Loss of LKB1 Leads to Alteration of the Immune Microenvironment in Non-Small Cell Lung Cancer

by

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ABSTRACT

The majority of non-small cell lung cancer (NSCLC) patients (70%) are diagnosed with adenocarcinoma versus other histological subtypes. These patients often present with advanced, metastatic disease and frequently relapse after treatment. The tumor suppressor, Liver Kinase B1, is frequently inactivated in adenocarcinomas and loss of function is associated with a highly aggressive, metastatic tumor (1). Identification of the mechanisms deregulated with LKB1 inactivation could yield targeted therapeutic options for adenocarcinoma patients. Re-purposing the immune system to support tumor growth and aid in metastasis has been shown to be a feature in cancer progression (2). Tumor associated macrophages (TAMs) differentiate from monocytes, which are recruited to the tumor microenvironment via secretion of chemotaxic factors by cancer cells. We find that NSCLC cells deficient in LKB1 display increased secretion of C-C motif ligand 2 (CCL2), a chemokine involved in monocyte recruitment. To elucidate the molecular pathway regulating CCL2 up-regulation, we investigated inhibitors of substrates downstream of LKB1 signaling in A549, H23, H2030 and H838 cell lines. Noticeably, BAY-11-7082 (NF-κB inhibitor) reduced CCL2 secretion by an average 92%. We further demonstrate that a CCR2 antagonist and neutralizing CCL2 antibody substantially reduce monocyte migration to NSCLC (H23) cell line conditioned media. Using an *in vivo* model of NSCLC, we find that LKB1 deleted tumors demonstrate a discernible increase in CCL2 levels compared to normal lung. Moreover, tumors display an increase in the M2:M1 macrophage ratio and increase in tumor associated neutrophil (TAN) infiltrate compared to normal lung. This M2 shift was significantly reduced in mice treated with anti-CCL2 or a CCR2 antagonist and the TAN infiltrate was significantly reduced with the CCR2 antagonist. These data suggest that deregulation of the CCL2/CCR2 signaling axis could play a role in cancer progression in LKB1 deficient tumors.

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CHAPTER 1

INTRODUCTION

LUNG CANCER

Lung cancer accounts for up to 27% of all cancer related deaths and despite aggressive chemotherapeutic treatments, there has been little improvement in patient survival (3). Lung cancer consists of two broad classes: Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). 85% of lung cancers are NSCLC, which can be subdivided into histologically distinct types: adenocarcinoma (glandular), squamous (inner lining of the bronchial tubes) and large cell carcinoma. 70% of NSCLC are adenocarcinomas and although there have been advances in targeted therapeutics for Epithelial Growth Factor Receptor (EGFR) and EML4-ALK mutations, those mutations represent a low frequency in adenocarcinoma populations (*3*). The tumor suppressor Liver Kinase B1 (LKB1) is the second most mutated gene (behind *TP53*) in NSCLC (*4*). Loss of LKB1 in conjunction with oncogenic activation of KRas promotes aggressive, metastatic tumors in transgenic models (1). Notably, inactivation of LKB1 is commonly present in the background of oncogenic KRas mutations in patients. This cancer subtype represents a genetically unique tumor population (5). The standard care for these patients is chemo-radiation therapy, which results in little improvement in survival (6). Due to the high incidence of metastatic disease in NSCLC patients, effective treatment options are a significant challenge, indicating a considerable need for specific therapeutics in LKB1/KRas cancers.

LKB1 was initially recognized for its role in a heritable cancer disorder, Peutz-Jeghers syndrome, but has been recently acknowledged to be mutated in other spontaneous cancers, including 30% of NSCLC (*7*). LKB1 phosphorylates AMP-activated protein kinase (AMPK), a major sensor of cellular energetic stress. The LKB1-AMPK pathway is active under energetic stress or hypoxic conditions, causing cellular arrest to ensure survival (*7*). AMPK also regulates metabolism by stimulating glucose uptake and inhibiting protein synthesis, leading to energy conservation at the expense of cell growth and proliferation. Although AMPK is mostly known for its effects on cell metabolism, it also regulates mitochondrial biogenesis and disposal, autophagy and cell polarity. Loss of LKB1- AMPK function allows tumors to escape the pathway's restraining effects on cell

growth and proliferation. By targeting downstream effectors of LKB1-AMPK that are deregulated in LKB1 null cancers, further therapeutic options can be identified. Furthermore, the broad regulatory functions of LKB1 suggest that additional avenues could be utilized. LKB1 also has other targets in addition to AMPK such as MARK, SIK, and SAD that play an essential role in cell polarity. Loss of LKB1 has been shown to de-regulate these substrates and play a role in tumorigenesis (7).

