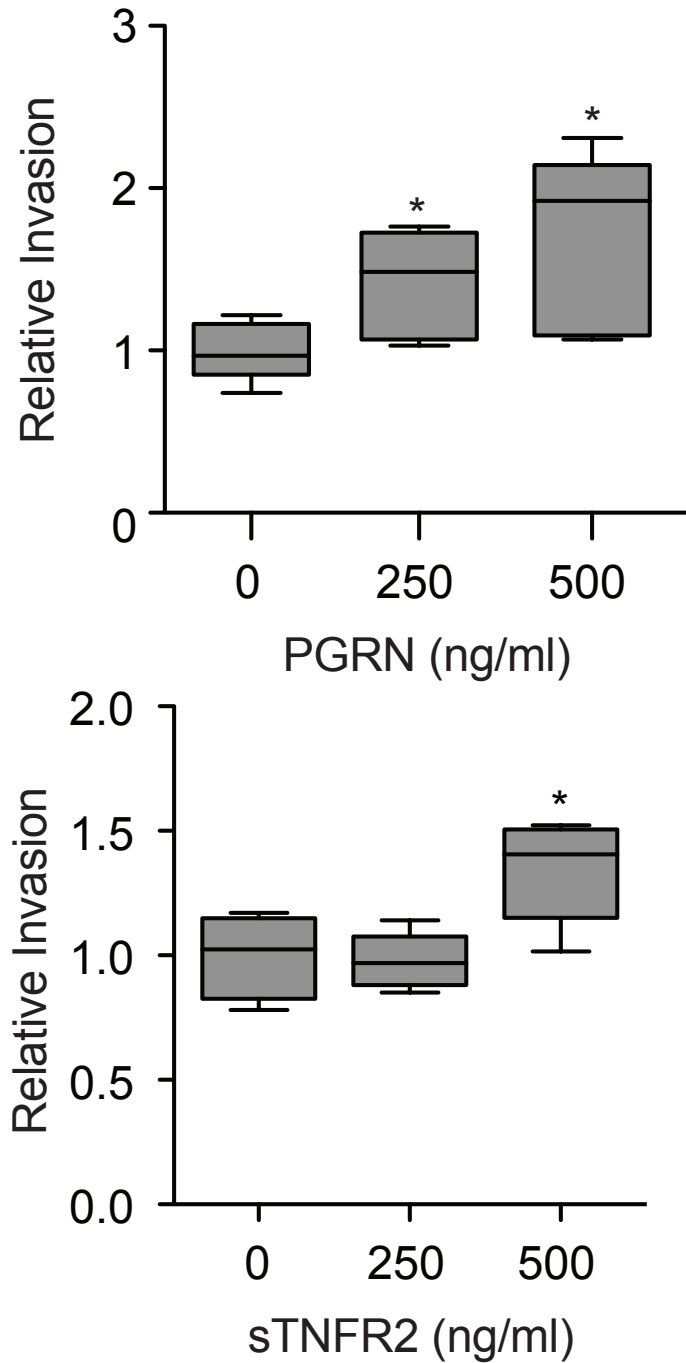
**Figure 22: Scatter plot comparing protein secretion reduced in TAMs from RKIP tumors and rescued in CCL5 over-expressing tumors**

To determine which proteins were reduced by RKIP in TAMs and rescued in CCL5-recruited TAMs, we plotted expression of all proteins measured by the L308 array on a scatter plot. Relative protein is shown for each protein from the cytokine array; x-axis: RKIP TAMs relative to control TAMs, y-axis RKIP+CCL5 TAMs relative to RKIP TAMs. Both axes are shown on a log 2 scale.



**Figure 23: Validation of gene expression of *Grn*, *Mmp12*, and *Slpi* in CCL5-recruited TAMs relative to those in RKIP tumors.**

We validated gene expression of the top genes reduced in TAMs from RKIP expressing tumors and rescued in CCL5-recruited TAMs. Relative mRNA was measured from three independent TAM samples per group. Relative mRNA was calculated as compared to control TAMs, with *Gapdh* as the reference gene.



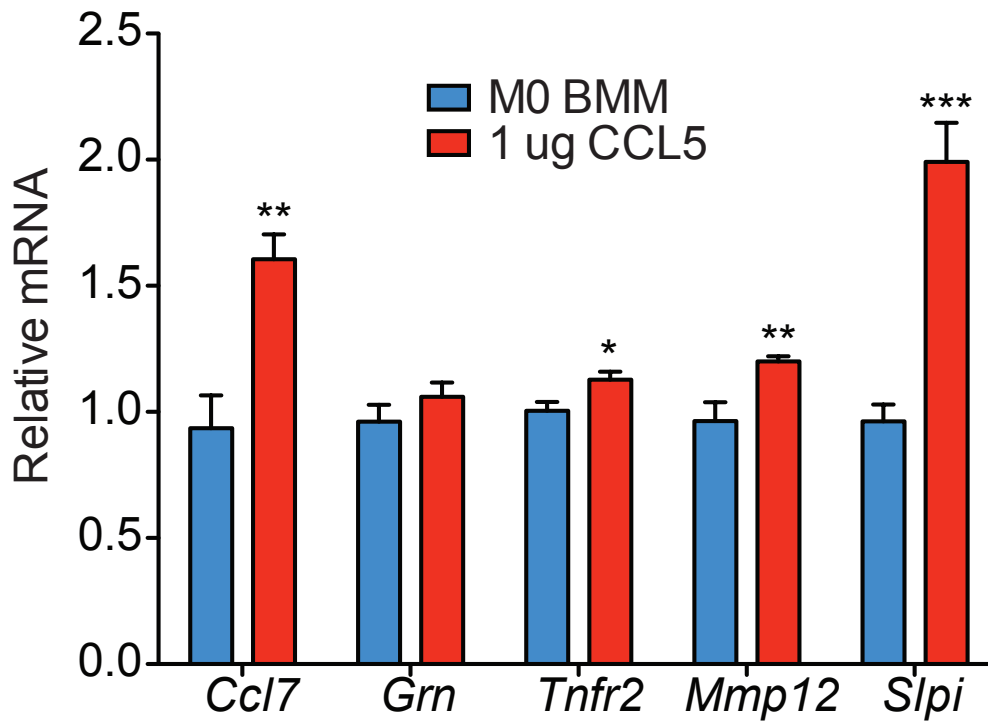
**Figure 24: Grn and sTNFR2 alone are able to potentiate BM1 tumor cell invasion**

To determine if TAM secreted factors could potentiate BM1 tumor cell invasion, we pretreated tumor cells for 24 hours with the indicated concentration of recombinant protein in serum free media before measuring invasion. Relative invasion for BM1 cells pretreated with various concentrations of GRN or sTNFR2 for 24 hours, results are plotted relative to an untreated control. Statistical significance was determined using a Student's T-test, n=5 per group.

We then determined whether CCL5 overexpression in BM1+ RKIP tumors could enable recruitment of TAMs that secrete pro-metastatic factors. Analysis of proteins in the CM of isolated TAMs by cytokine arrays revealed robust induction of a number of factors that were suppressed in TAMs recruited to non-metastatic tumors (Figure 20 - 21). For example, RKIP suppressed VEGF-A, VEGF-D, OPN, LGALS3, SLPI, MMP-12, sTNFR2, and GRN expression by TAMs, and extracellular levels of these proteins were restored or even elevated in RKIP+CCL5 tumors relative to control tumors (Figure 22). We confirmed the induction of *Mmp12*, *Slpi*, and *Grn* in TAMs isolated from RKIP+CCL5 tumors by qRT-PCR (Figure 23).

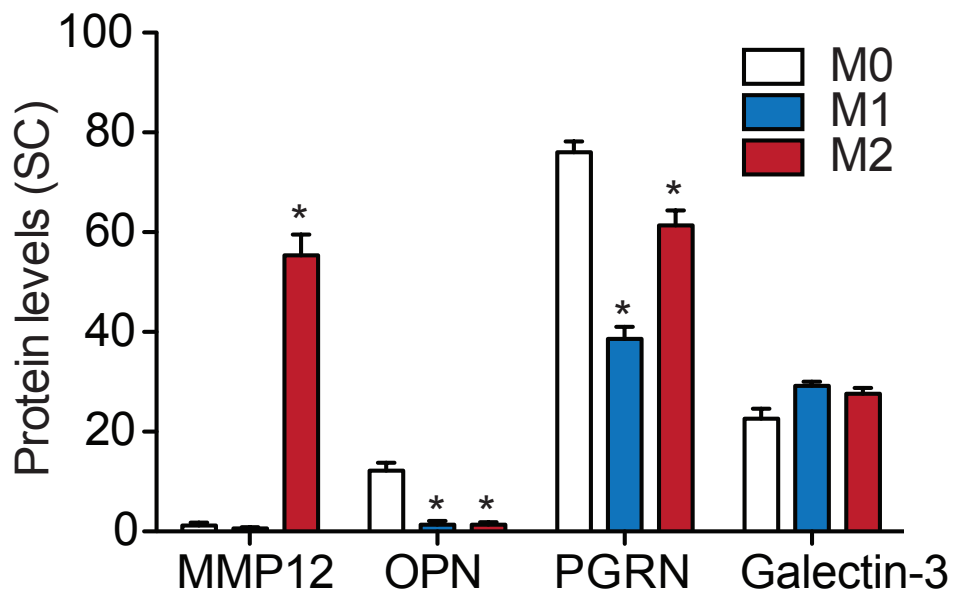
The group of TAM proteins suppressed by RKIP and induced by CCL5 included a number of potentially pro-metastatic factors. To confirm this possibility, we investigated whether PRGN or sTNFR2 were sufficient to drive invasion of TNBC cells *in vitro*. Treating BM1 cells with 500ng/mL recombinant sTNFR2 or GRN significantly induced tumor cell invasion (Figure 24). These results provide empirical evidence that pro-metastatic factors counter-regulated by RKIP and CCL5 directly promote the invasiveness of human breast cancer cells.

The finding that CCL5 restored the pro-metastatic function of TAMs was surprising given that it is generally believed that CCL5 acts as a chemotactic agent to recruit macrophages to tissues<sup>44</sup>. This observation led us to hypothesize that CCL5 might have direct actions on TAMs to promote the expression of pro-metastatic factors. Treating bone marrow-derived macrophages with CCL5 *in vitro* compared to control serum free treated cells significantly induced the expression of several pro-metastatic factors including *Ccl7*, *Tnfr2*, *Mmp12*, and *Slpi* (Fig 25). Thus, overexpressing CCL5 might overcome metastasis suppression in non-metastatic (RKIP+) tumors both by recruiting TAMs and directly programming them to overexpress pro-metastatic factors.



**Figure 25: CCL5 induces gene expression of *Ccl7*, *Tnfr2*, *Mmp12*, and *Slpi***

To examine whether CCL5 alone could induce expression of pro-invasive genes seen in TAMs, we treated BMDMs with 1  $\mu\text{g/ml}$  of recombinant CCL5 in serum free media for 24h compared to a serum free media control. Relative mRNA levels for bone marrow derived macrophages (BMMs) treated with or without 1  $\mu\text{g/ml}$  of recombinant CCL5 for 24h. *Gapdh* is used as a reference gene.



**Figure 26: *Mmp12* is M2 specific, while *Opn*, *Grn*, & *Galectin-3* are expressed in M0 or M1**

To determine if proteins found highly expressed in CCL5-recruited TAMs were of an M1 or M2 phenotype we compared expression of the above genes in proteomic data of M0, M1, and M2 derived BMDMs. Protein levels (spectral counts) in the conditioned media collected from BMMs (M0), LPS -induced BMMs (M1), and IL-4-induced BMMs (M2) were quantified by mass spectrometry.

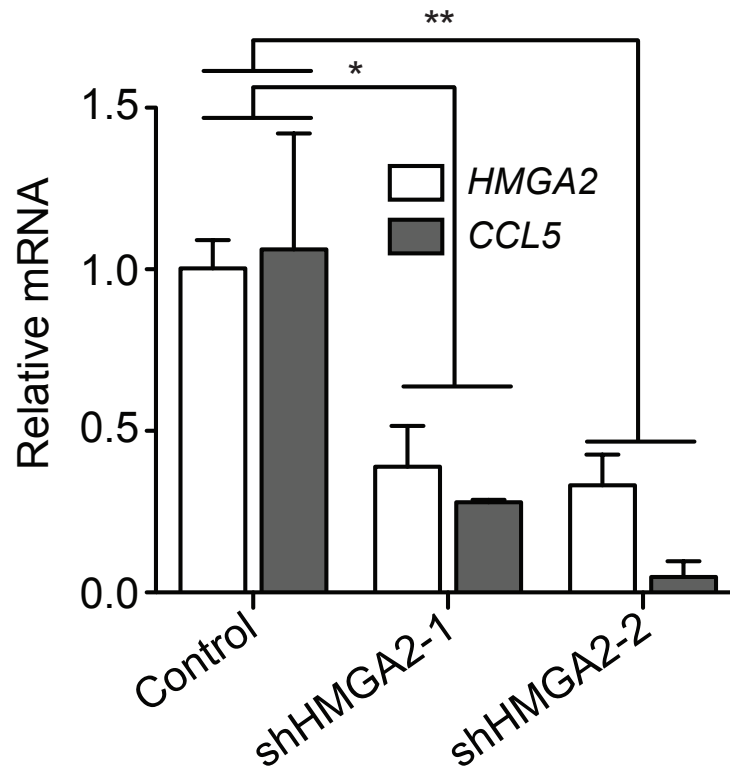
The difference between TAMs from metastatic (BM1 or BM1 RKIP+CCL5) and non-metastatic (RKIP+) tumors could reflect a switch from an M2 to an M1 phenotype. To examine this possibility, we analyzed proteins secreted by bone marrow-derived macrophages (M0), M1 macrophages (activated by LPS/IFN $\gamma$ ), and M2 macrophages (activated by IL4) using mass spectrometry. When we compared them to factors secreted by CCL5-recruited TAMs, MMP12 was significantly increased in M2 compared to M0 and M1 macrophages; however, GRN and LGALS3 were broadly expressed, and OPN was selectively expressed in M0 macrophages (Figure 26). These results suggest that the markers expressed in the CCL5-recruited TAMs are not indicative of a classic M1 or M2 phenotype.

Collectively, these findings demonstrate that CCL5 overexpression can promote macrophage infiltration, macrophage function, and intravasation on a non-metastatic (RKIP+) background, suggesting that downregulation of CCL5 by RKIP, and the concomitant reduction in TAMs, may be an important mechanism by which RKIP suppresses metastasis.

### **Suppression of metastasis and TAMs by RKIP is coordinated through HMGA2 signaling**

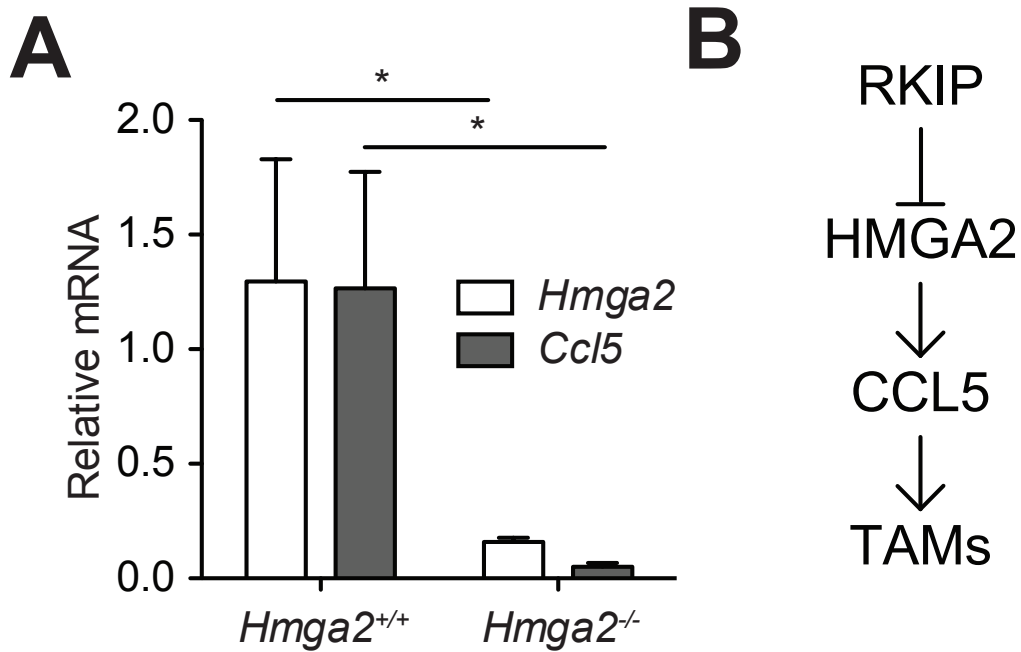
Our previous work showed that RKIP suppresses breast cancer metastasis in part by inhibiting the architectural transcription factor High-mobility group AT-hook 2 (*HMGA2*)<sup>18,97</sup>. We therefore determined whether RKIP suppresses macrophage recruitment via a similar mechanism. *HMGA2* depletion in BM1 cells led to a significant decrease in *CCL5* expression *in vitro* (Figure 27). To test whether *HMGA2* regulates macrophage accumulation *in vivo*, we crossed *Hmga2*<sup>-/-</sup> mice with the invasive *MMTV-Wnt1* genetically engineered mouse (GEM)<sup>108</sup>. Similar to the RKIP<sup>-</sup> phenotype, *Hmga2*<sup>-/-</sup> GEM mice (relative to *Hmga2*<sup>+/+</sup>) had decreased *Ccl5*

expression in the mammary tumors (Figure 28A) and a marked reduction in the number of macrophages present both in the tumor tissue as well as in the surrounding stroma (Figure 29). Together with previous findings<sup>18,97</sup>, these results suggest that RKIP suppression of tumor cell CCL5 expression, macrophage recruitment and metastasis is coordinated through HMGA2 signaling (Figure 28B).



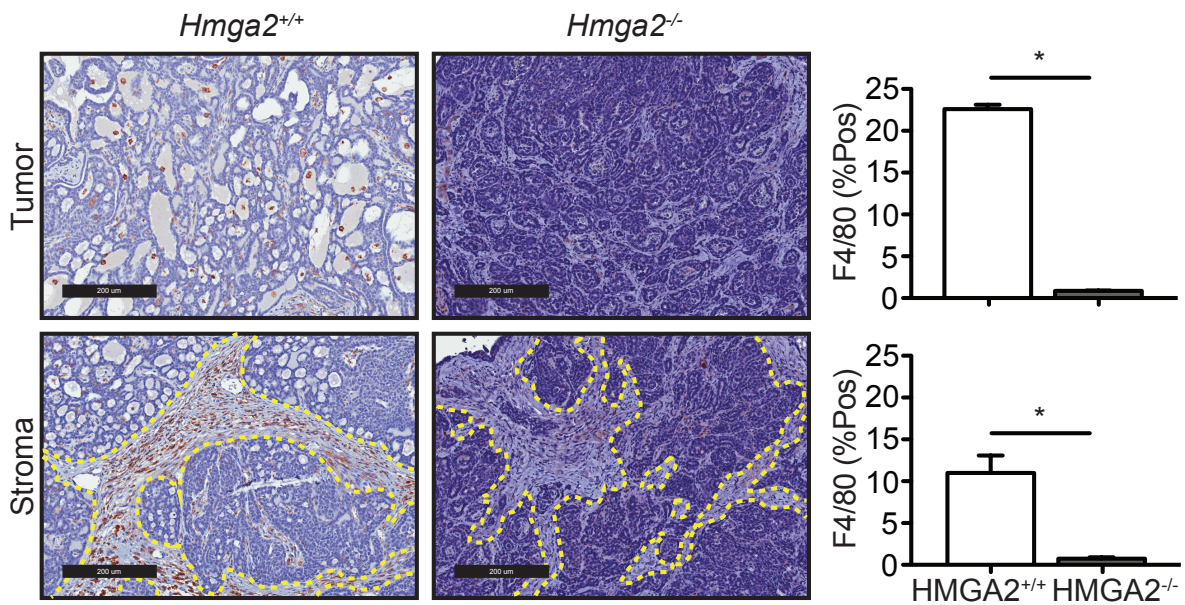
**Figure 27: Suppression of HMGA2 using shRNA lentiviral transduction reduces CCL5 expression in BM1**

BM1 cells were transduced with two separate shRNAs targeting HMGA2. Relative expression of *HMGA2* and *CCL5* was quantified by qRT-PCR and normalized to *GAPDH*. Results (n=3 per group) are relative to BM1 cells transduced with control shRNA.



**Figure 28: Reduced expression of CCL5 in an HMGA2<sup>-/-</sup> in the Wnt GEM model of breast cancer**

A) qRT-PCR analysis of *Hmga2* and *Ccl5* gene expression in tumors isolated from control and *Hmga2*<sup>-/-</sup> Wnt GEM mice (n=4). B) A schematic showing the regulation of CCL5 by RKIP through HMGA2.



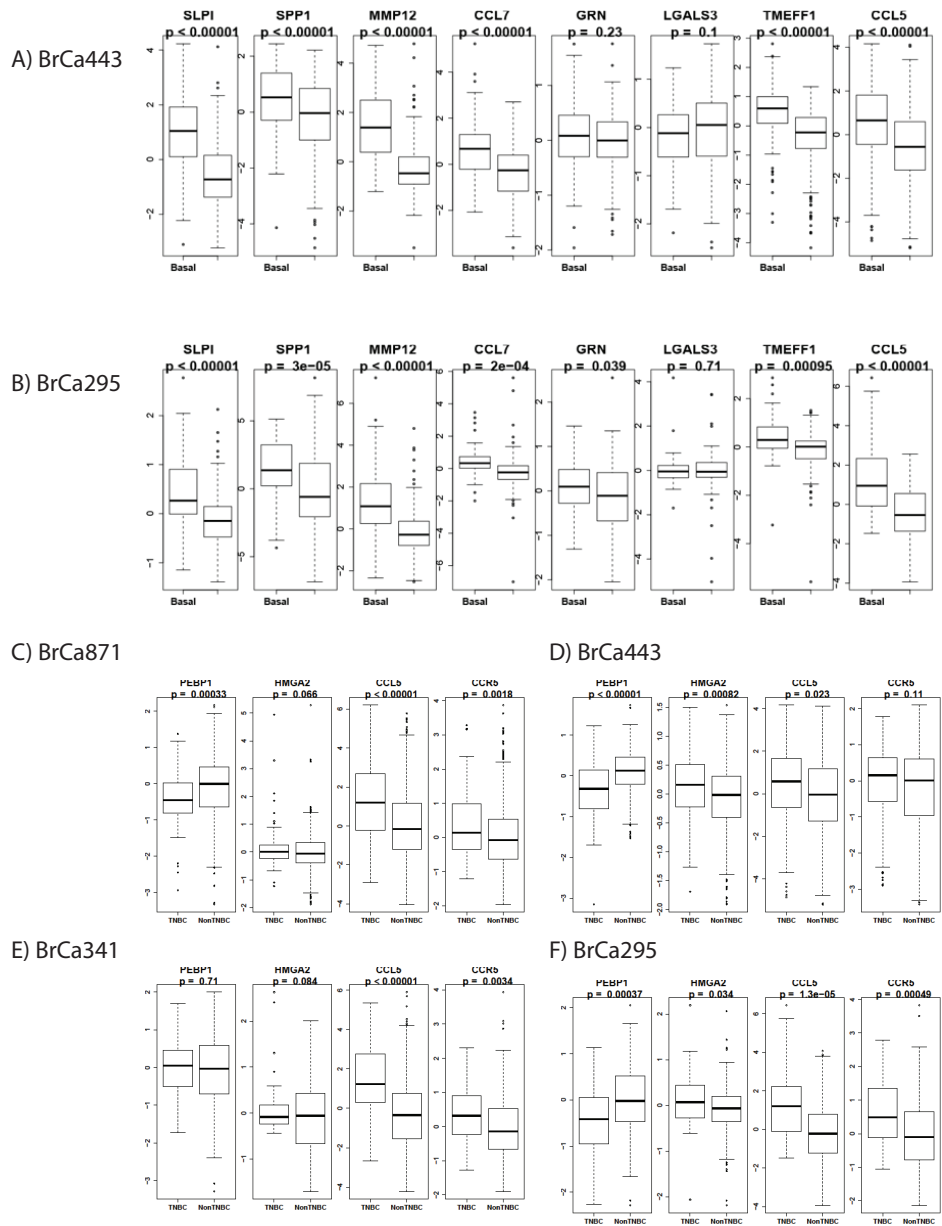
**Figure 29: HMGA2 <sup>-/-</sup> Wnt GEM mice show reduced macrophage infiltration**

Representative images of macrophage infiltration in wild type and *Hmga2*<sup>-/-</sup> mice as determined by F4/80+ staining. Stromal regions are delimited by the dashed lines. Macrophage infiltration was quantified as the %F4/80+ area in the tumor and stromal regions.

### **An RKIP-macrophage gene signature predicts metastasis-free survival**

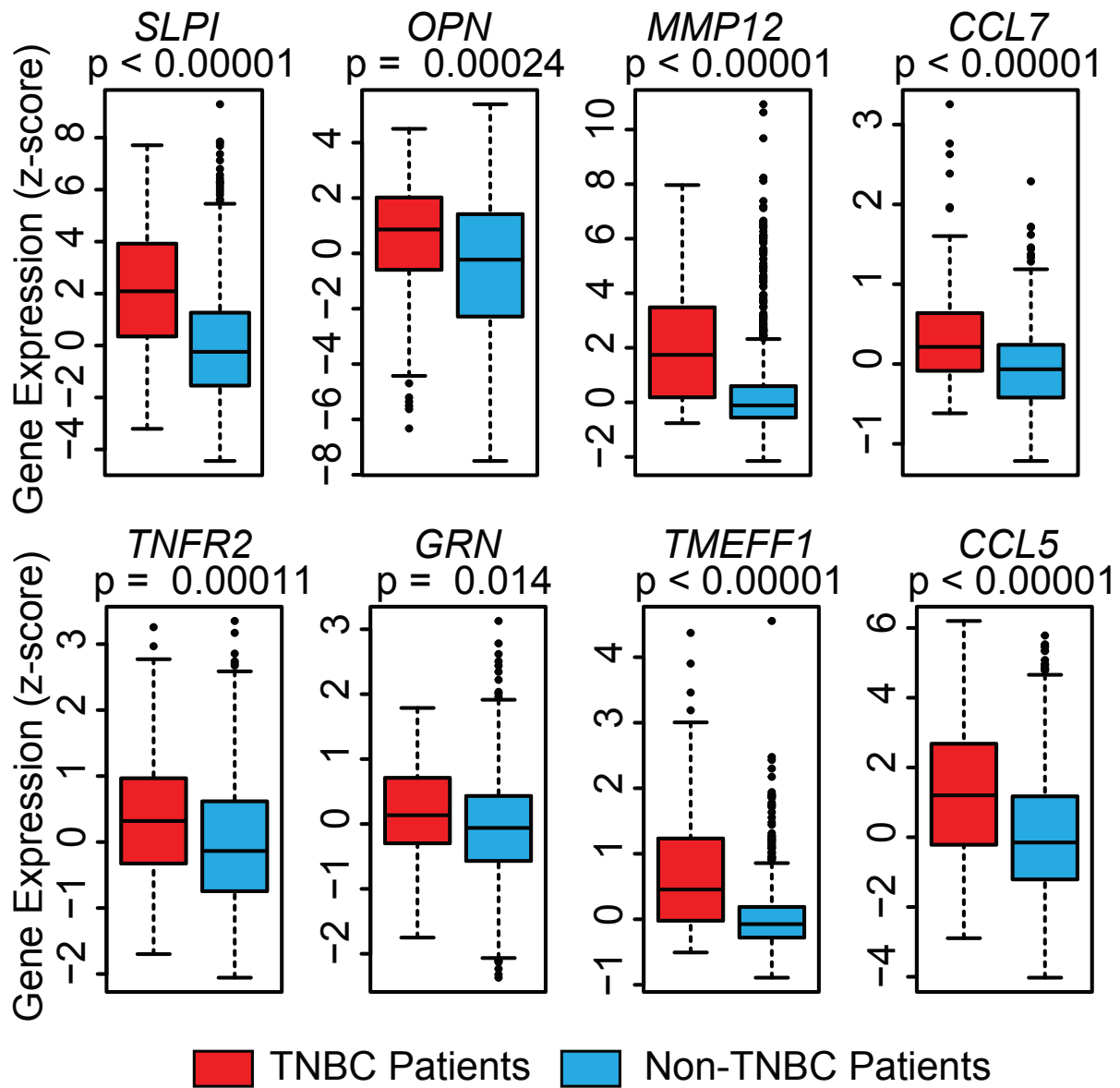
Our data suggest that an RKIP-HMGA2-CCL5 pathway regulates recruitment of a TAM population that promotes tumor metastasis in mice. To begin to validate this pathway in humans, we analyzed gene expression in human tumors obtained from TNBC (n=319) and non-TNBC (n=1631) patients. When we examined gene expression across 4 independent data sets from breast cancer patients, we found that *RKIP* was suppressed and *HMGA2*, *CCL5* and *CCR5* were induced in TNBC tumors relative to non-TNBC tumors (Figure 30). Thus, an RKIP- HMGA2-CCL5 pathway can be used to classify metastatic versus non-metastatic tumors in both mouse models and human patients.

Since TAMs secrete regulators of metastasis in mice (Figure 4), we examined the gene expression of these TAM-secreted proteins in human breast cancer patients and found that *SLPI*, *OPN*, *MMP12*, *CCL7*, *TNFR2*, *GRN*, *TMEFF1*, and *CCL5* were all significantly increased in TNBC compared to non-TNBC patients (Figure 31). Our results show that CCL5 recruits TAMs that secrete these factors. Therefore, we performed gene set analysis (GSA) as previously described<sup>18</sup> to identify which of these factors were consistently co-expressed with *CCL5* in human TNBC tumors and found a strong correlation between the gene expression levels of *CCL5* with *TNFR2*, *GRN*, and *CCL7* in TNBC patients in all 4 datasets (Figures 32 - 33). These results raise the possibility that the signaling pathway from RKIP to the three factors secreted by TAMs (*TNFR2*, *GRN*, and *CCL7*) defines a set of linked events that are prognostic for patient outcome.



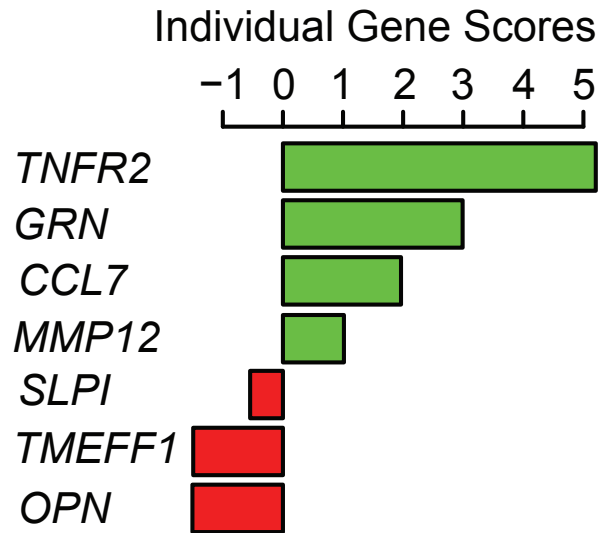
**Figure 30: Expression of SLPI, SPP1, MMP12, CCL7, TNFR2, GRN, LGALS3, TMEFF1, PEBP1, HMGA2, CCL5, and CCR5 in human tumor samples**

Expression estimates of genes are from a breast cancer patients, stratified into either TNBC or non-TNBC patients. SLPI, OPN (SPP1), MMP12, CCL7, TNFR2, GRN, LGALS3, TMEFF1, and CCL5 or PEBP1 (RKIP), HMGA2, CCL5 and CCR5 expression levels we compared between TNBC and non-TNBC patients using a Student's T-test.



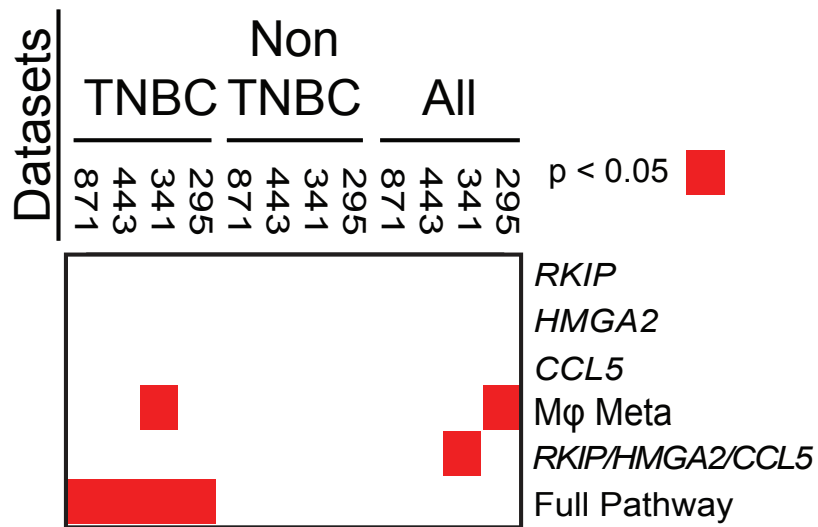
**Figure 31: CCL5-recruited TAM genes more highly expressed in TNBC patients compared to non-TNBC patients**

Gene expression estimates from a set of 871 breast cancer patients, stratified into either TNBC or non-TNBC patients.



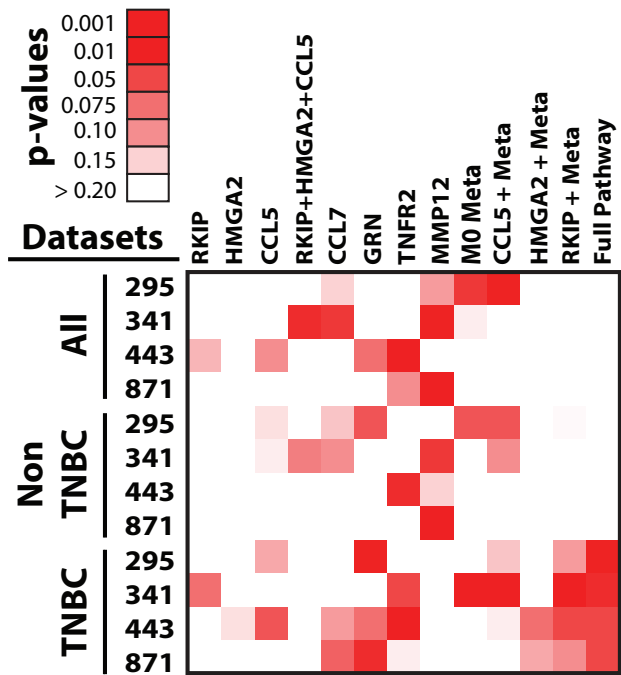
**Figure 32: TAM secreted proteins most correlated to CCL5 in TNBC patient tumor expression**

Individual scores for each gene in comparison to CCL5 expression are plotted for 12 separate genes.



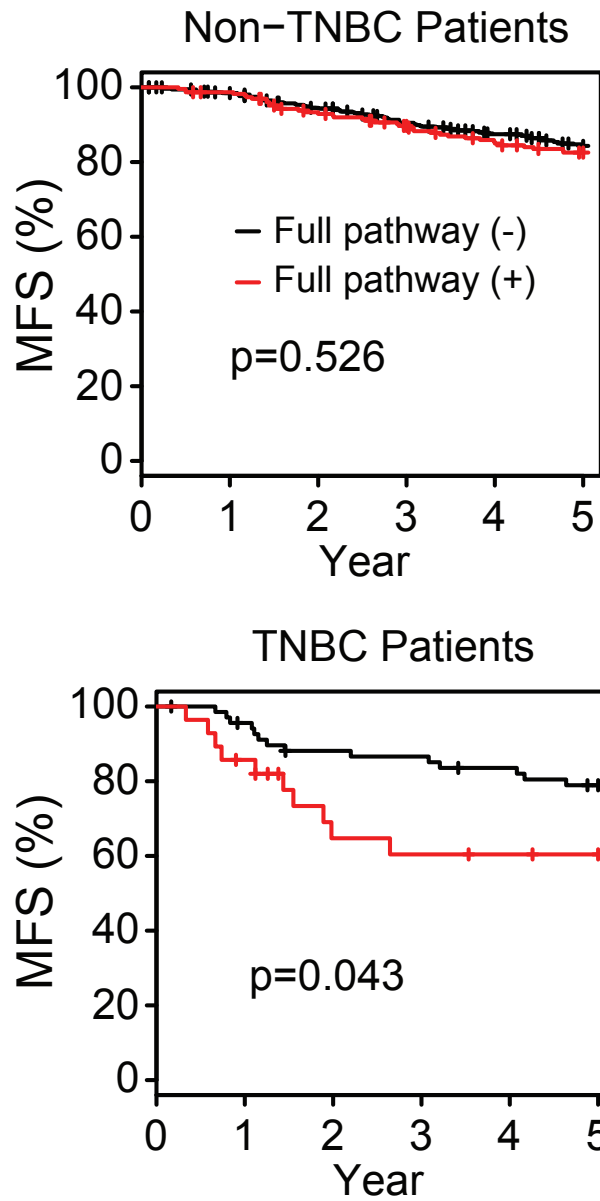
**Figure 33: Full Tumor-TAM pathway signature is predictive of outcome in TNBC patients**

Heatmap identifying data sets (top) where breast cancer metastasis free survival is significantly stratified by classifier (right)



**Figure 34: Full Pathway alone is predictive compared to all other combinations of genes included in TNBC-TAM signature**

To determine which components of the TNBC-TAM signaling pathway were most important for patients' outcome we compared the full pathway to each gene alone in addition to combinations of TNBC or TAM gene expression alone. Heat-map identifying data sets (left) where breast cancer metastasis free survival is significantly stratified by classier (top).



**Figure 35: Full TNBC-TAM signature stratifies TNBC but not non-TNBC patient survival**

Kaplan-Meier plots are shown for a set of all 871 breast cancer patients. Patients (+) for the Full Pathway signature are shown (red line) compared to those (-) for the Full Pathway signature (black line)

To determine the clinical value of these genes, we developed a signature utilizing the expression levels of tumor genes regulating TAM recruitment (*RKIP*, *HMGA2*, *CCL5*) in combination with stromal TAM-secreted genes (a TAM metagene derived from *TNFR2*, *GRN*, and *CCL7*). When we examined all patients in the data sets or those categorized as non-TNBC using molecular phenotypes as classifiers<sup>109</sup>, no significant relationship to clinical outcome was observed (Figures 33 - 34). However, when we limited analyses to TNBC patients, a gene signature based upon the combination of *RKIP*<sup>low</sup>, *HMGA2*<sup>high</sup>, *CCL5*<sup>high</sup>, and TAM-metagene<sup>high</sup> expression was significantly prognostic for poor metastasis-free survival (MFS) (Figure 35). When considered alone, both the tumor-based gene signature (*RKIP/HMGA2/CCL5*) and the TAM genes (*TNFR2*, *GRN*, *CCL7*) were poor prognostic indicators for breast cancer outcome. Only the gene signature based on the combined tumor and TAM regulatory modules was significant across four independent sets of TNBC patients (Figures 33 - 34). These results highlight the importance of tumor-stromal crosstalk in the metastatic progression of TNBCs.

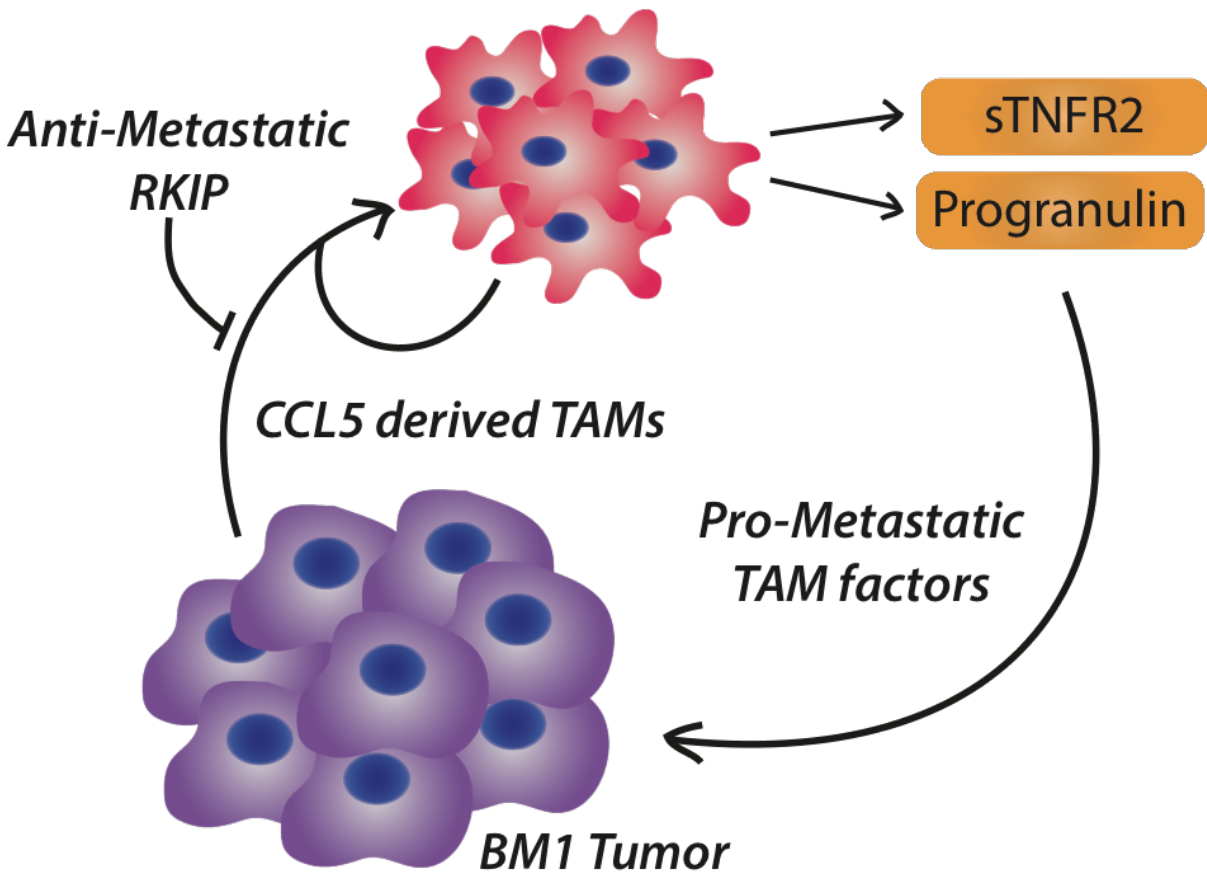


Figure 36: Schematic summarizing the circular interplay between TNBC cells and TAMs.

## Discussion

In this study, we identified a novel mechanism whereby RKIP regulates tumor invasiveness by inhibiting infiltration of a subset of TAMs that secrete pro-metastatic factors. We showed that TAMs recruited to metastatic RKIP<sup>-</sup> tumors, relative to non-metastatic RKIP<sup>+</sup> tumors, had reduced ability to drive tumor invasion and decreased secretion of numerous pro-metastatic factors. We demonstrated that RKIP inhibits TAM recruitment by reducing CCL5 expression. CCL5 overexpression was sufficient to rescue recruitment of pro-metastatic TAMs and tumor cell intravasation on a non-metastatic RKIP<sup>+</sup> background, and CCL5 inhibition reduced TAM infiltration. Interestingly, a gene signature based on the RKIP regulatory pathway combined with pro-metastatic TAM factors regulated by RKIP was prognostic for metastasis-free survival of TNBC patients. Thus, suppression of RKIP, through direct effects in tumor cells and indirect on TAM recruitment in the microenvironment, may partially explain the aggressive tumors observed in TNBC patients (Figure 36).

Our demonstration that RKIP expression in tumors markedly attenuates infiltration of pro-metastatic TAMs suggests that metastasis suppressors play a more extensive role in regulating the tumor microenvironment than previously realized. TAMs are known to promote metastatic progression through secretion of growth factors, MMPs, and suppression of the immune system<sup>37</sup>. Our protein array analysis of secreted factors suppressed in TAMs from RKIP<sup>+</sup> tumors and restored in TAMs from RKIP<sup>+</sup>CCL5 tumors revealed similar categories including angiogenesis, extracellular matrix organization, growth factor activity, immune system development and regulation of locomotion (Tables S1,2)<sup>110</sup>. Thus, one important mechanism by

which RKIP suppresses metastasis is by reducing the number and pro-metastatic phenotype of TAMs.

On a molecular level, RKIP regulates TAM recruitment, in part, by attenuating CCL5 expression. Although the CCL5-CCR5 axis has been implicated in breast cancer metastasis, the role of macrophages in this process and the molecular and cellular mechanisms of action have been controversial<sup>44,106,107</sup>. Our study shows that paracrine CCL5 signaling recruits TAMs and perhaps directly programs their pro-metastatic function. This process is further promoted by an autocrine loop leading to CCL5 expression in the TAMs themselves. Expression of CCL5 and the presence of CCL5-recruited TAMs are insufficient by themselves to predict outcome, consistent with the fact that CCL5 cannot completely rescue the TAM phenotype. However, the combination of the RKIP tumor-signaling pathway with the CCL5-TAMs enables generation of a prognostic gene signature for TNBC patients. This result highlights the crosstalk between tumor cells and TAMs (Figure 7) and suggests that taking into account both tumor and stromal factors may be effective for prognosis and therapeutic efficacy in TNBC patients.

Consistent with this hypothesis, previous work has shown that shed TNFR2 (sTNFR2) protein is higher in the plasma of pancreatic, endometrial, and breast cancer patients<sup>111-113</sup> and is associated with an increased risk of cancer<sup>113</sup>. Granulin (GRN) expression blocks TNFR2-mediated inflammation and has been shown to drive migration, invasion and VEGF expression in breast cancer<sup>114,115</sup>. GRN is highly expressed in a number of tumors including breast, and has also been targeted using biologics in hepatocellular carcinoma<sup>114,116</sup>. Moreover, we provide direct evidence that GRN and sTNFR2 can promote the invasiveness of human TNBC tumor cells *in vitro*.

Because of the strong evidence of pro-invasive action and the clinical relevance of these factors, CCL5, GRN, and sTNFR2 are all potential targets for anti-TNBC drug treatment.