IMMUNE SYSTEM AND CANCER

The integration of immunology in the study of cancer progression is becoming a critical connection, as the immune system has a significant role in the development of a tumor. Whether tumors evade recognition by the adaptive immune system, or utilize innate cell function to the advantage of the tumor, it is important to incorporate the influence the immune system has on cancer progression.

TUMOR ASSOCIATED MACROPHAGES

Macrophages are a type of white blood cell that primarily phagocytoses cellular debris, foreign substances and microbes. Monocytes, the pre-cursor to macrophages, circulate the body and are recruited to tissue based on the chemical gradients of a wide variety of chemokines and cytokines. Monocytes can polarize to classical M1 macrophages in response to Interfeuron gamma (IFNγ), microbial stimuli, such as Lipopolysaccharides, or cytokines such Tumor Necrosis Factor alpha (TNF-α) and Colony Stimulating Factor 2 (CSF-2) (8). M1 macrophages play a role in clearance of microbes as well as mediating resistance to intracellular parasites (9). Monocytes can also differentiate into alternative M2 macrophages by Transforming Growth Factor Beta (TGFβ) and cytokines such as Interleukin 4 or 10 (IL-4 ,IL-10). M2 macrophages play a role in wound healing by reducing the inflammation, remodeling tissue, promoting angiogenesis and stimulating cell proliferation (10).

Tumor associated macrophages (TAMs) generally exhibit an M2 phenotype and are thought to be particularly important in tumorigenesis, tumor growth and metastasis. Monocytes can migrate to the tumor environment in response to cytokines such as CCL2, CCL5, CCL20 and then differentiate into TAMs in response to factors such as TGFβ and IL-10. TAMs then secrete pro-

tumor factors important in tumor growth and metastasis, such as Vascular Endothelial Growth Factor (VEGF), Extra-Cellular Matrix (ECM) proteases, Fibroblast Growth Factor (FGF) (11). By programming M2 polarization, cancer cells can utilize the innate immune system to sustain tumor growth and proliferation, aid in metastasis and recruitment of nutrients, and evade adaptive immunity. However, little is understood as to the mechanisms that govern these events in tumors, including in NSCLC.

TUMOR ASSOCIATED NEUTROPHILS

Tumor associated neutrophils (TANs) are derived from granulocytic cells that are produced in the bone marrow. Neutrophils are short-lived phagocytes that are the first responders to inflammatory sites. Like macrophages, neutrophils infiltrate tissue in response to cytokines such as CCL2, CCL3, IL-1β and IL-6 (12). Neutrophils are primarily known for their anti-bacterial functions, however, TANs are a distinct population differing significantly in their functional activity (13). A study done by Fridlender et. al demonstrated the importance of TGFβ in TAN polarization. Blockade of TGFβ resulted in an increase influx of anti-tumor (N1) neutrophils and a reduction of pro-tumor (N2) neutrophils (14). N2 neutrophils secrete products such as reactive oxygen species, matrix membrane proteinases (MMPs), collagenases, and aid in translocation of the tumor cells through vasculature. TANs have been relatively uninvestigated until recently, but some studies have defined roles for TANs in regulating tumor cell proliferation, angiogenesis, and metastasis (15).

HYPOTHESIS

To investigate the effects of LKB1 loss, we ran a global gene expression array on a human NSCLC adenocarcinoma cell line H23 re-expressing either a non-functional (kinase dead, KD) LKB1 or functional LKB1. Among the genes with altered expression, CCL2 and other chemokines were found to be highly expressed in the Kinase Dead LKB1 cell line versus the full length LKB1 cell line (Table 1). Based upon the reported role of these chemokines in the recruitment of pro-tumorigeneic immune cells, we hypothesized that increased expression and secretion of CCL2 associated with LKB1 inactivation may result in a distinct immune microenvironment in LKB1-deficient NSCLC tumors. Characterization of the immune cell

populations associated with LKB1 inactivation could provide a mechanism towards the aggressive nature of LKB1- NSCLC.