While our studies revealed a role for RKIP in macrophage recruitment to tumors in both xenograft and syngeneic mouse models, other metastasis suppressors might have unique roles in regulating additional cell types in the stroma. Furthermore, we identified factors secreted by pro-metastatic TAMs in this study using immune-compromised nude mice that lack mature T-cells. It is possible that, in TNBC patients, RKIP might also play a role in regulating T-cells through factors such as CD80 (upregulated in RKIP+ BM1-derived TAMs) and is a potential therapeutic tool in the treatment of breast cancer patients<sup>104</sup>.

Understanding how the tumor cells and TAMs interact could lead to novel strategies for blocking TAM recruitment to TNBCs and tumor metastatic progression. In the case of CCL5 - recruited TAMs, there is an array of pro-metastatic genes that support the tumor. Elegant work on TAMs in breast cancer by Pollard and others has implicated CCL2 and GM-CSF in TAM recruitment in breast cancer. However, the models used to study CCL2 are largely based on the luminal/HER2+ MMTV-PyVT GEM model<sup>43</sup> and are likely to display a unique set of molecular interactions. In the present study, no inhibition of CCL2 or GM-CSF expression by RKIP was observed by RNAseq analysis. Instead, we show that expression of a metastasis suppressor in tumor cells regulates recruitment of a CCL5-responsive TAM population secreting pro-metastatic factors. Future studies will be necessary to determine which inhibitors of these pathways will be most effective therapeutically either alone or in combination in triple-negative breast cancer.

## CHAPTER 4: Tumor Exosomes are required for Programming of Tumor-Associated Macrophages

### Abstract

Triple-negative breast cancer (TNBC) patients have the highest risk of recurrence and metastasis. Infiltration of macrophages into the tumor promotes tumor growth, survival, therapy resistance, and metastasis. We previously demonstrated that the metastasis suppressor Raf Kinase Inhibitory Protein (RKIP) regulates the number and phenotype of tumor-associated macrophages (TAMs) through CCL5. While we demonstrated that CCL5 is sufficient to recruit pro-metastatic TAMs, the mechanism by which recruited TAMs are programmed to a pro-metastatic phenotype is not known.

To determine the mechanism of macrophage programming by TNBC cells, we treated bone marrow derived macrophages (BMDMs) with conditioned media (CM) from TNBC cells, CM depleted of exosomes, or tumor exosomes alone. Only exosomes alone showed increased levels of pro-invasive genes. CCL5 expression in tumor cells increased the pro-invasive phenotype of macrophages via tumor exosomes. Additionally, expression of RKIP or knock out of CCL5 blocked pro-invasive programming of macrophages through exosomes. Finally, we found that resistance to the CCL5 inhibitor Maraviroc could be transmitted to macrophages through exosomes.

For *in vivo* validation, mice were injected with exosome-depleted tumor cells using shRab27a. While shRab27a in tumors did not change TAM number, it decreased the ability of TAMs to drive TNBC invasion and reduces expression of pro-invasive cytokines including CCL3, CCL7, CCL19, CXCL1, CXCL3, Flt-1, OPN, SLPI, TGF-  $\beta$ 3, TNFSF14, and Thrombospondin. Co-injection of exosome programmed TEMs, which also express these pro-invasive cytokines,

resulted in both an increase in number and size of lung metastases. Taken together, these results indicate that exosome are essential for the pro-metastatic programming of TAMs within the microenvironment as well as transferring therapeutic resistance.

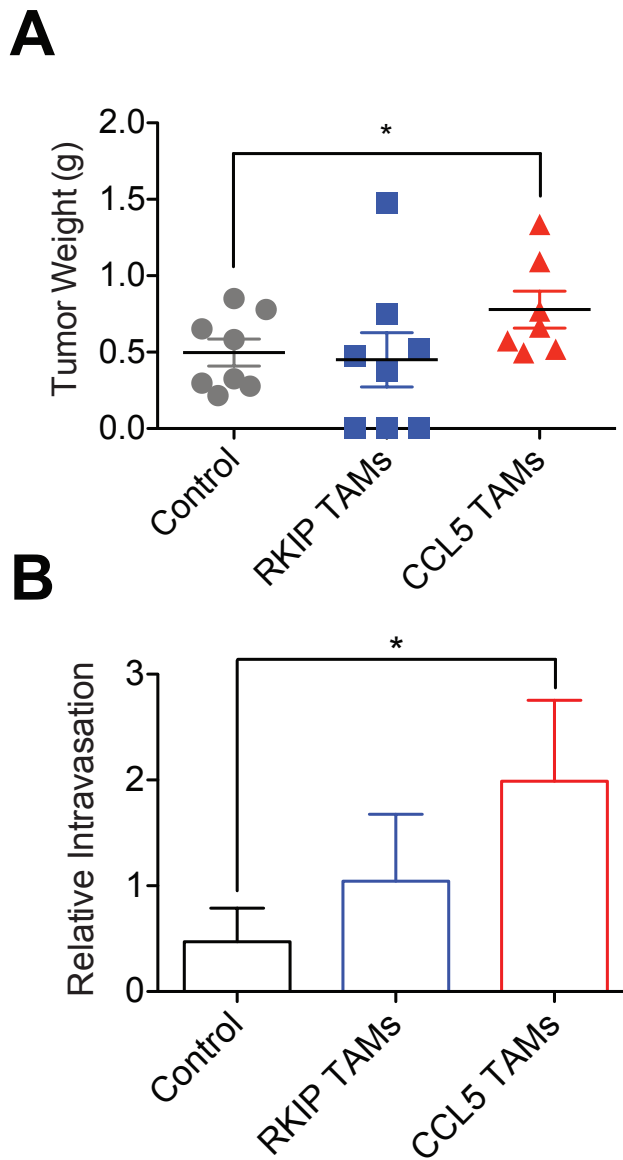
## **Introduction**

One out of every eight women in the United States will develop breast cancer in her lifetime. Of these women, 15-20% will have basal-like or triple-negative breast cancer (TNBC). Triple-negative tumors lack expression of estrogen receptor (ER), progesterone receptor (PR) and amplification of human epidermal receptor 2 (HER2). This disease disproportionately affects African American and low-income women, with rates ~3 times higher than the rest of the population<sup>3</sup>. Because of the poor response to chemotherapy and lack of currently approved targeted therapies for TNBC patients, their 5 year survival rate is only 24%; by contrast, patients who can be targeted with estrogen receptor antagonists or HER2 antibodies have 5 year survival rates of ~98%<sup>4</sup>.

Our previous work elucidated the role of CCL5 in recruiting tumor-associated macrophages (TAMs) to TNBC tumors. CCL5 recruited TAMs showed increased expression of growth factors, cytokines, and other pro-metastatic factors including CCL7, MMP-12, Osteopontin, Progranulin, SLPI, and shed TNFR2, leading to an increased ability to drive tumor invasion *in vitro* and an increase in tumor intravasation *in vivo*. Recent work has shown that the level of M2 macrophages in the tumor stroma (determined through cluster of differentiation 163 (CD163) staining) correlates with outcome in patients, as well as with tumor type. More specifically, M2 macrophage recruitment positively correlates with TNBC tumors while negatively

correlating with ER+ tumors<sup>27</sup>. Our previous work established that a gene signature based on tumor CCL5 signaling and TAM gene expression indicative of CCL5-TAM recruitment, was able to significantly stratify specifically TNBC patients at high risk.

We sought to elucidate if CCL5 recruited macrophages were physiologically important for metastasis as well as the method by which tumor cells program TAMs once they were recruited within TNBC, focusing on soluble factors and exosomes secreted by tumor cells. While the role of exosomes has been well established in the creation of a pre-metastatic niche<sup>78,79</sup>, less is known about their role in programming TAMs toward a pro-tumor phenotype. Previous work has established that exosome are taken up by macrophages, likely through a phagocytic pathway<sup>78,117,118</sup>, and that exosomes can stimulate macrophage cytokine production through activation of TLR2, TLR3, or TLR4 receptors, leading to NF- $\kappa$ B and STAT3 signaling<sup>93,119</sup>. However, whether tumor exosomes are required for macrophage polarization by tumors is not known, and no studies have been performed *in vivo* to study TAM polarization by tumor exosomes. In this work, we show that tumor exosomes are both necessary and sufficient for the programming of macrophages to an M2-like, TAM phenotype capable of driving invasion and metastasis.



**Figure 37: CCL5 recruited TAMs increase tumor growth and intravasation**

1 million MDA-MB-231 tumor cells were injected into each mouse. In the indicated groups, 0.5 million TAMs were injected. TAMs were isolated from tumors either over-expressing RKIP (RKIP TAMs, blue) or RKIP & CCL5 (CCL5 TAMs, red). A) Final tumor weights are shown per group. B) Relative intravasation was measured by quantifying the ratio of human *GAPDH* (tumor) to mouse *Gapdh* by qRT-PCR (n=10 per group) from blood taken immediately prior to sacrifice.

## Results

### ***CCL5-recruited TAMs are sufficient to increase intravasation in vivo***

Our previous work had demonstrated that CCL5 expression in tumors was sufficient to recruit TAMs, restore tumor cell intravasation, as well as generate a clinically prognostic gene signature for TNBC patients when combined with TAM gene expression<sup>120</sup>. To determine the effect of CCL5-recruited TAMs on metastasis, we isolated TAMs from RKIP expressing (non-metastatic) and RKIP+CCL5 expressing (CCL5-rescued) tumors. We then injected one million MDA-MB-231 cells along with 0.5 million TAMs into nude mice. To address the effect on metastasis in this xenograft model, we assayed the ability of tumor cells to intravasate into the blood stream by comparing tumor GAPDH to mouse *Gapdh* expression by qRT-PCR as previously described<sup>21,22,120</sup>. We found that CCL5-recruited TAMs showed an increase in both tumor growth (Figure 37A) as well as tumor cell intravasation into the blood stream (Figure 37B). By contrast, TAMs from RKIP over-expressing tumors were not able to potentiate tumor growth or intravasation (Figure 37). These results suggest that TAMs recruited by CCL5 are programmed by tumor cells to regulate the metastatic properties of tumor cells.

### ***Secreted CCL5 protein cannot program macrophages***

To address how tumor cells program macrophages toward a pro-tumor TAM phenotype, we developed an assay based upon the ability of tumor cell conditioned media (CM) to educate bone marrow-derived macrophages (BMDMs) to promote tumor cell invasion. We termed these tumor-educated macrophages (TEMs) (Figure 38A). We first asked if CCL5 protein secreted from tumors could directly program TEMs by altering gene expression. Our previous work had

demonstrated that, when BMDMs were treated with a high dose of recombinant human CCL5 (1 ug/ml) compared to a BMDM control under serum-free conditions, *Grn*, *Mmp12*, and *Ccl7* expression were all induced<sup>120</sup>. However, when 20 ng/ml of mouse M-CSF is added to support macrophage survival in culture during TEM programming, stimulation of macrophages with even this high dose of CCL5 on top of M-CSF stimulation was not able to program TEMs to a pro-invasive phenotype (Figure 38B) nor induce the expression of the pro-metastatic genes *Ccl7*, *Grn*, or *Mmp12* in the TEMs (Figure 38C). Figure 38A summarizes the method for programming TEMs, tumor cell treatment with TEM CM, and invasion assay. The present results suggest that M-CSF alone is capable of stimulating low-level expression of many pro-invasive genes, and addition of CCL5 in the presence of M-CSF is not capable of further inducing expression above these levels.

#### ***Exosomes are a necessary component of TNBC CM programming of TEMs***

We then asked whether the CM of the tumor cells is sufficient to generate TEMs that further promote tumor cell invasion. As shown in Figure 39A, TNBC CM was initially collected and used to program TEMs, which were assayed for changes in cytokine expression. CM from the programmed TEMs was subsequently collected and used to pre-treat TNBC cells which were then assayed for invasion. When TEMs are programmed using CM from the bone-metastatic variant of the human TNBC cell line MDA-MB-231 (BM1) or the mouse TNBC cell line E0771-LNB (LNB), expression of *Ccl7* is induced in TEMs (Figure 39B) and their CM is able to increase tumor cell invasion (Figure 39C).

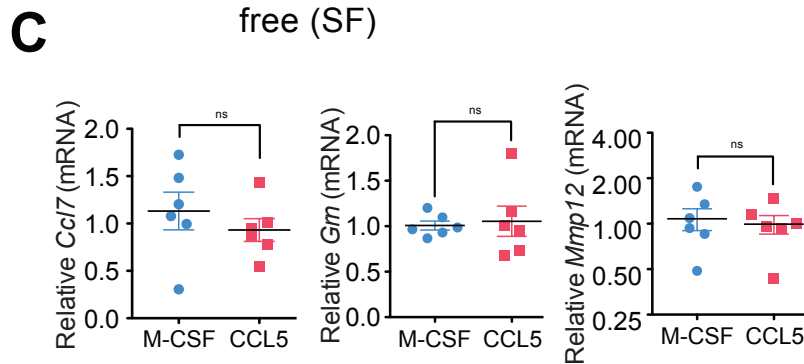
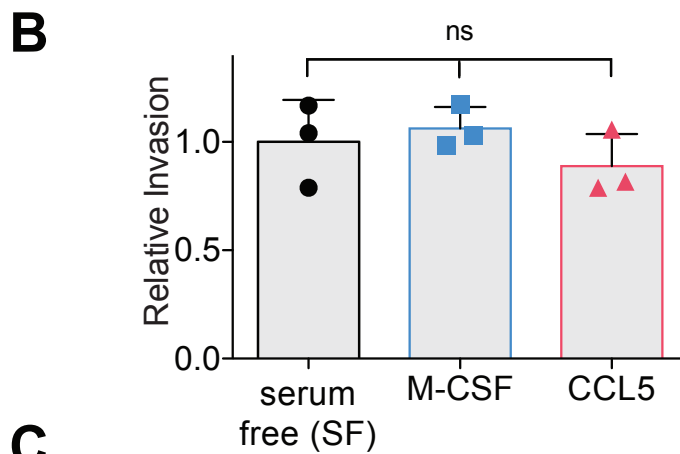
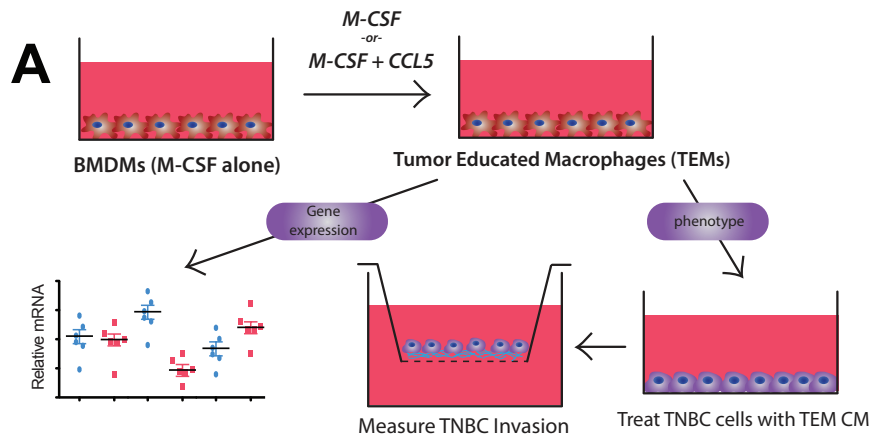
Because CCL5 was not able to act directly on macrophages to program them, we next determined if CCL5 could act indirectly in TEM programming, by stimulating tumor cells. BM1

cells were treated with serum free media as control or serum free media with 100 ng/ml of recombinant human CCL5 for 24 hours. After 24 hours, cells were washed and CM was collected over 24 hours to assay its ability to program TEMs. When we tested CM from the TEMs programmed with CM of BM1 stimulated with CCL5, we saw an increased induction of tumor cell invasion compared to control (Figure 39D).

Because CCL5 was not directly affecting TEM programming, we determined if exosomes within the CM are responsible for TEM programming (described in Figure 40A). Upon centrifugation at 100,000 x g for 1 hr to deplete exosomes, the CM of TNBC cells was no longer able to program TEMs that potentiated TNBC invasion (Figure 40B). These results indicate that soluble factors in TNBC CM are not mediating the programming of TEMs.

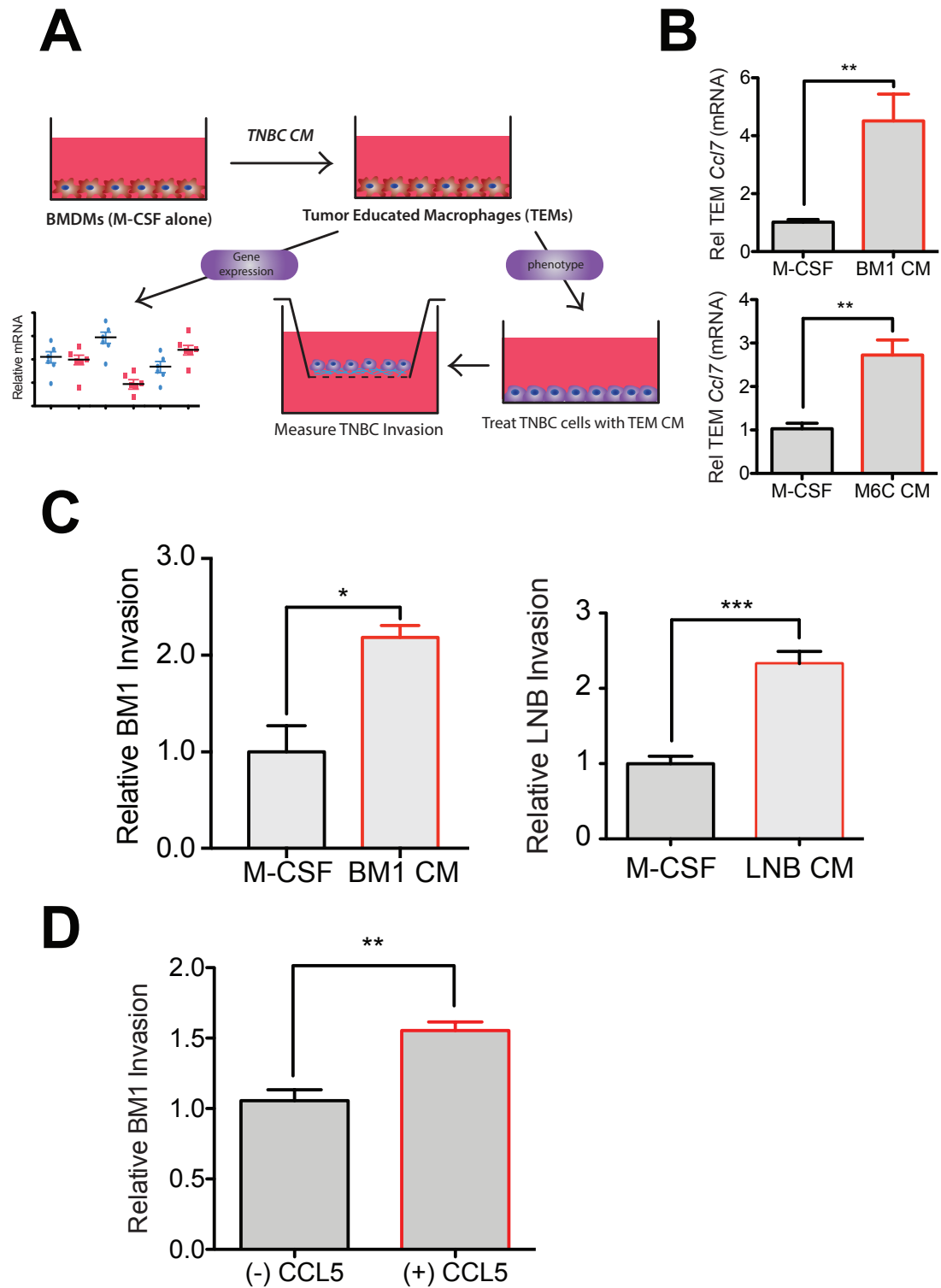
#### ***IZon qEV size exclusion columns are best for exosome isolation***

Before directly testing the role of exosomes in TEM programming, we first evaluated different methods of exosome isolation to identify the one that gave the best and most consistent results. NanoSight analysis of ultracentrifuged samples, along with those isolated using ExoQuickTC reagent, showed larger aggregates relative to those isolated using the qEV size-exclusion columns (Figure 41). Exosomes isolated using the qEV columns were also the most consistent and effective at programming TEMs to drive TNBC invasion (Figure 42).



**Figure 38: CCL5 does not directly program TEMs capable of potentiating TNBC invasion**

A) Schematic demonstrating treatment scheme of experiment. TEMs were programmed with M-CSF alone or M-CSF with CCL5 for 48 hrs. After 48 hrs, TEMs were washed three times and TEM CM was collected in SF media for 24 hrs. The indicated TNBC cells were incubated in TEM CM for 24 hrs, followed immediately by an invasion assay. P-values were calculated using a Student's T-test with N = 5 per group. B) Relative invasion is shown for BM1 cells that were pre-treated with TEM CM. TEMs were programmed with either 20 ng/ml of M-CSF or 20 ng/ml of M-CSF plus 1  $\mu$ g/ml of CCL5 for 48 hrs. C) Relative expression in TEMs is shown for *Ccl7*, *Grn*, and *Mmp12*, measured by qRT-PCR, with *Gapdh* as the loading reference.

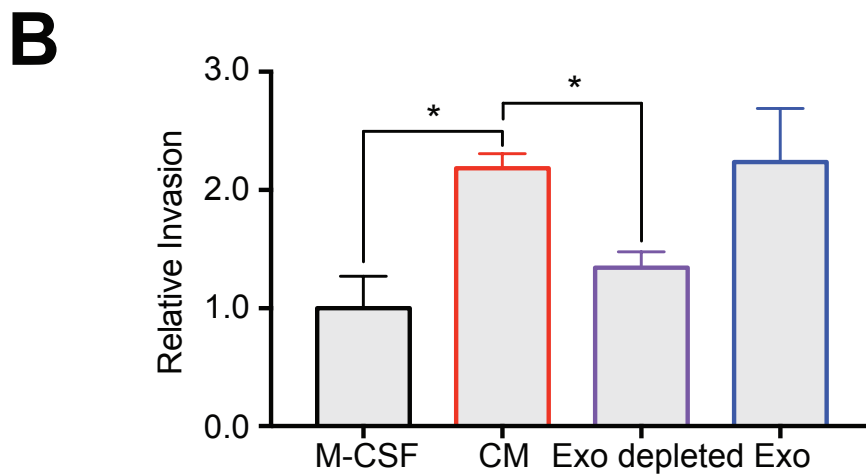
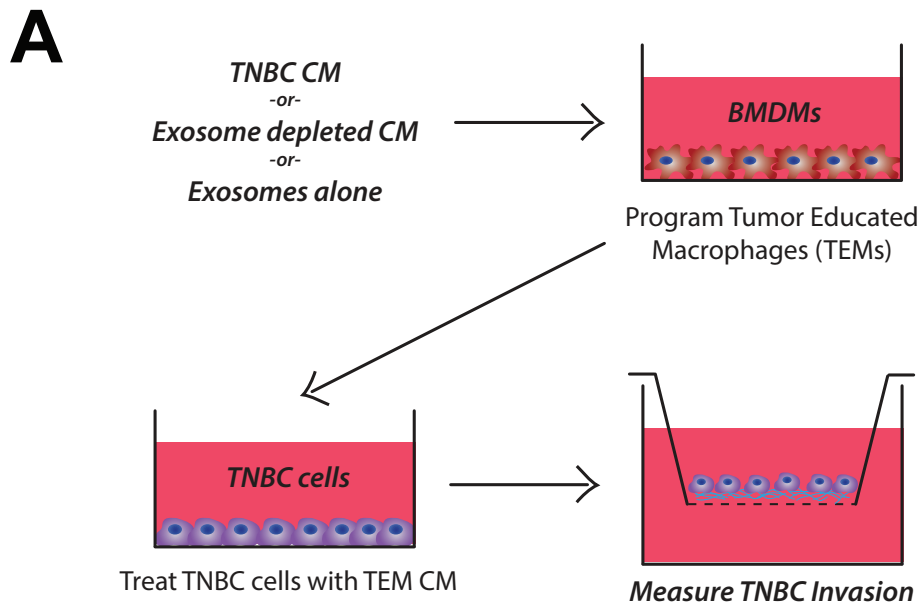


**Figure 39: TEMs programmed with TNBC CM are able to potentiate TNBC tumor cell invasion**

A) Schematic demonstrating treatment scheme of experiment. B) qRT-PCR of *Ccl7* in TEMs programmed with TNBC CM compared to M-CSF alone with *Gapdh* as a loading control.

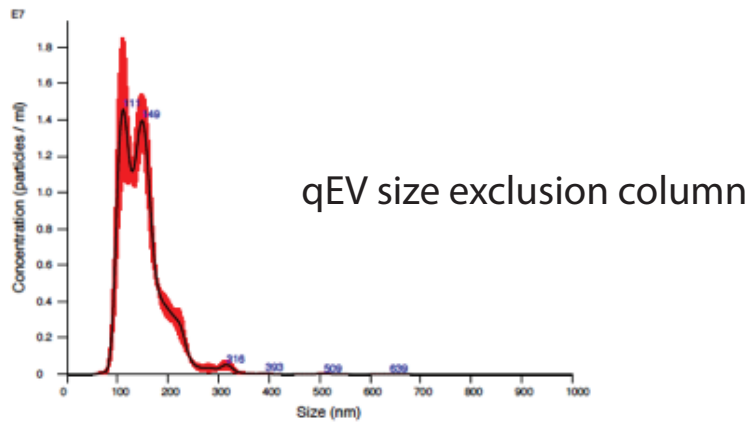
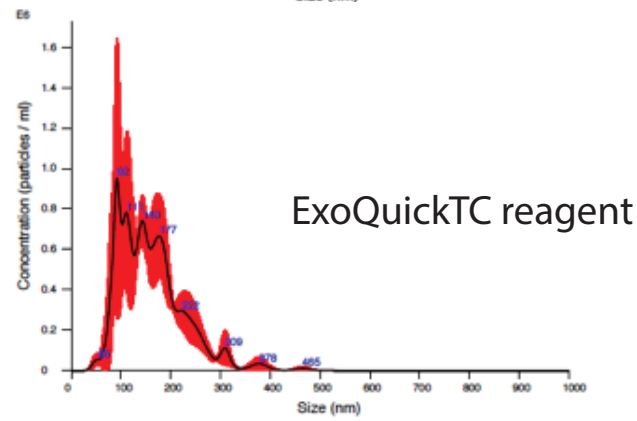
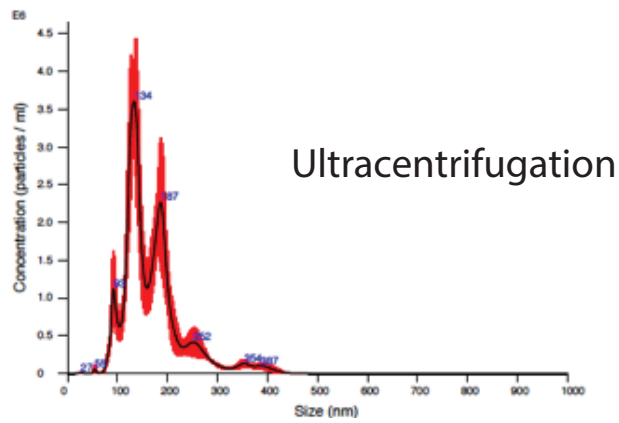
**Figure 39: Continued:**

C) TEMs were programmed with CM from the indicated tumor cells for 48 hrs. After 48 hrs, TEMs were washed three times and TEM CM was collected in SF media for 24 hrs. The indicated TNBC cells were incubated in TEM CM for 24 hrs, followed immediately by an invasion assay. P-values were calculated using a Student's T-test with N = 5 per group. D) BM1 cells were treated for 24 hrs with 100 ng/ml of CCL5. Cells were then washed three times and placed in SF media to collect BM1 CM for 24 hrs. TEMs were programmed with CM from the indicated tumor cells for 48 hrs. After 48 hrs, TEMs were washed three times and TEM CM was collected in SF media for 24 hrs. BM1 cells were incubated in TEM CM for 24 hrs, followed immediately by an invasion assay. P-values were calculated using a Student's T-test with N = 5 per group.



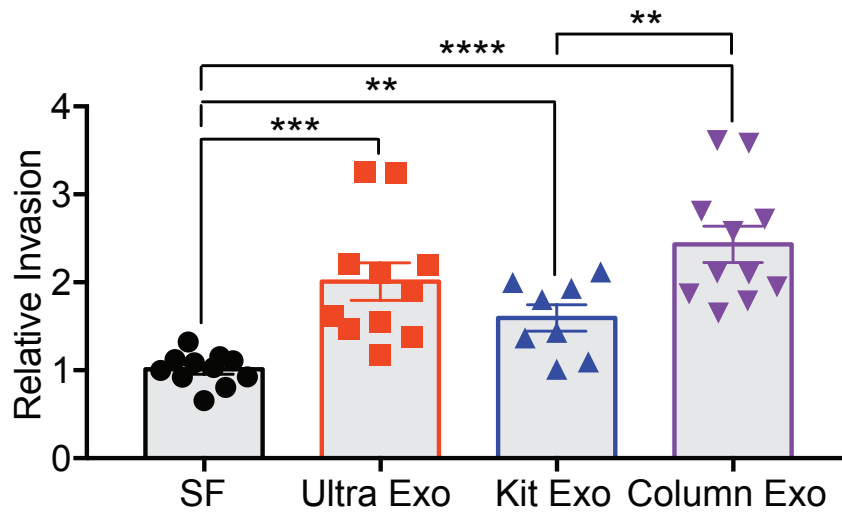
**Figure 40: TEM potentiation of TNBC invasion depends on exosomes in CM**

A) Schematic demonstrating treatment scheme of experiment. B) BM1 cell invasion is shown for samples treated with TEM CM. TEMs were programmed with M-CSF alone, BM1 CM, BM1 CM with exosomes eliminated by ultracentrifugation at 100,000 x g for 1 hour, or BM1 ultracentrifuge isolated exosomes alone. P-values were obtained using a Student's T-test, N=5 per group.



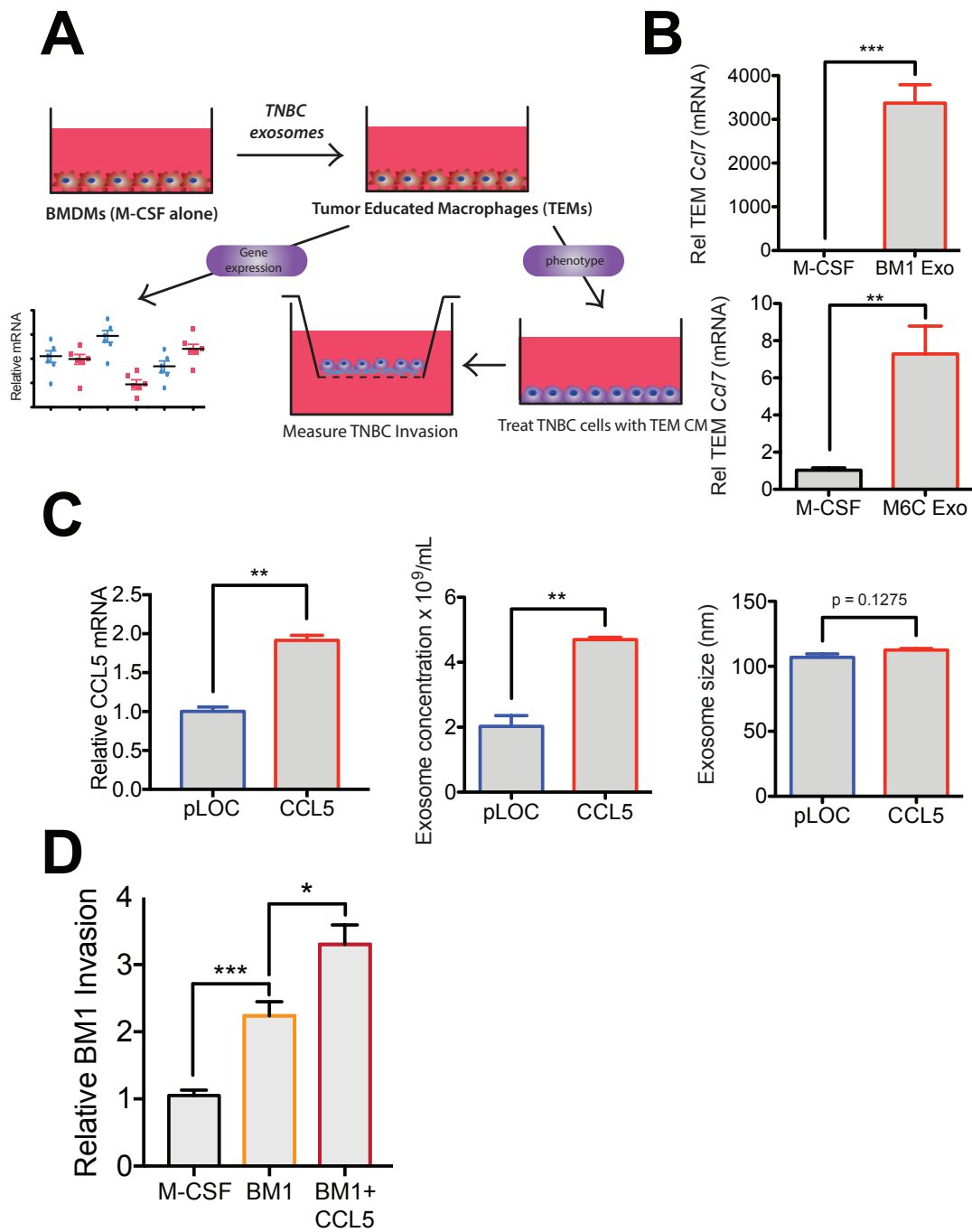
**Figure 41: qEV column gives purest exosome isolation free of larger exosome aggregates**

Isolated exosomes were diluted 20x and their concentration and size were measured using a nanoSight instrument from Malvern. Graphs show concentration on the y-axis and particle size on the x-axis.



**Figure 42: qEV size exclusion columns isolate most consistent and potent exosomes for macrophage programming**

To determine which method of exosome isolation would be best for future studies, we compared macrophage programming with ultracentrifuge, ExoQuickTC Kit, and qEV size exclusion columns.  $1 \times 10^8$  exosomes/ml from each condition were used to treat BMDMs for 48 hrs. After 48 hrs media was removed, cells washed 3 times, and CM was collected in SF media over 24 hrs. TEM CM was then used to treat BM1 cells for 24 hrs, followed immediately by an invasion assay as described in Chapter 2, Materials and Methods.



**Figure 43: CCL5 expression in tumors is able to alter exosome programming of TEMs, altering their potentiation of tumor cell invasion**

A) Schematic describing programming of TEMs with TNBC exosomes. B) qRT-PCR expression of Ccl7 in TEMs treated with M-CSF or M-CSF and tumor exosomes. C) BM1 cells were transduced with a CCL5 over-expression plasmid or control pLOC plasmid. CCL5 expression was

**Figure 43 Continued:**

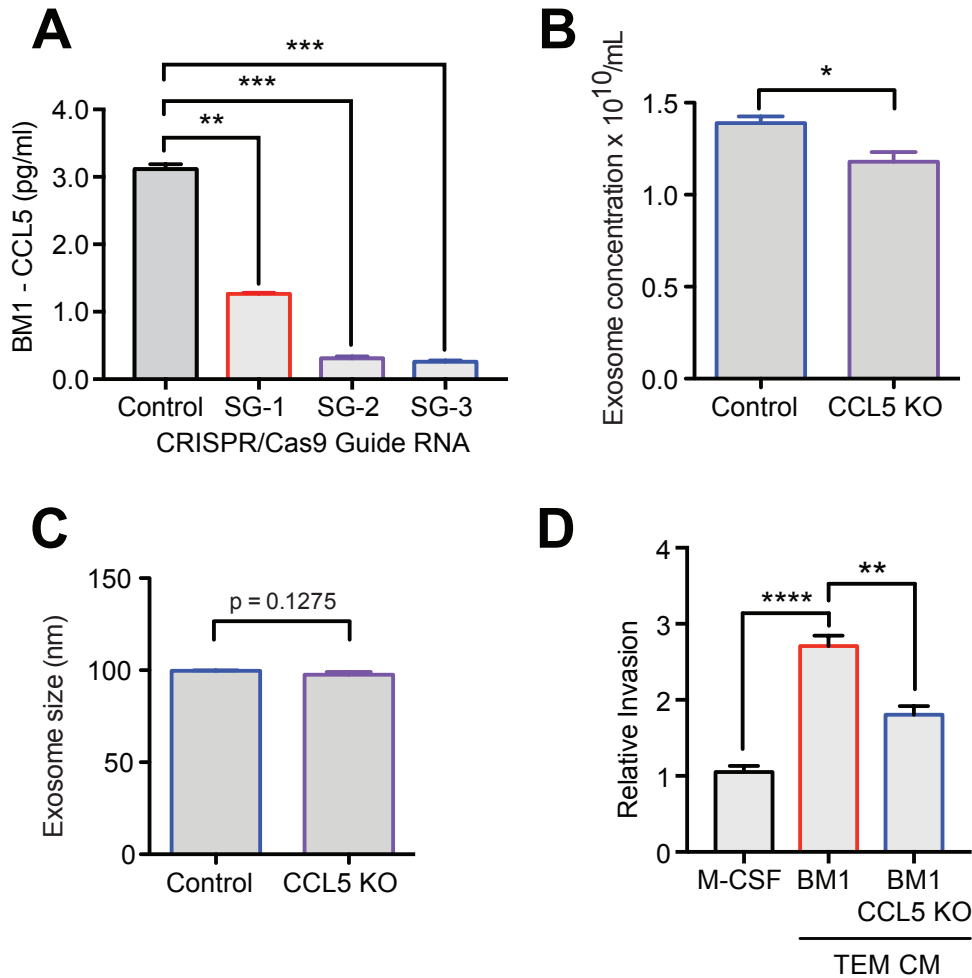
measured by qRT-PCR, using GAPDH as loading control, in samples just prior to exosome isolation (N=3). Exosome concentrations and mean size are shown for exosomes following qEV isolation. Concentration and size were measured using a nanoSight microscope (N=3). D) BM1 exosome programmed TEMs were compared to those programmed with exosomes from BM1 over-expressing CCL5 and macrophages with M-CSF BMDMs as a negative control. TEM CM was collected as described previously and in Chapter 2: Materials and Methods. Programmed macrophage CM was incubated with BM1 tumor cells for 24 hours followed by an invasion assay. M-CSF only macrophage CM treated BM1 cells were used as a negative control, against which to calculate relative invasion.

### ***Exosomes are sufficient for programming of TEMs***

Once we had established size exclusion columns as the best method for isolating tumor exosomes, we asked if the tumor exosomes were sufficient to program TEMs. To determine if exosomes in the tumor CM were responsible for TEM programming, we isolated them using qEV size exclusion columns and then added them directly to the BMDMs as described in Figure 43A. The results show that exosomes directly program BMDMs to secrete *Ccl7* (Figure 43B), and exosome programmed TEM CM is sufficient to increase TNBC cell invasion (Figure 43D). Because we demonstrated CCL5 acts indirectly on tumor cells to potentiate TEM programming through TNBC CM, we asked if CCL5 expression also impacts exosome programming of TEMs. A 2-fold increase in CCL5 expression resulted in an ~2.5-fold increase in exosome number (Figure 43C). No change in exosome size was observed upon overexpression of CCL5 (Figure 43C). We then programmed TEMs with the same number of exosomes from BM1 and BM1+CCL5 cells and found that BM1+CCL5 exosome TEMs could potentiate tumor cell invasion at a significantly greater level than TEMs programmed with BM1 exosomes (Figure 43D). This suggests that CCL5 not only affects the secretion of tumor cell exosomes, but also regulates their cargo.

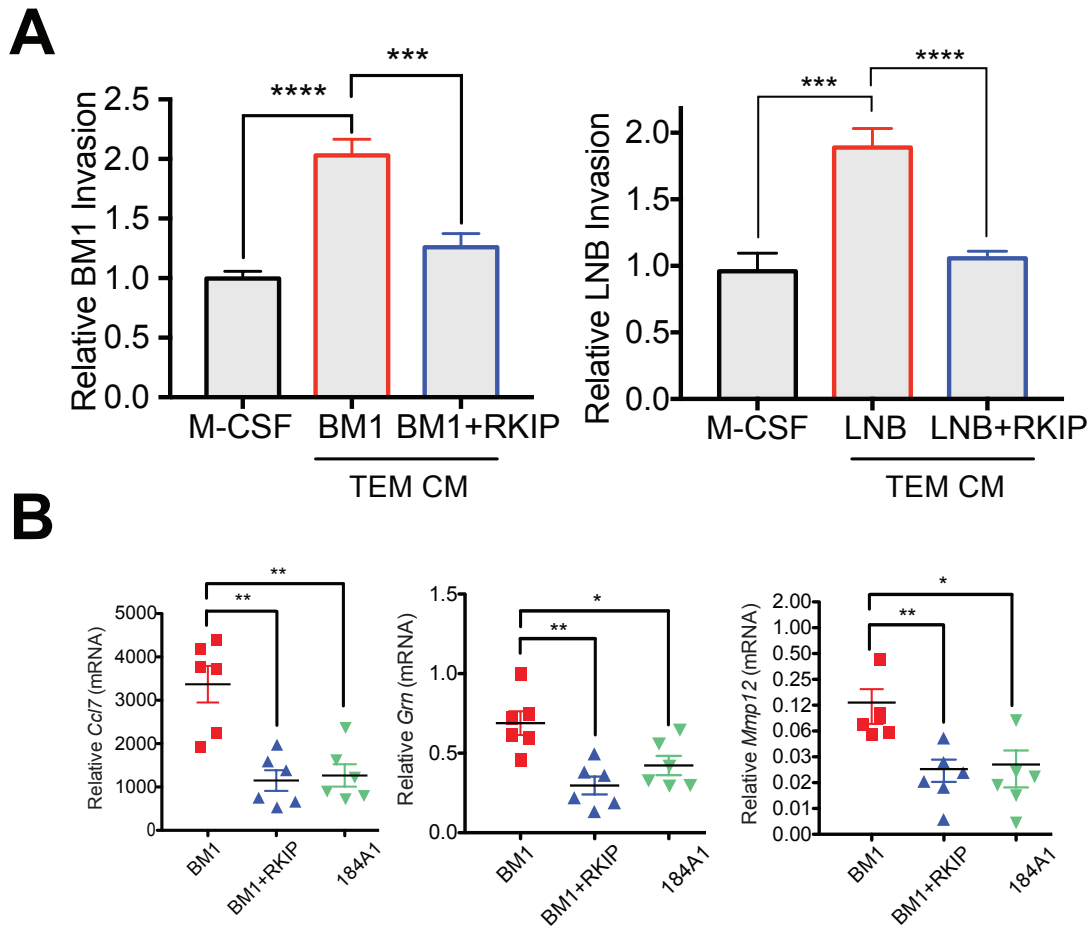
Because CCL5 is necessary for TAM recruitment<sup>120</sup> and can regulate exosome programming of TEMs, we next determined if CCL5 expression in tumors was necessary for the complete programming of TEMs by tumor exosomes. Using transduction of BM1 with a CRISPR/Cas9 lentivector and guide RNAs targeting CCL5, we efficiently knocked out (KO) the expression of CCL5 in BM1 (Figure 44A). CCL5 KO did not change the size of exosomes secreted by BM1 cells (Figure 44C), but did yield a small but significant decrease in exosome secretion (Figure 44B). CM from TEMs programmed with exosomes from BM1 CCL5 KO cells was no longer

able to potentiate TNBC invasion to the same degree as CM of TEMs programmed from control BM1 cells (Figure 44D). This suggests that CCL5 is necessary for the programming of TEMs via exosomes, and that CCL5 expression regulates exosome secretion.



**Figure 44: Tumor CCL5 is necessary for full programming of TEMs capable of potentiating tumor cell invasion**

To determine if CCL5 is necessary for the full programming of TEMs by BM1 exosomes, we examined whether a CCL5 KO would affect TEM programming by tumor exosomes. A) CCL5 was knocked out (KO) using a CRISPR/Cas9 lentiviral vector with targeting guide RNAs for CCL5. CCL5 expression was measured in the CM of BM1 tumor cells collected over 24 hrs in SF media. Media was concentrated 10X to ensure we could detect the smallest amount of CCL5 in KO using an ELISA from Ray Biotech. B-D) Exosomes were isolated from control BM1 cells and BM1 with CCL5 KO. B-C) Isolated exosome concentration (B) and size (C) were measured using a nanoSight microscope following isolation from CM using qEC size exclusion columns (N=3). D) TEMs were programmed as described before, and TEM CM was used to treat BM1 tumor cells and immediately assay BM1 invasion.