CHAPTER 2

METHODS

Cell Culture

Human lung non-small cell carcinoma cells (ATCC; H23: CRL-5800, H2030: CRL-5914, H2009: 5911, H358:CRL-5807, H441: HTB-174, H838: CRL-5834, A549:CCL-185) were maintained in RPMI, 10% FBS supplemented with 5% penicillin streptomycin. Peripheral Blood Mononuclear cells were used fresh (ZenBio; SER-PBMC). Monocytes (CRL-9855) were maintained in Iscove's modified Dulbecco's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.05 mM 2-mercaptoethanol, 0.1 mM hypoxanthine and 0.016 mM thymidine, 90%; fetal bovine serum, 10%.

Migration Assay/Flow Cytometry

Conditioned media was placed in the lower chamber, 4 μM polycarbonate transwell filter was placed in the transwell and PBMCs were placed in the upper chamber. Cells were incubated at 37°C for two hours. Media from the lower chamber was collected and cells were trypsinized from lower chamber and the bottom of the filter using 5mM EDTA/PBS. Cells were suspended in an antibody cocktail containing CD3, CD19, CD56, HLA-DR, CD14, CD11c (ebioscience cat# 11-0039-42, 11-0199-42, 11-0569-42, 12-9952-42, 11-45-0149, 17-0016-42 respectively) and run through Accuri C6 flow cytometer.

ELISA

Cells were dosed with vehicle, Dasatinib 250nM, LY294002 10mM, U0126 10μM, Rapamycin 10nM, Bortezomib 100nM or Bay-11-7082 5μM (Selleck,Cat No.S1021, S1105, S1102, S1039, S1013, S2913 respectively) for 24 hours. Cell culture supernatant was collected and a quantitative CCL2 ELISA performed (R&D cat# DCP00). Cell culture supernatant was also collected from H23 KD LKB1 and full-length LKB1, CMT64 and CRISPR LKB1 CMT64.

siRNA depletion of HIF1-α

5nM si-HIF1-α (QIAGEN, SI02664431) was diluted into Opti-MEM and HiPerFect transfection reagent added (QIAGEN, 301702) in H23 and H2030 cell lines. Cell lysates and media were collected after 24 hours.

CRISPR mediated deletion of LKB1

6µg of Lenti Viral plasmid (U6-gRNA/EFa-puro-2A-Cas92Z-GFP) is mixed with Lipofectamine 2000 and Lentiviral Packaging Mix (Sigma, SHP001) and added dropwise to 293T cells. Media is changed after 24 hours, then collected at 72 hours. Viral media is filtered through a .45µM strainer onto H2009 and CMT64 cells. Transdux at 1:1000 is added (Systems Bio, LV850A-1)**.** Cells are selected with 3µg/ml of puromycin 48 hours post infection.

NF-kB p50/p65 Transcription Factor Assay Kit

Nuclear and Cytoplasmic lysates were collected from H23 and H2030 cell lines. The nuclear fraction was run on the transcription factor assay kit (Abcam, ab133128).

Transgenic Mice

The KRas/LKB1-null/Luciferase transgenic mouse is a well-characterized model of LKB1-deficient NSCLC (1). Expression of Cre recombinase in the lung, via intra-tracheal introduction of the Cre-adenovirus, results in biallelic deletion of the floxed LKB1 gene,activation of the KRas oncogene and expression of Luciferase. Luciferase will bind Luciferin, which can be visualized using a Xenogen. Deletion of LKB1 and over activation of KRas results in an aggressive tumor burden on the mice, with an average survival time of 9 weeks. Tumor burden is visualized on a Xenogen and relative optical intensity measured. 4 weeks post infection, mice were (check tense) randomized to vehicle, CCL2 neutralizing antibody at 5mg/ml twice per week (R&D; AB-479-NA) or a CCR2 antagonist (Sigma; SML0711-25mg) at 2mg/kg twice per day. Tissues were digested using Collagenase IV (Life Tech; 17104-019).

CHAPTER 3

RESULTS

Molecular Pathway/Mechanism

To investigate the effects of LKB1 deficiency on gene expression, an mRNA array was performed on the human lung adenocarcinoma cell line, H23 (LKB1 $^{-/-}$). Functional LKB1 or a Kinase Dead LKB1 (KDLKB1) were re-expressed into the H23 cell line to elucidate the role of LKB1 upon gene expression. Notably, CCL2 mRNA expression was 242 fold higher in the KDLKB1 compared to LKB1 (Table 1).

Gene Symbol	Genebank ID	Fold change (KDLKB1 vs LKB1)
CCL2	NM_002982	242.71196
CDH11	NM_001797	161.68156
WNT2	NM_003391	114.95994
GRIK3	NM_000831	65.504135
FAM46A	NM_017633	50.594418
CYP24A1	NM_000782	42.663696
SPOCK1	NM_004598	40.943287
AJAP1	NM_001042478	39.710697
CCL20	NM_004591	37.46643
MEOX1	NM_004527	37.394913
CYP24A1	NM_000782	30.10249
MEG3	NR_003531	28.918484
CCL5	NM_002985	27.542534
CXCR4	NM_001008540	10.280791

Table 1 Gene expression array on H23 Human Lung Adenocarcinoma cell line with the LKB1 re-expression or Kinase Dead-LKB1 (KDLKB1).

Consistent with these data, quantitative ELISA results demonstrated that H23 KDLKB1 cells secreted 7868pg/ml of CCL2 compared to 63 pg/ml in the H23 LKB1 (Figure 1). Conversely, using the Lenti-virus CRISPR system, LKB1 was knocked down in CMT 64 cells (mouse lung carcinoma, LKB1$^{+/+}$) and CCL2 was increased by 11 fold (Figure 2). Lysates were collected and blotted for LKB1 to ensure knockdown (S4). Furthermore, other LKB1 null (H838, A549) NSCLC cell lines secreted significantly higher CCL2 levels compared to LKB1 expressing (H2009, H358, H441) NSCLC cell lines (Table 2).

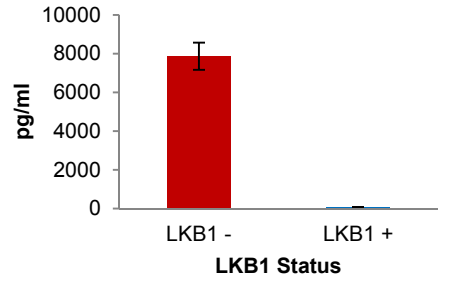


Figure 1 Quantitative ELISA on H23 isogenics, expressed with full length LKB1 (LKB1 +) or Kinase Dead LKB1 (LKB1 -) *p=0.002.

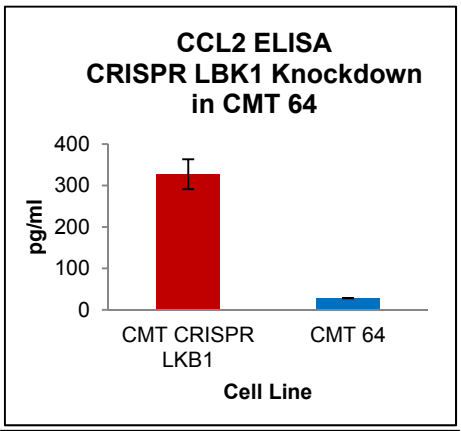


Figure 2 Quantitative ELISA on CMT 64 parentals and CMT 64 with CRISPR deletion of LKB1 *p=0.03.

Immunohistochemistry was performed on transgenic KRAS^{+}/LKB1^{+} vs. KRAS^{+}/LKB1^{-} NSCLC mouse tumors using an

anti-murine CCL2 antibody. The LKB1 null tumors displayed distinctly higher levels of CCL2 compared to LKB1 wild type (Figure 3).

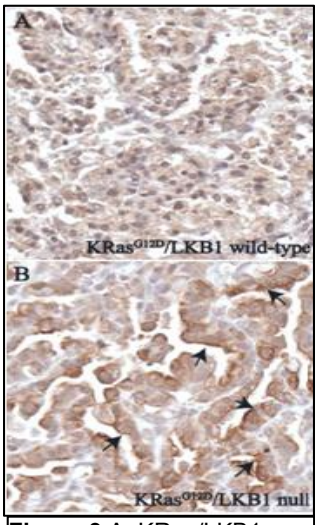


Figure 3 A. KRas/LKB1 wild-type CCL2 IHC staining samples from transgenic mice. B. KRas/LKB1 null sample.