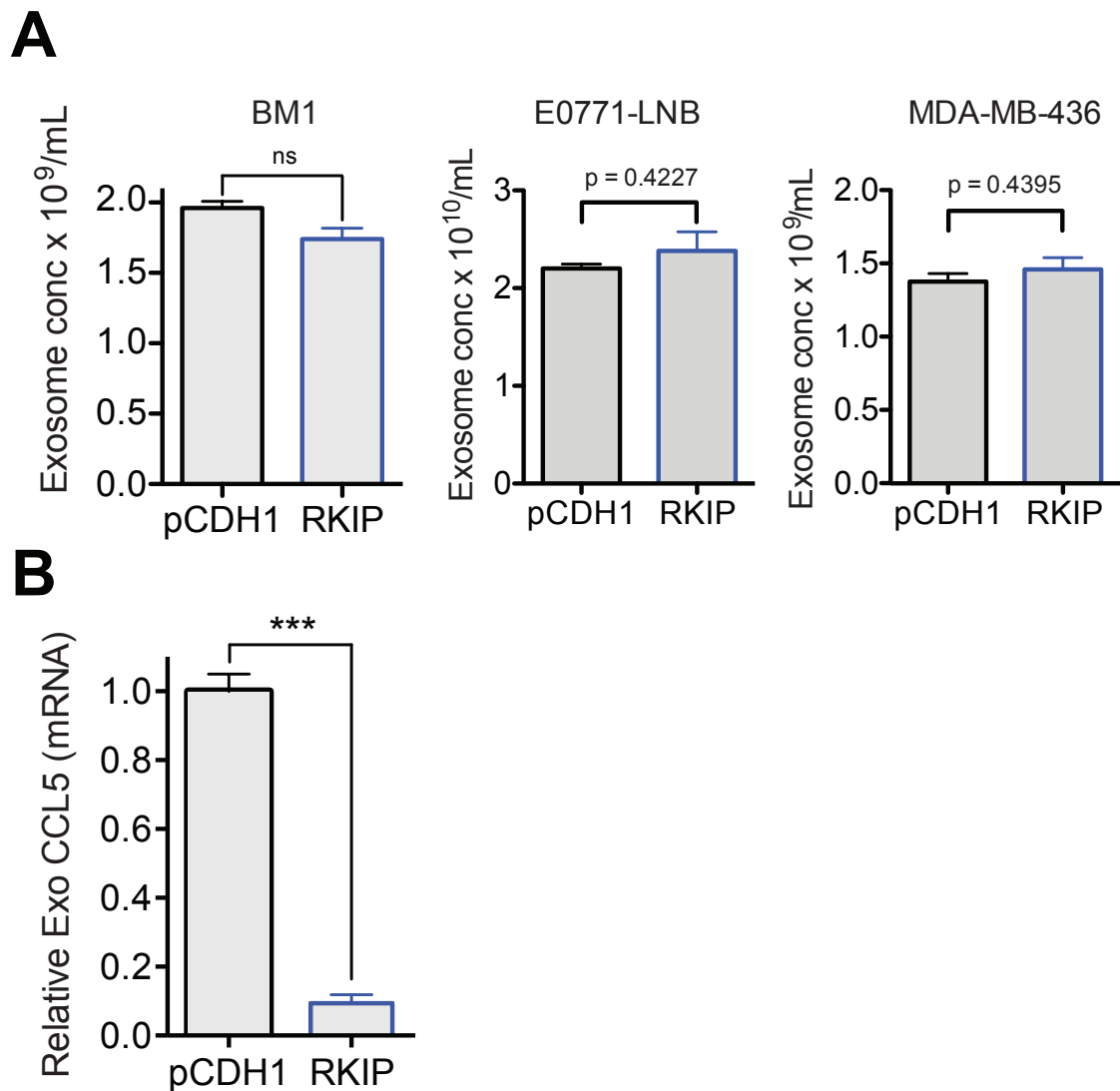
**Figure 45: RKIP regulates exosome programming of TEMs *in vitro***

A) Exosomes from control BM1, BM1+RKIP, LNB, and LNB+RKIP cells were used to program TEMs. TEM CM was then assayed to determine its effect on invasion as described previously. B) Expression of *Ccl7*, *Grn*, and *Mmp12* was measured by qRT-PCR with *Gapdh* as the loading control in TEMs programmed with exosomes from BM1, BM1+RKIP, or 184A1 cells.

### ***TEM programming reflects metastatic state of TNBC cells***

Our previous work established the role of metastasis suppressors in both recruiting TAMs as well as altering their function once they were recruited to the tumor<sup>98</sup>. Because RKIP blocks both TAM recruitment as well as pro-invasive TAM CM, we tested whether RKIP also regulates exosome programming of TEMs. As can be seen in Figure 45A, exosomes from TNBC tumors program TEMs that are able to potentiate tumor cell invasion, while those from RKIP over-expressing tumor cells do not. This suggests that the metastatic ability of the tumor cell dictates the programming of the microenvironment to reflect the invasive state of the tumor. Pro-metastatic genes *Ccl7*, *Mmp12*, and *Grn* are all expressed at lower levels in TEMs programmed by RKIP over-expressing cells (Figure 45B). Levels of these genes in BM1+RKIP exosome programmed TEMs are comparable to TEMs programmed with exosomes from the normal human mammary epithelial cell line 184A1. This suggests RKIP blocks exosome programming from tumor cells.

Because we observed changes in exosome secretion upon alterations in CCL5 expression, we measured the change in exosome number coming from cells over-expressing the metastasis suppressor RKIP and saw no significant change from control TNBC cells (Figure 46A). We therefore suspected that RKIP could be affecting the cargo of exosomes. Because our previous work showed that RKIP suppresses levels of CCL5 in the tumor cells<sup>120</sup>, we examined CCL5 mRNA levels in exosomes. As in the tumor cells, CCL5 mRNA was significantly reduced in exosomes from BM1+RKIP cells compared to control (Figure 51B), indicating that the RNA content of the exosomes is indeed altered by expression of the metastasis suppressor in tumor cells.



**Figure 46: RKIP does not regulate exosome secretion, but does regulate levels of mRNA in exosomes**

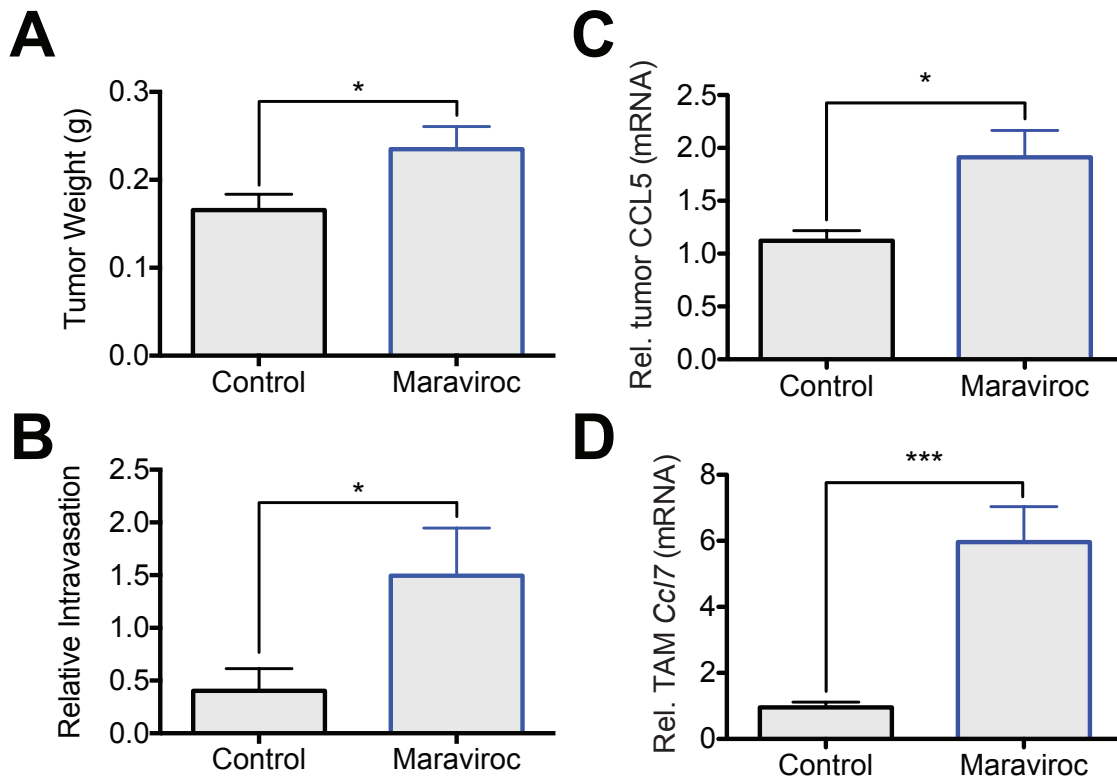
A) Exosome concentrations were calculated using nanoSight in the CM of BM1 and MDA-MB-436 cells. 0.5 million cells per 6-well were plated so cells were confluent the following morning. For BM1 and MDA-MB-4356, cells were washed 3 times in PBS and exosomes were collected in SF media over 24 hrs. (N=3 per group) E0771-LNB exosome concentrations were measured post purification using an iZon qEV column. B) RNA was isolated from exosomes isolated using ExoQuickTC. Relative CCL5 was measured using qRT-PCR with GAPDH as a loading control.

### ***Exosomes transmit tumor cell resistance to CCL5-targeting drugs***

Because of the previous work suggesting that the blockade of CCL5 could efficiently reduce metastasis<sup>44,107</sup> and our data showing the critical role of CCL5 in exosome programming, we tested the CCR5 inhibitor Maraviroc in our system. Our previous study showed that limited Maraviroc treatment of xenograft mice could reduce tumor growth and TAM infiltration<sup>120</sup>. To determine whether Maraviroc is still inhibitory after long term treatment, we increased our treatment period to a full 21 days and began treatment earlier. However, after treating tumor-bearing mice with Maraviroc by oral gavage twice daily for 21 days, we found that tumors were significantly larger (Figure 47A). We also observed an increase in tumor cell intravasation into the blood stream (Figure 47B). This mode of tumor resistance to therapy was associated with an increased expression of CCL5 in tumor cells isolated from treated mice (Figure 47C) in conjunction with higher expression of the pro-metastatic gene *Ccl7* in the TAMs isolated from these mice (Figure 47D). *Ccl7* is known to bind CCR1, CCR2, and CCR3, which have been implicated in TAM recruitment and promotion of metastasis.

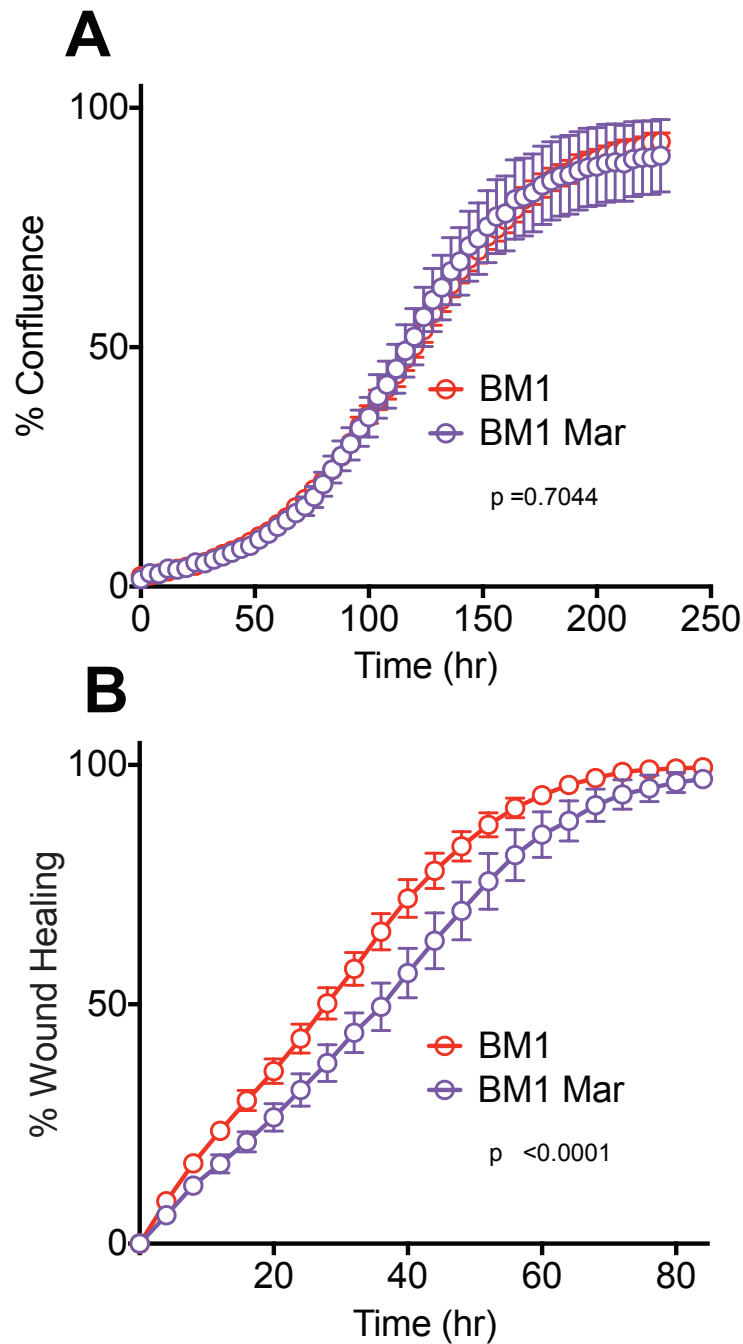
This work demonstrates that exosomes are able to reflect the metastatic state of tumors, and that CCL5 expression in tumors regulates exosome programming of TEMs. We next asked if exosomes could transmit resistance to Maraviroc to macrophages. To test this question, BM1 cells were treated *in vitro* with a low dose of drug for four weeks. We then assayed growth and migration, and found that Maraviroc did not increase growth (Figure 48A) and showed only a slight decrease in migratory ability of the cells (Figure 48B). However, we found an almost 3-fold increase in the expression of CCL5 in the Maraviroc-treated tumor cells, similar to increases we observed after *in vivo* treatment (Figure 49A). When we isolated exosomes from the Maraviroc -

treated cells and used them to program TEMs, we found that the CM from these TEMs showed an increased ability to drive invasion in BM1 cells (Figure 49B). This demonstrates tumor exosomes are capable to transmitting resistance from tumor cells to macrophages.



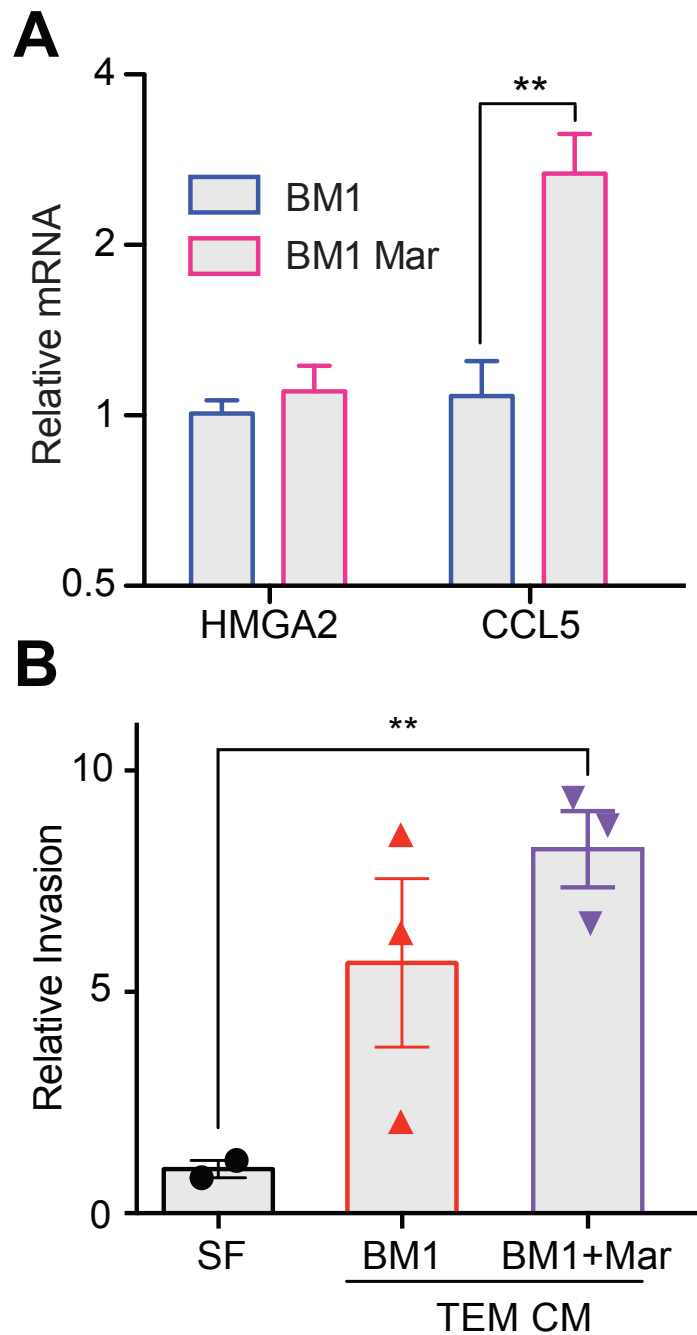
**Figure 47: Maraviroc induces resistance through CCL5 expression in tumors and *Cc17* in TAMs**

Mice were treated starting at day 3 for 21 days with 8 mg/kg of Maraviroc twice daily by oral gavage A) Tumors were weighed immediately after being removed. B) Relative intravasation was measured by quantifying the ratio of human *GAPDH* (tumor) to mouse *Gapdh* by qRT-PCR (n=8 per group) from blood taken immediately prior to sacrifice. C) Tumor cells were isolated from digested tumors following Ficoll-PAQUE gradient centrifugation using the Miltenyi Biotech mouse cell removal kit. *CCL5* expression was measured in isolated tumor cells using qRT-PCR with *GAPDH* as a loading control D) TAMs were isolated as previously described and in Chapter 2: Materials and Methods. *Cc17* expression was measured by qRT-PCR with *Gapdh* as a loading control.



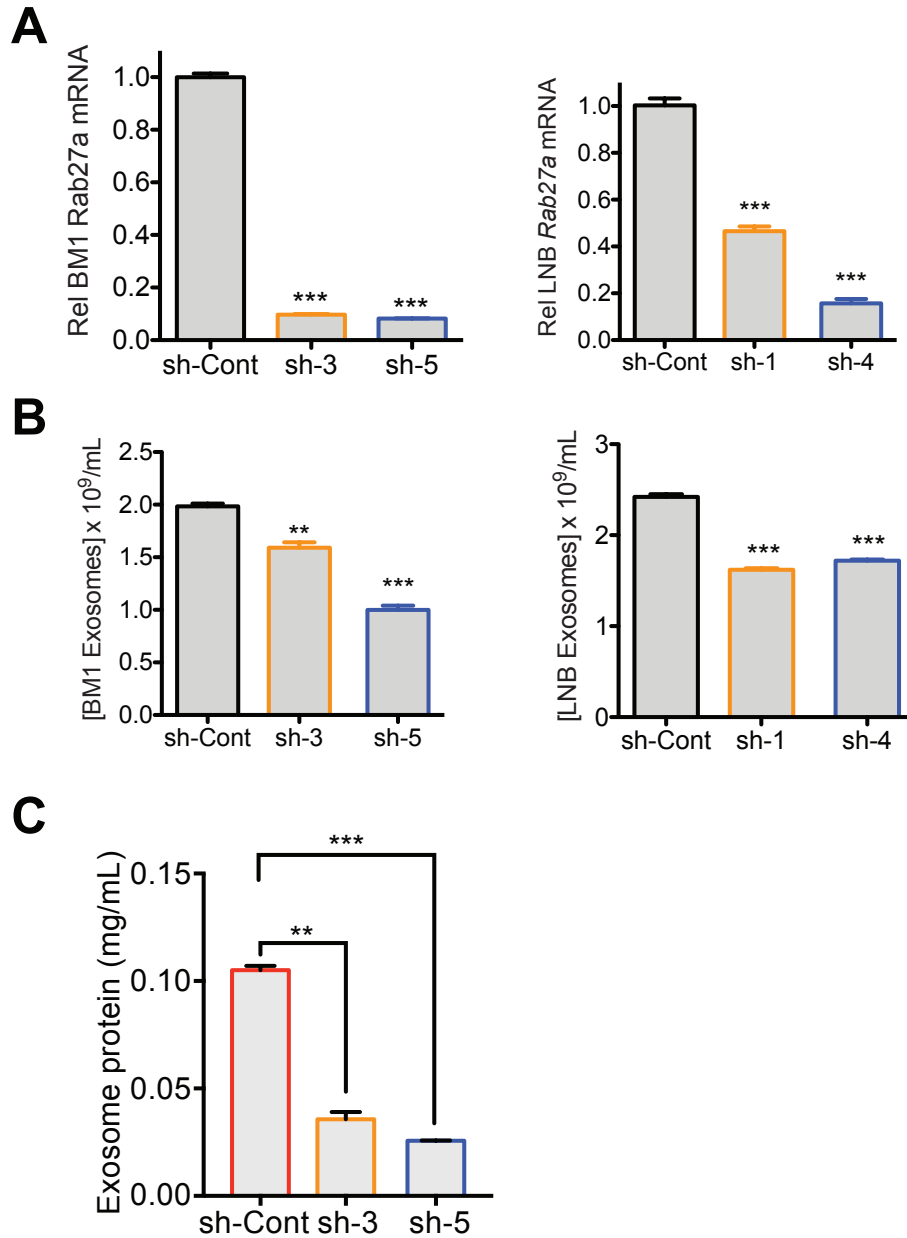
**Figure 48: Maraviroc does not increase tumor cell growth rates in migration in vitro**

A) 3,000 cells per well were plated in 96-well plate and percent confluence was measured every 4 hours using Inccucyte B) 30,000 cells were plated and allowed to adhere overnight. The following morning, a scratch was made to remove the cells in the center of the plate. % wound healing was measured every 4 hrs using Inccucyte.



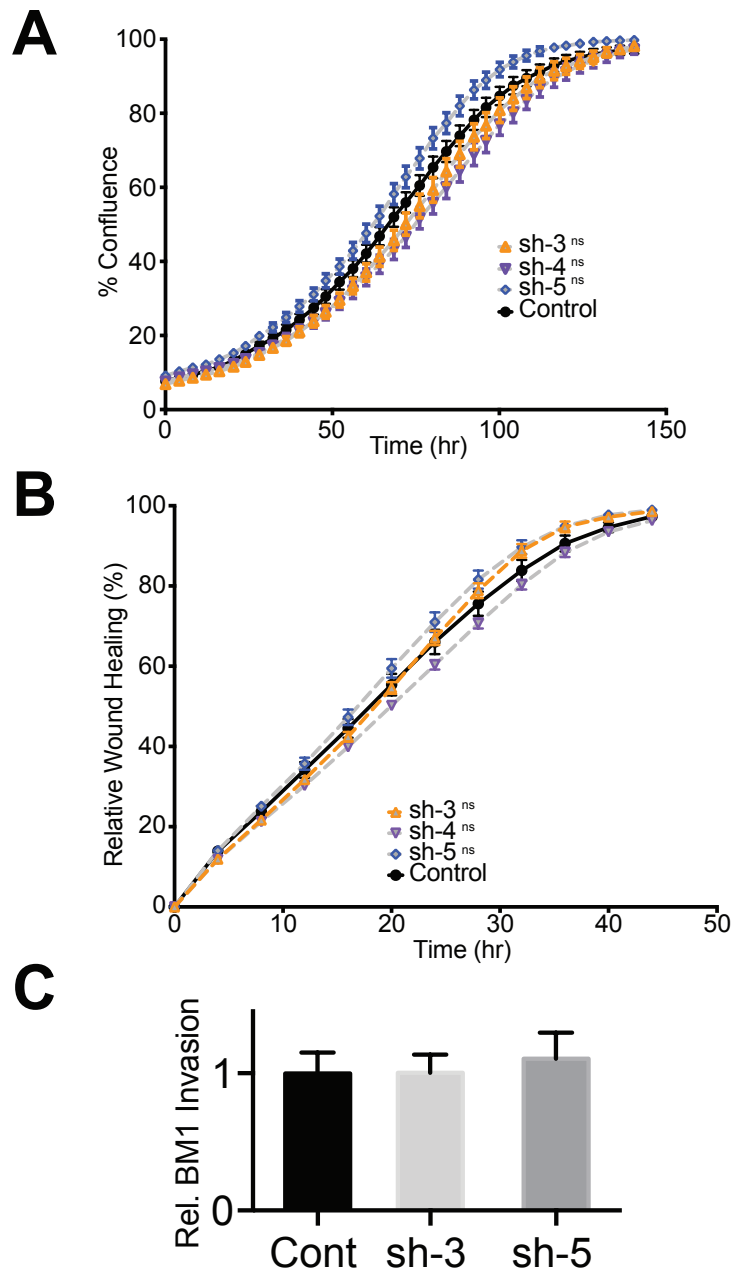
**Figure 49: Maraviroc induces CCL5 expression in vitro and modulates exosome programming of TEMs**

A) CCL5 expression was measured by qRT-PCR using GAPDH as a loading control B) Exosomes were isolated from BM1 cells resistant to Maraviroc and used to program TEMs. Invasion assays were performed as described previously on BM1 cells following treatment with the indicated TEM CM.



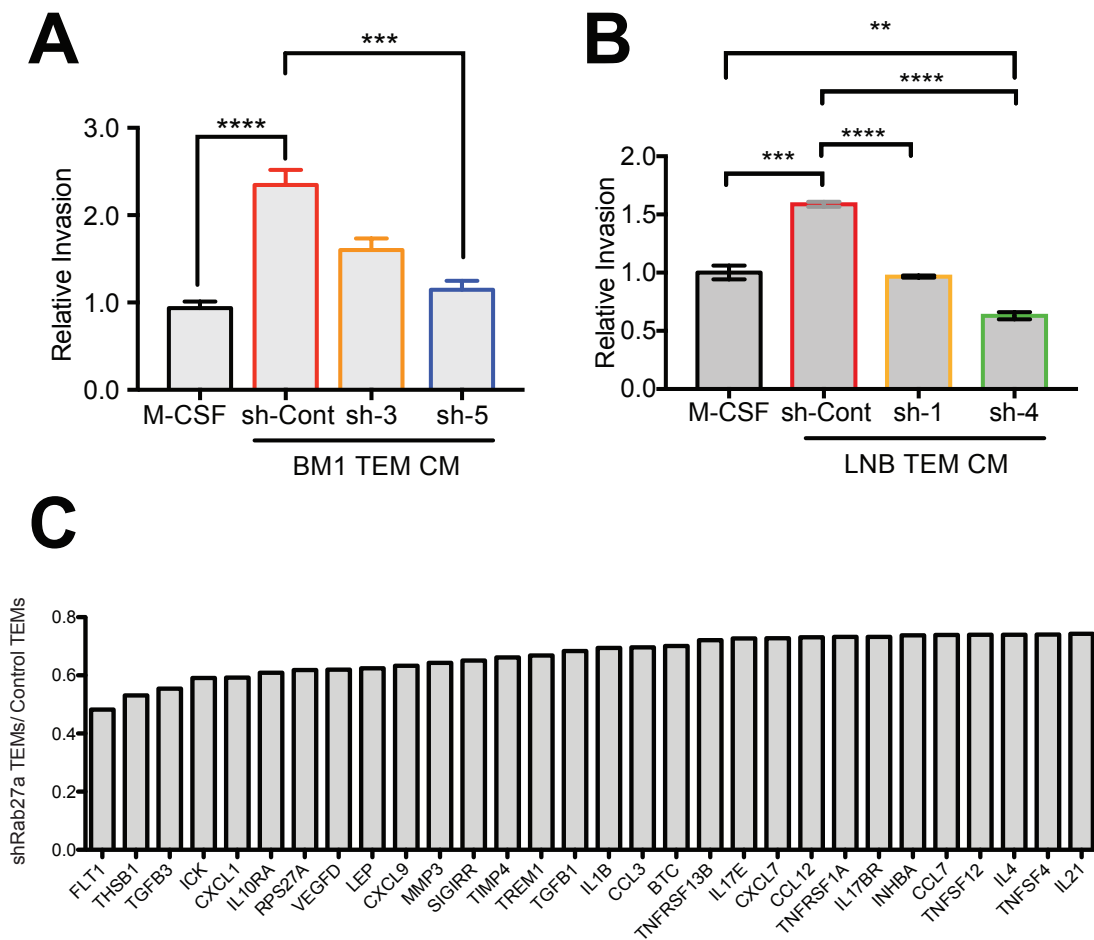
**Figure 50: Rab27a knock-down reduces exosome numbers and exosome protein**

Rab27a was knocked down using a shRNA lentiviral vector, or a non-specific shRNA control. A) Rab27a expression was measured by qRT-PCR normalized to GAPDH expression. B) Exosome numbers were measured in the CM of tumor cells after 24 hours using nanoSight. C) Exosome protein was determined by pelleting exosomes using ExoQuickTC from the tumor CM collected in serum free media. Protein amount was quantitated in RIPA lysates using a BCA assay.



**Figure 51: Rab27a KD does not affect intrinsic tumor cell growth, migration, or invasion**

A) 3,000 BM1 cells were plated per 96-well plate. Images were taken every 4 hrs using an Essen Incucyte and % Confluence was measured over the time course (N=8). B) 30,000 BM1 cells were plated per 96-well plate and allowed to adhere overnight. Scratches were made to remove cells using an Essen WoundMaker. Images were taken every 4 hrs using an Essen Incucyte and % Wound Healing was measured over the time course (N=8). C) Relative invasion was measured as described previously in BM1 tumor cells transduced with shRNAs targeting Rab27a.



**Figure 52: Blocking exosome secretion blocks *in vitro* TEM exosome programming**

A-B) Exosomes were isolated from control BM1 (A) and LNB (B) cells or cells with the corresponding shRNA targeting Rab27a, and then used to program TEMs.  $1 \times 10^8$  exosomes/ml were used for BM1 programming and  $1 \times 10^9$  for LNB exosome programming. CM from BM1 (A) or LNB (B) exosome programmed TEMs was used to treat the corresponding tumor cell and then immediately measure invasion using a Boyden chamber. C) Cytokine array comparison of relative expression between shRab27a TEMs and Control TEMs. Lowest 30 expressing proteins shown.

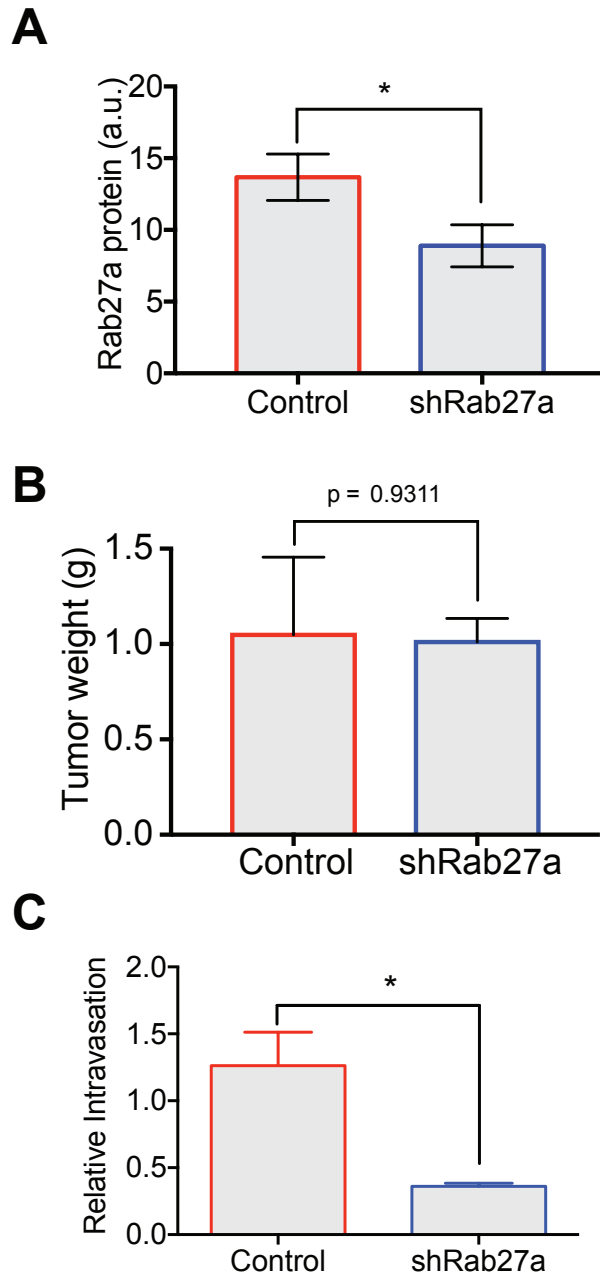
### ***Exosomes are required for TEM programming in vitro***

Previous work has established the critical role of Rab27a in the fusion of multi-vesicular bodies to the plasma membrane and exosome secretion<sup>71</sup>. To examine if tumor exosomes were essential for programming of TAMs, we knocked out expression of Rab27a in human BM1 and mouse LNB tumors using lentiviral shRNAs (Figure 50A). We saw a corresponding lowering of exosome numbers in the CM media of cells after transducing with multiple shRab27a clones (Figure 50B). When we measured exosome protein, we saw a similar decrease with Rab27a depletion (Figure 50C). As the Rab family of proteins are critical regulators of endosomal sorting and membrane fusion, we examined if Rab27a KD interfered with autonomous cellular growth, migration, or invasion. In multiple clones of shRab27a, no change was observed in tumor cell growth, migration, or invasion when Rab27a was knocked down (Figure 51A-C).

To examine if we observe a loss of programming upon reduction in tumor exosome secretion, we tested exosome programming of TEMs by control and shRab27a tumor cell exosomes. Programming of TEMs from tumor exosomes was blocked when cells had Rab27a knocked down (Figure 55A - B), providing additional evidence that, *in vitro*, exosomes are essential for programming TEMs to potentiate tumor cell invasion.

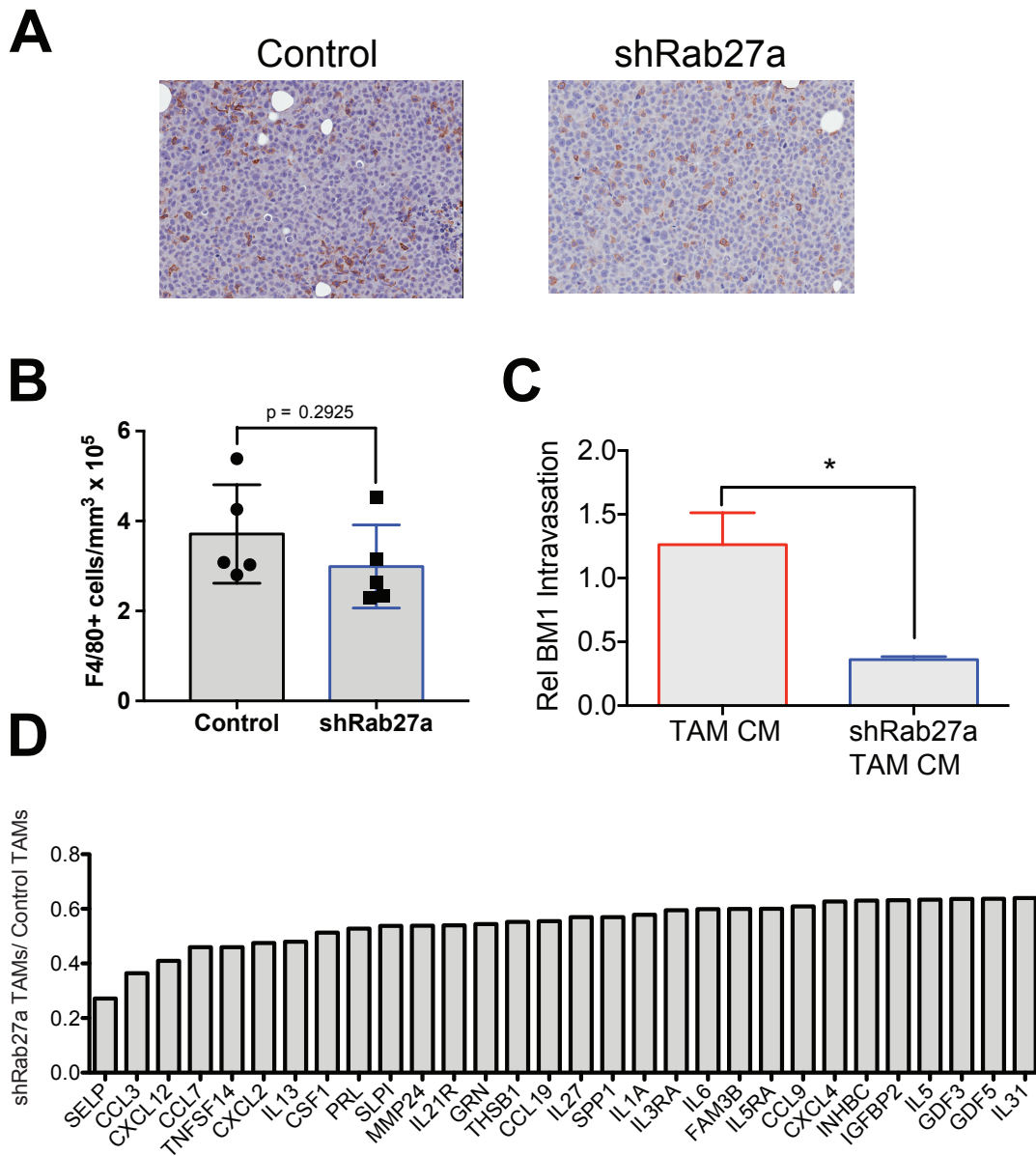
Because TEM CM programmed with exosomes from shRab27a BM1 cells were not able to drive tumor cell invasion, we determined which proteins secreted by TEMs were reduced compared to TEM CM programmed with control BM1 exosomes using an L308 mouse cytokine array. The 30 most decreased proteins are graphed in Figure 55C and include VEGFR1, TGF-  $\beta$ 3, CXCL1, VEGF-D, CXCL9, TGF-  $\beta$ 1, MMP3, IL-1 $\beta$ , CCL3, CCL12, TNFR1, CCL7, and IL-4. DAVID<sup>110</sup> analysis indicates that the genes with reduced expression in shRab27a TEMs are enriched in

cytokine signaling, inflammatory response, wound healing, and growth factor activity, all processes required for invasion and metastasis.



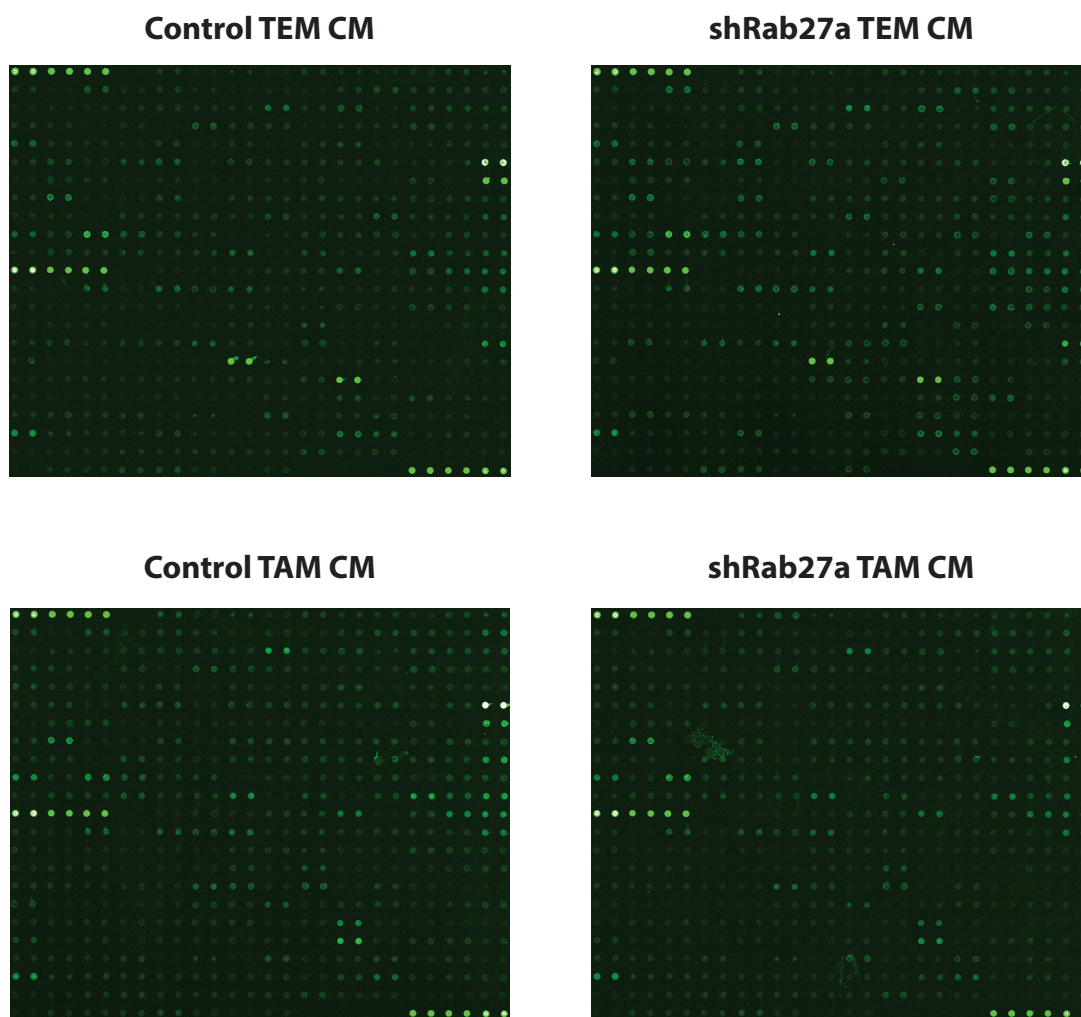
**Figure 53: Exosome depletion reduces tumor intravasation**

A) Rab27a amounts were quantified using a Li-Cor imager of immunoblots of tumor cell lysates. B) Tumor weights are shown for each group C) Relative intravasation was measured by quantifying the ratio of human *GAPDH* (tumor) to mouse *Gapdh* by qRT-PCR (n=5 per group) from blood taken immediately prior to sacrifice.



**Figure 54: b27a KD does not affect infiltration of macrophages into the tumor.**

IHC was performed as described in Chapter 2: Materials and Methods. Sections of tumor were stained for F4/80. A) Representative images of F4/80 stained tumor sections in *Control* and *shRab27a* tumors. B) Macrophage infiltration was quantified using Aperio as the number of positive cells corrected for area. Statistical difference between groups was tested using a Student's T-test (N=5 per group). C-D) TAMs were isolated from Control or shRab27a tumors as described previously. One million TAMs were plated per mL of SF media. SF media was collected and processed 24 hours later. BM1 tumor cells were treated with TAM CM and invasion was immediately assayed (C) or cytokine expression was compared between the two groups using an L308 cytokine array, lowest 30 expressers shown.



**Figure 55: L308 Cytokine Array of TEM and TAM CM**

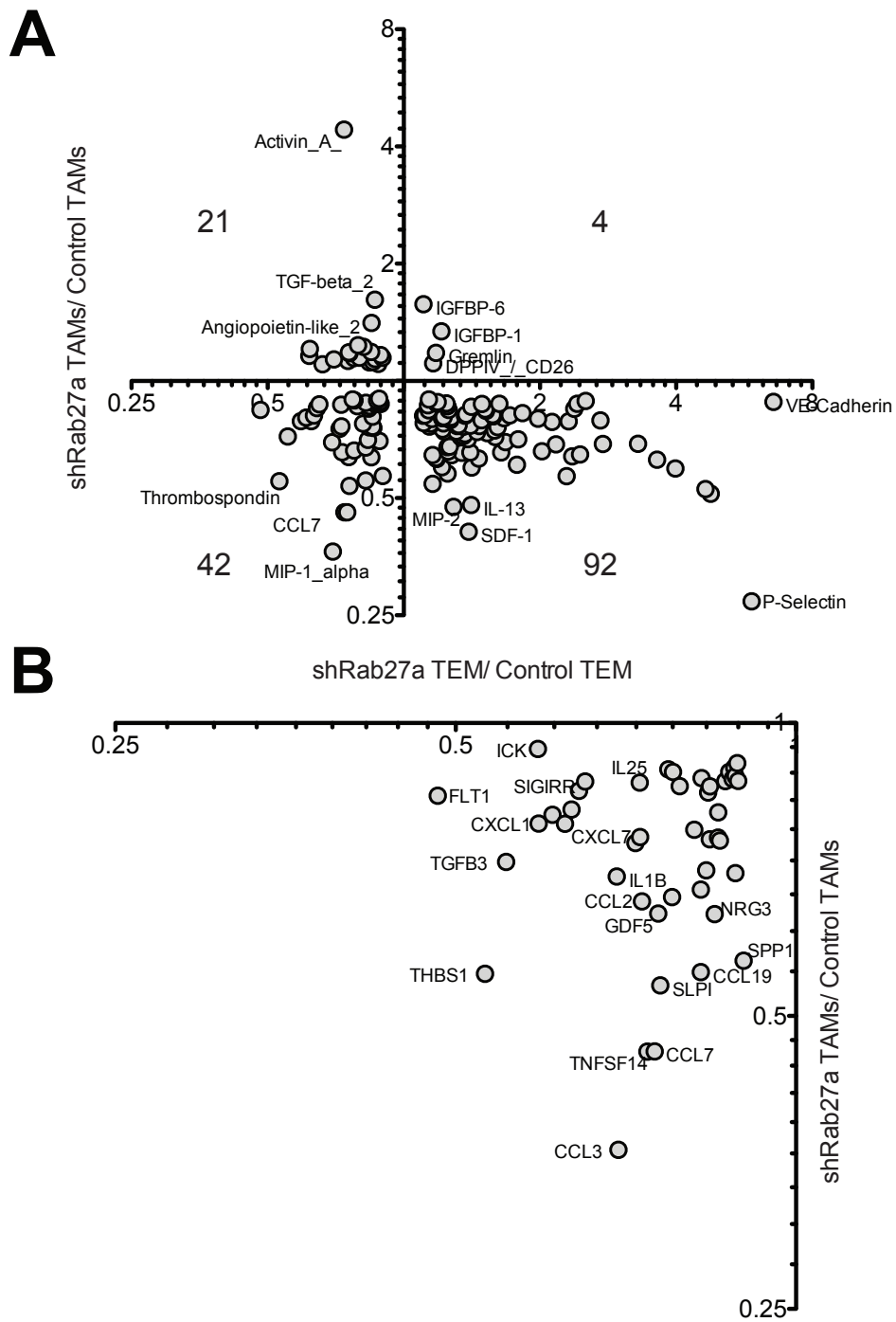
L308 Cytokine array analysis of both TEMs and TAMs derived from control or exosome depleted (shRab27a) tumor samples.

### ***Exosomes are required for TAM driven invasion and intravasation***

We next determined whether tumor exosome secretion affected recruitment and/or phenotype of TAMs. Rab27a knock-down did not significantly change the recruitment of TAMs to the tumor core (Fig 54A-B). When we isolated TAMs from tumors, TAMs from shRab27a tumors were not able to potentiate tumor cell invasion at the same level as those from control tumors (Figure 54C).

To determine if exosomes are necessary for programming of TAMs *in vivo*, we chose BM1 shRab27a tumor cells with the lowest exosome number (sh-5). Tumors grown with shRab27a expressed significantly less Rab27a as determined by immunoblotting (Figure 53A). Knocking down Rab27a did not affect the size of BM1 tumors (Figure 53B). However, it did decrease the ability of tumor cells to intravasate into blood vessels (Figure 53C). As previously noted in Figure 51C, shRab27a did not affect cell autonomous invasion, suggesting effects on intravasation are due to changes in the stroma.

To determine the mechanism by which exosome programming regulated the pro-invasive phenotype of TAMs, we ran the conditioned media of TAMs on an L308 mouse cytokine array. The proteins most decreased in TAMs when tumor exosome secretion was blocked included CCL3, CCL7, TNFSF14, IL-6, IL-13, SLPI, MMP-24, Progranulin, and Osteopontin (Figure 54D, 55). DAVID<sup>110</sup> analysis of genes with reduced expression in TAMs showed enrichment for cytokine signaling, ECM, immune response, inflammatory response, growth factor signaling, and chemotaxis. These categories correspond to a change in invasion and metastasis as well as a potential alteration of chemotaxis of other immune cells into the tumor microenvironment.



**Figure 56: Cytokine Array Results comparing control to shRab28a TEMs versus TAMs**

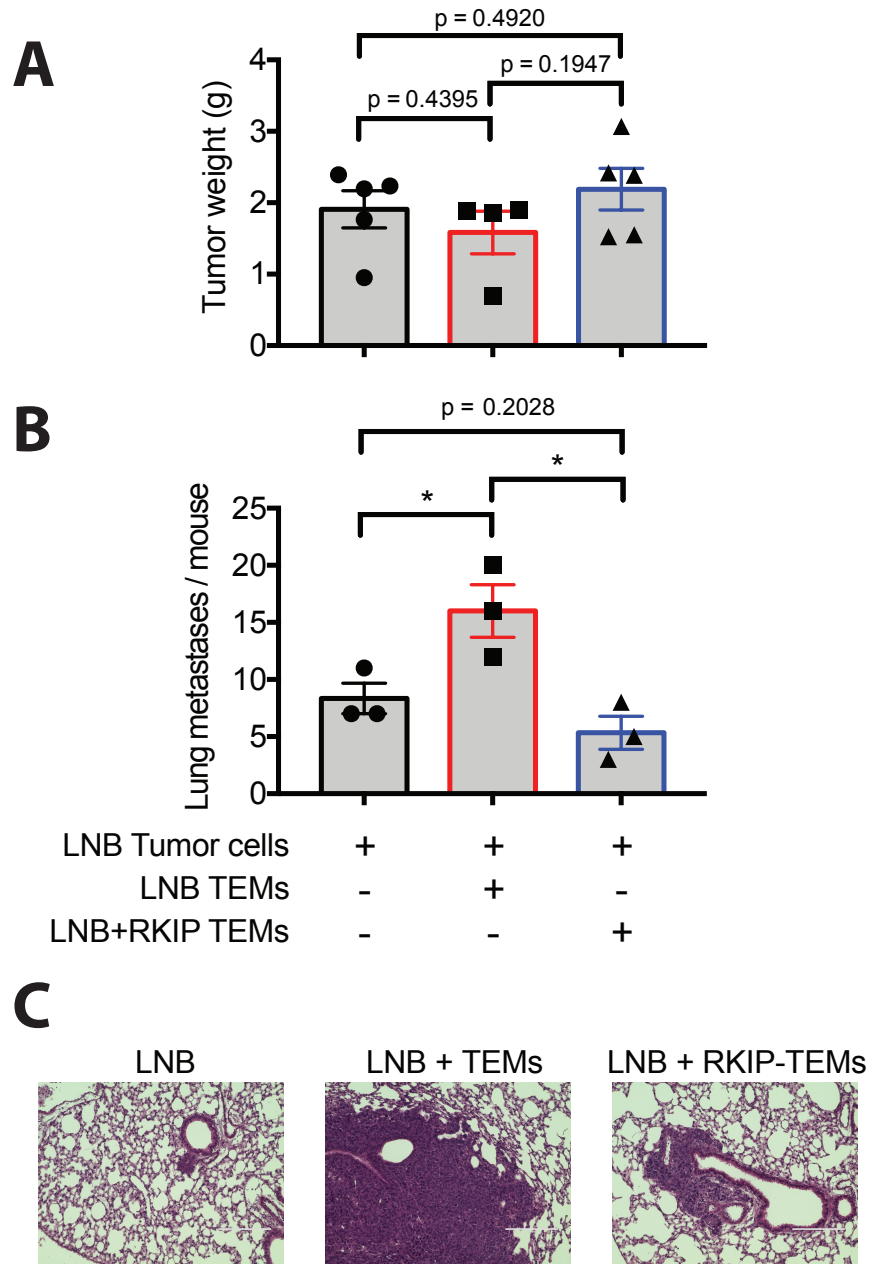
A) Comparison of relative expression of shRab27a TEMs to control TEMs (x-axis) to relative expression of shRab27a TAMs to control TAMs (y-axis). Numbers of differentially expressed proteins within each quadrant is denoted. B) Zoom in of lower-left quadrant showing the 42 proteins reduced by shRab27a in both TEMs and TAMs

### ***Differentially expressed TEM and TAM cytokines largely overlap***

To determine functionally how much overlap we see between exosome programmed TEMs and TAMs, we compared the difference in cytokine secretion from TEMs programmed *in vitro* by control BM1 exosomes to those from TAMs programmed *in vivo* by exosome-depleted shRab27a tumor cells (Figure 56A-B). Specifically, we examined how much overlap there was between proteins differentially expressed between control and shRab27a in both TEMs and TAMs (Figure 56A). To do this, we focused on proteins that showed relative expression levels that differed by <0.9- and >1.1-fold, resulting in 159 differentially expressed proteins. To determine the overlap between the groups we plotted differential expression of proteins in TEMs along the x-axis and differential expression of TAMs along the y-axis. We counted how many proteins fell into each quadrant to compare those reduced in both groups, increased in both groups, or showing opposite levels of expression.

Overall, we observed 46 of 159 differentially expressed proteins were positively correlated between the two groups (~ 30 percent overlap in differentially expressed genes within TEMs and TAMs), suggesting a significant fraction of the genes programmed in TAMs derive directly from tumor exosomes. Proteins reduced in both groups included CCL3, CCL7, CCL19, CXCL1, CXCL3, Flt-1, Osteopontin, SLPI, TGF-beta 3, TNFSF14, and Thrombospondin. DAVID<sup>110</sup> analysis of the genes with reduced expression in both TEMs and TAMs when tumor exosome secretion is reduced shows enrichment for chemotaxis, wound healing, growth factor signaling, and MAP kinase activity. This suggests that tumor exosomes are an essential component for programming macrophages toward a pro-metastatic TAM phenotype.

The majority (92 out of 159, or 58 percent) fell within the group up-regulated in TEMs, but down-regulated in TAMs. This suggests not only a reduction in cytokine secretion upon reduction of tumor exosomes, but also increased expression of pathways normally blocked by tumor exosomes. The fact that these same cytokines are reduced in TAMs suggests that, in the complete tumor microenvironment, induction of new cytokines upon tumor exosome depletion is inhibited in TAMs by other stromal and immune cells present, or through feedback onto tumor cells altering *in vivo* exosome secretion.



**Figure 57: TEMs increase metastasis but not tumor growth *in vivo***

0.5 million LNB tumor cells were injected per mouse. Either 0.5 million LNB exosome programmed or LNB+RKIP exosome programmed TEMs were co-injected with tumor cells as indicated. A) Final tumor weights are shown for each mouse (N=4 or 5). B) Number of metastases per mouse are shown (N=3 per group). C) Representative images of lung metastases.

### ***Exosome programmed TEMs increase metastasis***

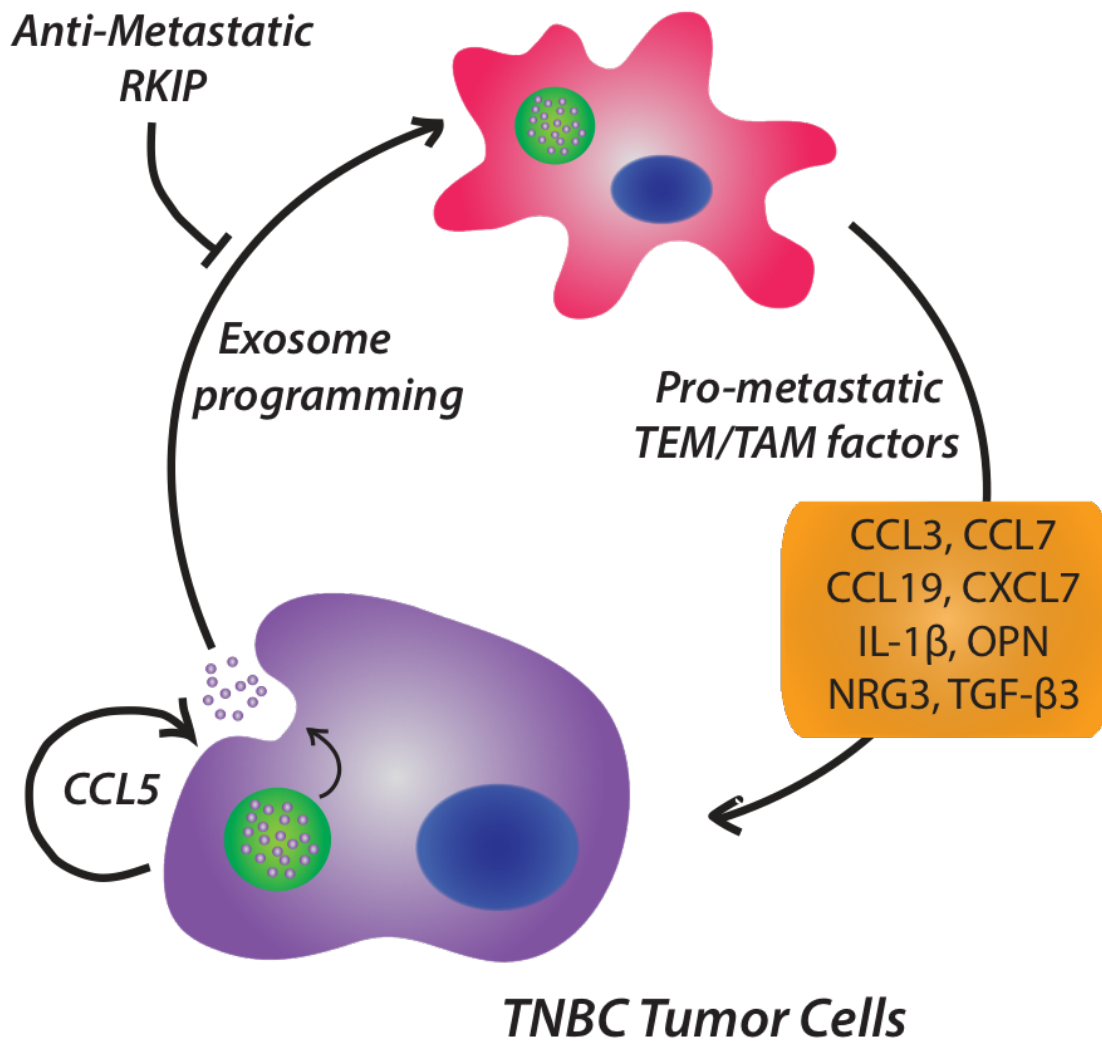
After establishing the ability of RKIP to regulated exosome cargo and subsequent programming of TEMs, we examined the effects of TEMs on metastasis *in vivo*. To do this, we co-injected 0.5 million TEMs programmed with either LNB or LNB+RKIP exosomes, with 0.5 million LNB tumor cells (LNB tumor cells alone were used as a control). Injection of TEMs with LNB tumor cells had no effect on tumor size at the end of the study (Figure 57A). However, compared to LNB tumor cells alone, tumors co-injected with LNB programmed TEMs showed an increase in both number and size of lung metastases (Figure 57B). By contrast, TEMs programmed by LNB+RKIP tumor exosomes showed no significant change in lung metastases compared to control. We compared these results to the co-injection of CCL5 recruited TAMs with MDA-MB-231 tumor cells. CCL5 recruited TAMs showed an increase in both tumor growth (Figure 52A) as well as tumor cell intravasation into the blood stream (Figure 53B). These results suggest that TEMs, like TAMs, regulate the invasive properties of tumor cells and the exosomes reflect the properties of the cells that secrete them.

### **Discussion**

Tumor exosomes are essential in the formation of a suitable pre-metastatic niche before the arrival of tumor cells<sup>78</sup>. Previous work on the role of exosomes and macrophage biology had shown that macrophages can take up exosomes *in vivo*<sup>78</sup>, and that exosome stimulation of mouse and human macrophage cell lines, mediated by TLR signaling and NF-κB activation, regulates cytokine production<sup>92-94</sup>. However, the function of these exosome programmed macrophages in tumor progression and metastasis has not been determined. In this study, we have established

that exosomes are necessary for *in vitro* programming of macrophages capable of potentiating tumor cell invasion. In contrast to previous work that is based on *in vitro* activation of macrophages to an M2 phenotype or the use of macrophage cell lines that already display a pro-tumor phenotype, our work shows that tumor exosomes alone are capable of programming BMDMs into pro-invasive TEMs. We demonstrate that the chemokine CCL5 has a non-chemotactic role in macrophage development by acting on tumor cells to alter their exosome secretion. We also show that the metastatic status of the tumor cell is phenocopied to the macrophages via exosomes. Finally, by blocking tumor cell exosome secretion, we found that TAMs recruited to the tumor microenvironment had a decreased ability to potentiate tumor cell invasion and, in return, tumor cells had a decreased metastatic ability. This decrease in the pro-metastatic secretome was associated with a decrease in numerous proteins that drive tumor metastasis and recruit & support TAMs such as CCL3, CCL7, CXCL2, & GRN. We identified many of these factors previously as associated with pro-metastatic TAMs in TNBCs including CCL7 and GRN, two of the three factors most correlated with CCL5 expression in TNBC tumors and part of a TNBC-TAM prognostic signature<sup>120</sup>. Co-injection of TNBC exosome programmed TEMs resulted in both an increase in number and size of lung metastases. Taken together, this work suggests that tumors are still capable of recruiting TAMs without exosome secretion, but exosomes are both necessary and sufficient for pro-metastatic TAM education.

## Exosome Programmed TEMs/TAMs



**Figure 58: Summary of tumor exosome programming of TAMs and pro-metastatic gene expression**

Summary of how tumor cells regulate programming of macrophages through exosomes. RKIP acts to block exosome programming, while tumor CCL5 expression feeds back to increase macrophage programming. Tumor exosomes regulated expression of pro-metastatic genes in TAMs and TEMs including CCL3, CCL7, CCL19, CXCL7, IL-1 beta, OPN, NRG3, and TGF-beta3

CCL5 has been well established as playing a role as a chemotactic factor for a variety of immune cells in the microenvironment including TAMs, T-cells, and T-regs. Work from Velasco-Velázquez et al established that CCL5 could play a role directly on tumor cells to affect growth and metastasis<sup>107</sup>. While our previous study demonstrated that CCL5 plays a critical role in the recruitment of TAMs that drive tumor metastasis, our present work suggests that CCL5 protein secreted from tumors does not have a direct effect on macrophage programming to a TAM phenotype. Instead, CCL5 secreted from the tumor cells or the microenvironment seems to directly impact the ability of the tumor to secrete exosomes that program TEMs to a more metastatic, TAM-like phenotype. This new role of CCL5 further demonstrates the importance of blocking CCL5 activity in tumors. However, our present work also shows that the CCR5 inhibitor Maraviroc alone drives tumor resistance, communicated in part through exosomes. Recent work using nanoparticle delivery to inhibit CCL5 at the transcriptional level may be an alternative therapy to Maraviroc<sup>121</sup>. Additionally, work has shown that the BRD4 inhibitor JQ1 regulates NFκB expression. This could be an additional alternative in TNBC to block the pro-metastatic effects of CCL5 on the tumor microenvironment.

Our data shows that, as expected, tumor exosomes alone do not program TEMs *in vitro* that are an exact mimic of their *in vivo* TAM counterparts (Figure 56). Given the complexity of the tumor microenvironment and the fact that multiple stromal cells secrete exosomes, the fact that we observed a significant overlap (~30 percent) supports the relevance of our findings. Many of the proteins that are reduced by tumor exosomes alone are increased in the complete immune and stromal environment. This suggests that many of the pathways repressed in macrophages by tumor exosomes can also be repressed by exosomes from other cells or feedback on tumor

cells altering tumor exosome secretion. However, the core group of proteins induced by tumor exosomes that are also induced *in vivo*. Proteins induced by exosomes in both groups included CCL3, CCL7, CCL19, CXCL1, CXCL3, Flt-1, Osteopontin, SLPI, TGF-beta 3, TNFSF14, and Thrombospondin. DAVID<sup>110</sup> analysis of the 42 genes induced by exosome secretion, both *in vitro* and *in vivo*, shows enrichment for chemotaxis, wound healing, growth factor signaling, and MAP kinase activity.

Interestingly, a number of the proteins induced in TAMs by tumor exosomes overlaps with proteins decreased in TAMs by RKIP relative to control tumors<sup>120</sup>. Similarly, we found here that RKIP regulates exosome programming of TEMs. Because RKIP did not alter the number of exosomes coming from tumor cells, it was clear that the cargo of the tumor exosomes must be changing with RKIP expression in tumor cells. This results in a change in TEM phenotype similar to that of TAMs from RKIP+ tumors. Because we see a similar difference between control and RKIP TAMs as between control and shRab27a TAMs, it suggests that part of the way RKIP can program TAMs is through regulating exosome cargo. Further studies will be required to determine the exact mechanism by which RKIP regulates exosome loading or content. Future studies should focus on whether surface proteins, internal protein cargo, or RNAs are regulated in exosomes by RKIP. Our results have shown dramatic alterations in cytokine production upon treatment with exosomes, as well as reduced in TAMs when tumor exosomes are reduced. Because TLR signaling and activation of NFkB are known to regulate expression of many of these cytokines, it is a likely mechanism that should be further explored.

## CHAPTER 5: SUMMARY & CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

### SUMMARY & CONCLUSIONS

#### *Recruitment of TAMs controlled through RKIP and CCL5*

Previous work related to the role of metastasis suppressors focused on the role they play in the tumor cells alone. The work presented here establishes that metastasis suppressors can have an added function in both recruitment of and, more importantly, function of TAMs. RKIP regulated the recruitment of TAMs to TNBC tumors by controlling expression of HMGA2, which in turn was required to induce expression of CCL5. Our work also elucidated the function of recruited TAMs, demonstrating that those present in RKIP expressing tumors differed not only in number but also in their function. Cytokine arrays of TAM CM showed that the pro-metastatic secretome differed drastically between the two TAM types, particularly with respect to the expression of pro-metastatic factors such as CCL7, GRN, MMP12, OPN, SLPI, and sTNFR2.

We demonstrated that these TAM proteins, highly expressed in CCL5-recruited TAMs, showed increased expression in the tumors of TNBC patients compared to non-TNBC patients. CCL7, MMP12, and TNFR2 correlated most highly with CCL5 in TNBC patient tumors, and when we formed a signature based on a CCL7-MMP12-TNFR2 TAM meta-gene in conjunction with tumor RKIP, HMGA2, and CCL5 expression, we found that it strikingly segregated patients in TNBC specifically, compared to non-TNBC patients. Previous work has demonstrated that shed TNFR2 (sTNFR2) protein is higher in the plasma of pancreatic, endometrial, and breast cancer patients<sup>111-113</sup> and associated with an increased risk of cancer<sup>113</sup>. Granulin (GRN) expression blocks TNFR2-mediated inflammation and has been shown to drive migration, invasion and VEGF

expression in breast cancer<sup>114,115</sup>. GRN is highly expressed in a number of tumors including breast, and has also been targeted using biologics in hepatocellular carcinoma<sup>114,116</sup>. Moreover, we provide direct evidence that GRN and sTNFR2 can promote the invasiveness of human TNBC tumor cells *in vitro*. Because of the strong evidence of pro-invasive action and the clinical relevance of these factors, CCL5, GRN, and sTNFR2 are all potential targets for anti-TNBC drug treatment.

### ***Exosomes are essential for programming pro-metastatic TAMs***

Previous work in the role of exosomes and macrophage biology had shown that macrophages can take up exosomes *in vivo*<sup>78</sup>, and that exosome stimulation of mouse and human macrophage cell lines, mediated by TLR signaling and NF- $\kappa$ B activation, regulates cytokine production<sup>92-94</sup>. However, the function of these exosome programmed macrophages in tumor progression and metastasis has not been determined. Here, we establish that tumor exosomes are the component of tumor CM responsible for programming TEMs toward a pro-tumor phenotype in TNBC. We also establish that CCL5 can regulate this process. Stimulation of tumor cells with recombinant CCL5 is able to modulate their programming of TEMs. However, direct stimulation of TEMs with CCL5, either recombinant or over-expressed in the CM of tumor cells, was not able to modulate TEM activity. This suggests that while CCL5 can directly recruit macrophages, it plays a role in modulating their function only indirectly through exosome secretion by tumor cells.

Additionally, we demonstrated that attempts to block TAM recruitment using the CCR5 inhibitor Maraviroc led to increased tumor growth and intravasation. This was in part through

increased CCL5 expression in tumors and increased CCL7 expression in TAMs. Maraviroc treatment of tumor cells *in vitro* modulated their exosome programming of TEMs, suggesting that resistance to targeted therapies can be transmitted through exosomes to macrophages in the stroma.

Examination of exosome programming when tumor invasion and metastasis was blocked using the metastasis suppressor RKIP showed that tumor invasiveness was phenocopied to TEMs through exosomes. RKIP did not regulate exosome secretion, but did elicit differences in exosome cargo, suggesting that the content of the exosomes is critical for determining TEM programming by tumor cells. Finally, we demonstrated that exosomes are essential *in vitro* as well as *in vivo* for education of TAMs. TAMs isolated from tumors expressing shRab27a (blocking exosome secretion) demonstrated reduced secretion of cytokines associated with migration, MAPK signaling, and chemotaxis. Additionally, many of the factors reduced in TAMs when Rab27a was used to block exosome secretion are involved in the recruitment of T-regs, neutrophils, and TAMs. This suggests that tumor exosome programming of TAMs is essential not only for the cross-talk between TNBC cells and TAMs to drive invasion, but also for the recruitment of other pro-tumor cells into the microenvironment.

## **DISCUSSION**

### ***Identification of patients with high TAM recruitment***

This work identified a gene based signature that identifies patients within TNBC that have a high level of genes associated with CCL5-recruited TAM infiltration. This signature has the potential to identify patients that could respond to a therapeutic targeting of CCL5 recruitment

of macrophages as well as factors secreted from TAMs including GRN and CCL7. TAMs within the microenvironment are critical for regulating the recruitment of T-regulatory cells (T-regs) as well as cytotoxic T-cells. In addition to the role of CCL5 in monocyte chemotaxis to the tumor, it has been shown to recruit T-cells<sup>122</sup>. However, the recruitment by CCL5 is generally thought to be a pro-inflammatory immune response<sup>123,124</sup>. Additionally, CCL5 has been shown to be essential for the recruitment of T-regs to the pancreatic tumor microenvironment<sup>125,126</sup>. This suggests that our signature identifying RKIP regulated CCL5 recruitment of TAMs to the microenvironment might also coincide with recent work to identify patients with highly T-cell inflamed tumors (those infiltrated by T-cells, T-regs, and TAMs), as TAM recruitment we observed likely coincides with cytotoxic T cell and T-reg recruitment<sup>127</sup>. How to properly target these patients creates a particularly interesting challenge. In the next discussion, I will further elucidate the issues with blocking CCR5 activity, as well as later discuss the potential for targeting NF- $\kappa$ B activation within the tumors to block CCL5 expression.

### ***Blocking recruitment of TAMs vs blocking function of TAMs***

When we examined blocking the recruitment of macrophages to the tumor core, our results at first pass seemed to conflict. Preliminary studies with a shorter treatment period of around 14 days showed some reduction in the number of macrophages to the tumor core (Figure 16). When we repeated this experiment, we increased our treatment period to a full 21 days and began treatment earlier. This time, we saw a clear increase in tumor size as well as intravasation of cells into the blood stream (Figure 46). Increased tumor size and tumor cell intravasation into the blood stream was also seen in conjunction with an increase in CCL5 expression in the tumors

and an increase in *Ccl7* in the TAMs. These results suggest that, while a brief treatment of mice with Maraviroc may result in a slight decrease in the number of TAMs infiltrating, long-term therapy is likely to alter the phenotype of the remaining TAMs to help the tumor survive.

From our work in Chapter 5, it is clear that exosome programming is controlled by tumor expression of CCL5. While Maraviroc may be sufficient to reduce the number of infiltrating macrophages, it will not have an effect on the programming of TAMs towards a pro-tumor phenotype. In fact, our data suggests that by increasing CCL5 expression, the tumors are overcoming the blockade of CCR5 at the tumor cell surface. This subsequent increase in CCL5 would likely lead to TAMs in the microenvironment that have an increased pro-tumor phenotype, with higher secretion of cytokines associated with increased tumor invasion and metastasis.

A better strategy than trying to block TAM recruitment would be to block the activity of TAMs. Early studies that were part of this work demonstrated that trying to block one TAM protein at a time yielded little results. In particular, the use of a GRN neutralizing antibody showed little effect on tumor intravasation. This was in part because the antibody targeted human GRN as opposed to the mouse isoform. Additionally, it is likely that the TAMs could compensate using one of the many other proteins they secrete. A potential way to consider blocking TAM phenotype would be to try to block their programming. This could be done by trying to block CCL5 at an expression level. Recent work from Ban *et al* demonstrated that utilizing *Ccl5* *-/-* stromal cells or targeting CCL5 expression (using nanoparticles) in conjunction with Maraviroc to block CCR5 resulted in reduced tumor growth and activation of the immune system<sup>128</sup>. This suggests that, if CCL5 can be reduced at the transcriptional level rather than by competition for receptor activation, the CCL5 recruited TAM phenotype could be controlled.

Particular methods for blocking CCL5 transcriptionally will be discussed further in Future Directions.

### ***Exosome programming of TAMs compared to CCL5 recruited TAMs***

We found that CCL5 and RKIP expression in tumor cells can regulate pro-invasive gene expression in TEMs programmed with tumor exosomes. MMP12, GRN, Galectin-3, CCL7, OPN, TNFR2, and SLPI were all identified as being highly differentially expressed between CCL5 recruited TAMs, and those in RKIP expressing tumors (Figure 21). We found that tumor exosomes also regulate expression of CCL7, OPN, and SLPI both *in vivo* and *in vitro* (Figure 56B). This suggests that many of the factors we see regulated by CCL5 and RKIP *in vivo* are also regulated by tumor exosome secretion both *in vivo* as well as *in vitro*. As the exosomes used in this study were from control BM1 cells, not those overexpressing CCL5, it is likely that we would not see all of the same genes differentially expressed in TAMs derived from shRab27a tumor samples compared to TAMs from CCL5 over-expressing tumors.

Data from Figure 39 demonstrates that while CCL5 does not act directly on TEM programming, it can affect how tumor cells program TEMs. It also suggests that for TAMs to be fully activated to express these genes, they must be in the presence of the tumor microenvironment to receive signals from other non-tumor cells. There are clear roles of exosome coming from other immune cells and fibroblasts in the environment.

### ***In vitro* exosome programming compared to *in vivo* TAMs**

Our data shows that, as expected, tumor exosomes alone do not program TEMs *in vitro* that are an exact mimic of their *in vivo* TAM counterparts (Figure 56). Given the complexity of the tumor microenvironment and the fact that multiple stromal cells secrete exosomes, the fact that we observed a significant overlap supports the relevance of our findings.

If we set a threshold of proteins with differential expression as < 0.9-fold or > 1.1-fold, we can determine how many of the genes may overlap as differentially expression between the two types of macrophages by shRab27a. If we remove proteins with 0.9 – 1.1-fold expression difference between shRab27a and control in both TEMs and TAMs, we see that 159 of the 308 proteins assayed are differentially expressed in both TEMs and TAMs. While there are obvious differences between macrophages stimulated *in vitro* with tumor exosomes alone and TAMs *in vivo*, they surprisingly show both a similar phenotype (their effect on driving tumor cell invasion and metastasis) as well as a 29% overlap in differentially expressed cytokines. This suggests that future studies into TAM biology can be done by studying TEMs.

## **FUTURE DIRECTIONS**

### ***Utilizing TEMs to study TAM biology***

The previous section of the discussion focused on the differences and similarities between TEMs and TAMs. Because they largely overlap, they provide a useful tool for the study of TAM biology and signaling *in vitro*. Our early attempts at proteomic analysis of TAMs isolated from tumors were problematic. Even once we had developed a reliable method for isolating TAMs, we found that surface labeling of TAMs with biotin for proteomics resulted in contamination of

cytoplasmic as well as nuclear proteins. This could have been for a number of reasons including perforations in cells resulting from mechanical and enzymatic disruption of the tumor tissue for isolations. It is also highly possible that proteins from dead or sheared cells were still present on the outside of isolated TAMs. Because we were utilizing non-tryptic enzymes for our digestions, proteins could be attached to segments of the extra-cellular matrix still attached to the outside of macrophages. Work from the Becker lab has established that proteomics of *in vitro* programmed macrophages can provide great insight into how selective stimulation affects their biology and secretion.

Because isolation of TAMs can be problematic for proteomic studies, TEMs offer an alternative approach to understand how they are functioning. When we examined the secretome of TEMs versus TAMs, many of the proteins were similarly expressed and many of the key cytokines regulating recruitment of pro-tumor cells or acting directly on tumor cells to modulate invasion and metastasis were reduced in both TEMs and TAMs when tumor exosome programming was blocked. Additionally, the amount of RNA needed for traditional RNAseq studies would be prohibitive for the amount that can be recovered from tumor isolated TAMs. Because a large number of TEMs can be programmed *in vitro*, they would offer an ideal model for scaling up for larger genomics or proteomics experiments to understand how proteins, RNAs, or epigenetic regulation occurred in macrophages in response to tumor exosome programming. Finally, TEMs have the most potential for doing mechanistic studies into how exosomes activate macrophage cytokine signaling and drive them toward a pro-tumor, pro-metastatic phenotype. Numerous knock-out mouse models are available from which bone marrow could be used to create BMDMs and examine their importance in tumor exosome programming of TEMs.

### ***Other methods of blocking recruitment of TAMs***

This work established that trying to block recruitment of TAMs using the CCR5 inhibitor Maraviroc leads to more highly metastatic tumors rather than decreasing TAM activity within the tumor. Another potential strategy that should be further investigated are transcriptional regulators of CCL5. One such mechanism would be through regulators of NF- $\kappa$ B activity. There are more than 750 potential NF- $\kappa$ B pathway inhibitors in development currently<sup>129</sup>, suggesting a large number that could be screened for decreased CCL5 expression. However, recent work has shown that NF- $\kappa$ B activity is essential within T-cells in the tumor microenvironment in order to see control of tumor growth<sup>130</sup>. Therefore, in subsequent work, it is essential to monitor both the blockade of macrophage recruitment as well as T-cell activity using syngeneic models.

An additional option is to use the BRD4 inhibitor JQ1. Recent work has shown that JQ1 can efficiently reduce the expression of the chromatin remodeler HMGA2<sup>131</sup>, which we showed in this work to be required for CCL5 expression. Preliminary studies have shown that JQ1 can efficiently block expression of both HMGA2 as well as CCL5 in BM1 cells (data not shown). JQ1 is also known to regulate the activity of NF- $\kappa$ B target gene expression<sup>132</sup>, which would again suggest the importance of monitoring the activity of T-cells in the tumor microenvironment when JQ1 is used. However, recent work has shown that JQ1 can also regulate the expression of PD-L1 on tumor cells. In this work, efficient tumor control was only seen in animals with an intact immune system<sup>133</sup>, suggesting that JQ1 can regulate tumor signaling and recruitment within the microenvironment, while not interfering with the host immune system.

### ***Metastasis suppressors as regulators of the microenvironment***

We have established for the first time that a metastasis suppressor, RKIP, can regulate the recruitment of TAMs to the tumor microenvironment. However, more than 40 experimentally verified metastasis suppressors that have been identified,<sup>134-136</sup> many with abilities to alter the extracellular space and potentially other cell types recruited to the environment<sup>137</sup>. Many investigators have utilized these genes for their ability to identify signaling pathways, molecules, and genes essential for different aspects of the metastatic cascade<sup>101,138</sup>. Because metastasis suppressors can affect so many different aspects of metastasis starting with invasion and ending with extravasation and outgrowth, their ability to regulate the microenvironment could help us understand what is necessary for cells to survive at each of these stages. Work from our lab has been fruitful in examining genes and pathways necessary for early intravasation as well as extravasation in metastasis<sup>18,21,23,97,98,139,140</sup>.

This work has established the involvement of RKIP in the primary tumor microenvironment through TAMs. Studying other metastasis suppressor genes that affect other signaling pathways in tumor cells could inform us about other cell types in the microenvironment that are both essential for metastasis, as well as how the tumor cell communicates with them to drive invasion and metastasis. Additionally, formation of the pre-metastatic niche also involves the recruitment of immune cells to this site. Utilizing the expression of a metastasis suppressor such as RKIP would allow one to study which cellular or molecular components were required for metastasis formation. Proteomic studies of exosomes secreted by the different organotropic metastatic derivatives of MDA-MB-231 demonstrated that integrins were essential for formation of the pre-metastatic niche. Understanding how RKIP or another metastasis suppressor is able to

alter the pre-metastatic niche formation would give further insight into which stromal cell types are most important for recruitment to these sites. It could also identify other molecular targets that could be exploited to help predict and prevent metastases.

### ***Metastasis suppressors and exosomes.***

Here, we demonstrate that RKIP regulates exosome function. Costa-Silva et al established that exosomes are essential in the formation of the pre-metastatic niche in pancreatic cancer<sup>78</sup>. Additional work from the Lyden group has also established that organotropic metastasis of MDA-MB-231 derivatives created by the Massagué group is controlled by alterations in exosome integrins and demonstrated that blocking integrin signaling could prevent formation of a pre-metastatic niche and metastatic lesions from forming<sup>79,80</sup>. Work from other labs has shown that exosomes can stimulate TLR3 in lungs through transmission of RNA, leading to recruitment of neutrophils and formation of the pre-metastatic niche<sup>119</sup>.

By utilizing RKIP and other metastasis suppressors, we could further investigate the molecular mechanisms that exosomes utilize to affect metastatic sites or stromal cells in the primary tumor. Proteomics analysis of exosomes from the Lyden group showed drastic differences in integrins. It is likely that proteomic comparisons of exosomes from control BM1 cells compared to those overexpressing RKIP or another metastasis suppressor are likely to identify targets that can regulate uptake in target cells. Studies of how RKIP or other metastasis suppressors regulate the RNA content of exosomes could give insight into potential targets transmitted to target cells, whether they be miRNA, lncRNA, mRNA, or other RNAs. Further study into how RKIP and other metastasis suppressors regulate exosome secretion and cargo could give

additional insight into the role exosomes play roles in both triggering the stroma toward a pro-tumor phenotype and priming sites for metastasis.

### ***Exosome stimulation of TLR signaling in macrophages***

Extensive work has demonstrated that toll-like receptor (TLR) signaling can play a significant role in the activation of macrophages toward either a pro-tumor or anti-tumor phenotype. Tumor exosomes in particular have been shown to activate TLR2 and TLR3 signaling in macrophages. Work from the Flavell lab was the first to demonstrate the necessity of TLR3 signaling in response to double stranded RNAs in macrophages<sup>94</sup>. This work established that double stranded RNAs could bind and activate TLR3 in macrophages. They found that the NF-κB adaptor protein MyD88 was not necessary for cytokine activation through exosome induced TLR3 signaling. Subsequent work has also shown that TLR2 can also be activated *in vitro* by exosomes. TLR2 based activation of NF-κB and STAT3, through the adaptor protein MyD88, leads to increased inflammatory cytokine signaling in macrophages<sup>92</sup>. This work has established that cytokine secretion in macrophages can be controlled by exogenous double stranded RNA or lipoproteins delivered to cells by exosomes through activation of TLR2/3 signaling.

It will first be essential to monitor if tumor cell exosomes from our cells activate TLR signaling in the same manner dependent on NF-κB, MyD88, or STAT3. By establishing if this is part of the mechanism of activation of macrophages it would be possible to establish if regulated TLR signaling could be responsible for controlling tumor growth and metastasis by controlling programming of macrophages to a pro-tumor TAM phenotype. It would then be essential to examine what role these pathways play in the control of exosome programming by the

metastasis suppressor RKIP. It is possible that RKIP regulates either the amount of total double stranded RNAs present in exosomes or the amounts of certain RNAs within the exosomes. If we see a change in RNA that corresponds to a lack of TLR induced NF- $\kappa$ B or STAT3 signaling, it would suggest that RNA levels in the exosomes play a critical role in activating macrophages, suggesting TLR3 rather than TLR2 signaling in macrophages.

STAT KO, TLR KO, and MyD88 KO models could all be employed to determine how cytokine activation that we observed being regulated by exosome treatment is regulated. Bone marrow from KO mice can be utilized to create TEMs and determine if these signaling pathways are required for exosome-mediated programming of our TEMs or whether any of these pathways are necessary for the differences we see between programming from exosomes of CCL5 over-expressing cells which show drastically different exosome programming compared to exosomes from control cells. Because RKIP derived exosomes are unable to program TEMs toward a pro-tumor phenotype, KO models that would abrogate exosome programming would likely not tell us anything. It would be important to measure carefully if RKIP is changing total RNA levels in exosomes that may differentially activate TLR signaling, or potentially regulating proteins that would activate TLR2, rather than TLR3.

However, CCL5 exosome programming shows an increase in programming above levels seen from control exosomes. Here it would be essential to see if TLR, MyD88, or STAT are required for this difference in programming. First, we would identify the components that are required for activation in macrophages by control TNBC exosomes. Then we can determine if exosomes from CCL5 overexpressing cells are able to program TEMs in KO BMDMs, which would suggest they are able to activate alternative pathways.

If we suspect particular genes are responsible for the differential programming of macrophages based on RNAseq of exosomes, it would be essential to study how over-expression or knock-down of those genes alters exosome programming. Additionally, we can monitor if the TLR pathway identified in control exosome programming also affects programming through that particular gene.

### ***Can exosome programming of TAMs alter the tumor microenvironment?***

Our data show that several of the proteins that are down-regulated in macrophages (both TEM and TAM) between control and shRab27a programming may serve as chemotactic factors for other cell types within the microenvironment such as neutrophils, TAMs, and T-regs. These proteins include CCL2, CCL3, CCL19, CXCL1, and CXCL7. CCL19 has been shown to be a chemotactic factor for CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>-</sup> T-regs to zones of T-cell infiltration in the tumor microenvironment<sup>141-143</sup>. Additionally, expression of CXCL1 recruits T-regs to the tumor microenvironment through CXCR2 signaling<sup>144</sup>. CXCL1 has also been shown to increase recruitment of CXCR2<sup>+</sup> neutrophils to the tumor environment, which correlates with poor prognosis<sup>145,146</sup>. CCL2, CCL3, and CXCL7 have all been shown to be chemotactic factors for macrophage recruitment into the tumor microenvironment and at metastatic sites<sup>147,148</sup>. This suggests that alterations of macrophages by tumor exosomes could not only have an impact on their direct pro-tumor phenotype, but also alter their ability to attract and interact with other cells within the tumor micro-environment. T-regs have been shown to have an essential role in blocking the anti-tumor effect of cytotoxic CD8<sup>+</sup> T cells that infiltrate within the tumor. Macrophages are among the first immune cells to arrive at tumor sites. If tumor cells have the

ability to regulate cytokine expression within macrophages in such a profound way, it is likely it may be one method by which they can control recruitment of other pro-tumor cells, including T-regs.

To better understand if tumor exosomes can play a role in T-reg recruitment it will be important to study them in two areas. First, do tumor exosome TEMs increase T-reg numbers when they are co-injected with tumor cells. This will allow us to understand if tumor exosomes alone are sufficient to drive TEMs to a phenotype that enables increased T-reg recruitment into the tumor. Additionally, it is likely that TEMs programmed by exosomes from RKIP expressing cells will lose this ability and can serve as a negative control to understand how tumor exosome programming alters TEM biology in the tumor environment *in vivo*. Additionally, it will be essential to see how blocking tumor exosomes secretion *in vivo* through shRab27a KD in tumors alters T-reg numbers in the tumor. Because TAM numbers are not altered, we can understand how alterations in TAM phenotype, including reduced cytokine secretion, alter T-reg recruitment. Because cytokines like CXCL1 and CCL19 are known to regulate the recruitment of T-regs, they will likely be reduced in shRab27A tumors.

Additionally, it will be important to monitor recruitment of neutrophils to the tumor microenvironment when exosome programmed TEMs are co-injected with tumor cells or when comparing control tumors to shRab27a KD tumors. Like macrophages, neutrophils can be alternatively activated when recruited to the tumor microenvironment, and are one of the first immune cells to hone to tumor sites<sup>149,150</sup>. Because of this, studies of neutrophils would require not only monitoring the number of neutrophils present in the tumor, but also determining the phenotypic difference between the groups. One potential marker for this activity would be

CXCR2+ neutrophils, which have been shown to be associated with poor prognosis<sup>146</sup>. It could also be necessary to isolate tumor infiltrating neutrophils and assay their secretome to determine if they have altered expression of cytokines or differential effects on tumor cell invasion.

### ***Exosomes as mediators of resistance***

In Chapter 4 of this work, we described the ability of exosomes to transfer resistance to the CCR5 inhibitor Maraviroc to macrophages. Other work has suggested that exosomes may also be a major mediator of resistance within the tumor microenvironment<sup>151</sup>. However, the full extent of the role of exosomes in transmitting resistance between the tumor and microenvironment is unclear. Exosomes have been shown to be essential players in crosstalk between the tumor and microenvironment. Resistance mechanisms within tumors are known to involve rewired expression networks as well as resistant mutations that could then be transferred to stromal cells. There are many examples of exosomes reprogramming different cell types within the stroma to affect tumor growth, invasion, and metastasis.

Resistance pathways are likely to then affect either secretion of exosomes or the cargo that is being loaded into exosomes. This would then in turn affect how stromal cells are activated. It is very likely that the mechanism we have observed in these studies can be applied to other mechanisms of resistance. One example that has been established is the role of exosomes in transferring chemoresistance to other cells, in part through the transfer of miRNA to recipient tumor cells<sup>152-154</sup>. Additionally, recruitment of TAMs is known to play a significant role in chemoresistance in numerous different tumor types<sup>153,155,156</sup>.

Therefore, it is very likely that transfer of exosomes to stromal cells that exhibit chemoresistance will further modulate their chemoresistant behavior. Additionally, it is likely that, in models of resistance to targeted therapies involving rewiring of the tumor cell<sup>157-160</sup>, this rewiring would lead to alterations in exosome secretion and cargo that could then program the stroma toward a pro-tumor resistance mechanism. One potential mechanism of rewiring through exosomes could utilize the stimulation of TLR on target cells by exosomes to regulate cytokine secretion by altering NF- $\kappa$ B activation. Cytokines could directly act on the tumor to drive growth, invasion, and metastasis or could act to recruit pro-tumor cells including T-regs, TAMs, or others. Because of the clear role exosomes can play in modulating the stroma as well as tumor cells, they represent a potent mechanism by which tumor cells can transmit resistance. While these mechanisms have begun to be studied in chemoresistance, their role in resistance to targeted therapies needs to be further elucidated.

### ***Exosomes as liquid biopsies***

This work has established that exosomes from tumor cells are essential for the programming of naïve macrophages to a TAM phenotype. When we reduced secretion of tumor exosomes by blocking expression of Rab27a, we saw a decrease in the pro-tumor phenotype of TAMs in the microenvironment. Additionally, when we modulated tumor signaling by expressing the metastasis suppressor RKIP or the chemokine CCL5, we observed drastic changes in the ability of tumor exosomes to program macrophages. This suggests that tumor signaling is affecting the cargo of exosomes such that they are no longer able to alter target cells to the same degree. This also suggests that, if we examine the exosome cargo between control tumor cells and those

overexpressing RKIP, we can determine which biomolecules, whether they be tumor DNA, mRNA, miRNA, or proteins, might be responsible for programming TAMs to a pro-tumor phenotype.

Our cytokine array results demonstrated that we see drastic regulation of many cytokines; approximately one third of those assayed were differentially expressed beyond a 50 percent change in expression. This suggests that NF- $\kappa$ B activation is being drastically altered between TAMs programmed in a control tumor versus those in proximity to shRab27a tumor cells. One likely regulator of NF- $\kappa$ B activity when macrophages are stimulated by exosomes is double stranded RNA stimulation of TLR. TLR2 and TLR3 activation and subsequent NF- $\kappa$ B activation. This mechanism involving exosome RNA has been well established for macrophages by previous work<sup>92,94,161</sup>. Therefore, a likely candidate as the mechanism driving differential activation of macrophages is an RNA signal. This could be due to a general down-regulation in RNAs by RKIP that lead to less RNA cargo being loaded into exosomes, or RKIP could be regulating specific RNAs that are loaded into exosomes.

Our previous work in Chapter 4 established that RKIP and CCL5 signaling along with TAM expression are potent predictors of outcome in patients. By examining the changes in cargo between exosomes from control and RKIP over-expressing samples, we can understand both what the changes are that alter the ability of exosomes to program the microenvironment in addition to discovering potential diagnostic targets. Proteins on the surface of the exosomes are an easy target. Mass spectrometry between the two sample types could identify proteins that are differentially expressed. Surface proteins could then be assayed by flow cytometry or by microfluidic methods. Surface proteins common to both could be utilized for isolation of exosomes from biofluids using anti-body enrichment in microfluidic devices. Because tumor

exosomes comprise such a small fraction of the total exosomes present in blood, biomarkers may be washed out if total exosomes are collected and assayed for expression. Methods to enrich tumor exosomes or flow cytometry could overcome these methods.

Another method for bioassays of exosomes would be the RNA cargo in the exosomes. If RNAseq studies of exosomes from control tumor cells compared to those over-expressing RKIP display genes differentially expressed by the two, they could be assayed in patient samples. RNAseq or arrays on each patient sample would be financially prohibitive for follow-up studies. However, an assay to multiplex genes or RNAs found regulated by RKIP at the RNA level in exosomes could provide a more cost-effective route to study their clinical utility. One such assay would be nanoString, which requires only a small amount of unpurified RNA to assay differential expression between samples at a high degree of efficiency and precision. An additional method would be digital droplet PCR (ddPCR). These assays separate qPCR reactions into ~20,000 droplets and assay how many droplets amplify the RNA target. Utilizing this method does not require normalization to a reference gene for comparisons of expression levels. In addition, a small fraction of the RNA needed for traditional qRT-PCR is required for ddPCR reactions, allowing one to assay many more genes from one patient exosome sample. Because RNAs can be assayed at much more precise and much lower levels, it may be possible to assay tumor cell exosomes in patient samples without enrichment. The feasibility of these studies would need to be investigated to understand whether enrichment of tumor exosomes from patient samples is needed for precise measurements of RNAs associated with poor outcome and programming of macrophages toward a pro-tumor TAM phenotype.

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