To investigate the mechanistic role of LKB1 deficiency on CCL2 secretion, signaling pathways de-regulated with LKB1 deficiency were targeted with inhibitory drugs in H23 and H2030 cell lines. mTOR, PI3K,SRC family and MEK inhibitors are therapeutic strategies for targeting in LKB1$^{-/-}$/KRas tumors. PI3K and SRC families up-regulate mTOR and MEK is de-regulated with oncogenic KRas (14). NF-κB was targeted due to its known inflammatory response cascade.

	Cell line	pg/ml CCL2
LKB1 +	H2009	34.25, ± 2.75
	H358	39.75, ± 7.75
	H441	98.25, ±5.75
LKB1-	H23	12866, ± 492
	H838	3242.5, ± 203
	A549	3256.75, ±479

Table 2 Human CCL2 quantitative ELISA from LKB1$^{-/-}$ and LKB1 $^{+/+}$ NSCLC cell lines.

AMPK ordinarily inhibits the 26s proteasome, which serves to degrade transcription factors involved in cell cycle progression and apoptosis (7). Dasatinib (SRC family inhibitor), LY294002 (PI3K Inhibitor), U0126 (MEK inhibitor) resulted in minimal reduction of CCL2 secretion. Rapamycin (mTOR inhibitor), significantly reduced CCL2 secretion in H23 ($p = 0.009$) cells but only an 8% reduction was seen in H2030 cells. However, Bortezomib (26s proteosome and NF-κB inhibitor) and BAY-11-7082 (NFκB inhibitor) substantially reduced CCL2 secretion in both cell lines ($p=0.002$) (Figure 4). Lysates were collected from the treatments and blotted for β-actin to ensure no change in global protein expression (S1).

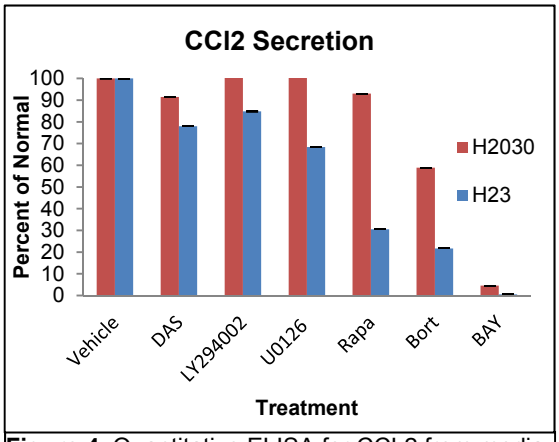


Figure 4 Quantitative ELISA for CCL2 from media collected from H23 and H2030 cells treated with vehicle, Dasatinib 250nM, LY294002 10nM, U0126 10μM, Rapamycin 10nM, Bortezomib 100nM, or Bay-11-7082 10μM for 24 hours.

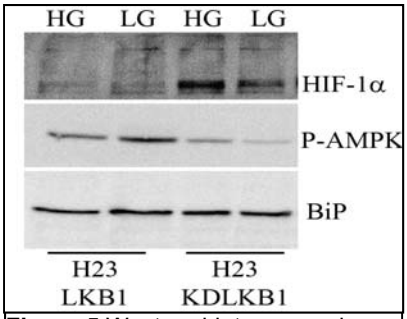


Figure 5 Western blots comparing H23 LKB1 and H23 KDLKB1. HIF1-α is higher in both high and low glucose in KDLKB1 compared to LKB1. P-AMPK demonstrates a decrease of activity in KDLKB1.

We further investigated the influence of mTOR signaling on CCL2 secretion to elucidate potential reasons why rapamycin treatment resulted in a drastic decrease in CCL2 in the H23 but not H2030. Loss of LKB1 has been shown to increase the transcription factor Hypoxia Inducible Factor 1 -alpha (HIF1-α) even under normoxic conditions, due to hyper activation of mTOR signaling in an animal model of Peutz-Jeghers syndrome (13,14). CCL2 expression has been found to be regulated by HIF-1α under hypoxic conditions (15). In H23KDLKB1 cells, under high and low glucose with normoxic conditions, HIF1-α is markedly higher than H23 LKB1 cells (Figure 5). To evaluate the role of HIF1-α on CCL2 regulation under normoxic conditions, we used 5nM siRNA for HIF1-α in H23 and H2030 cell lines and performed a quantitative ELISA. CCL2 secretion was reduced by 10% in H23 and 8% in H2030 treated with si HIF1-α (Figure 6). These findings suggest that while HIF1-α is upregulated with LKB1 deficiency, it does not have a critical role in CCL2 expression.

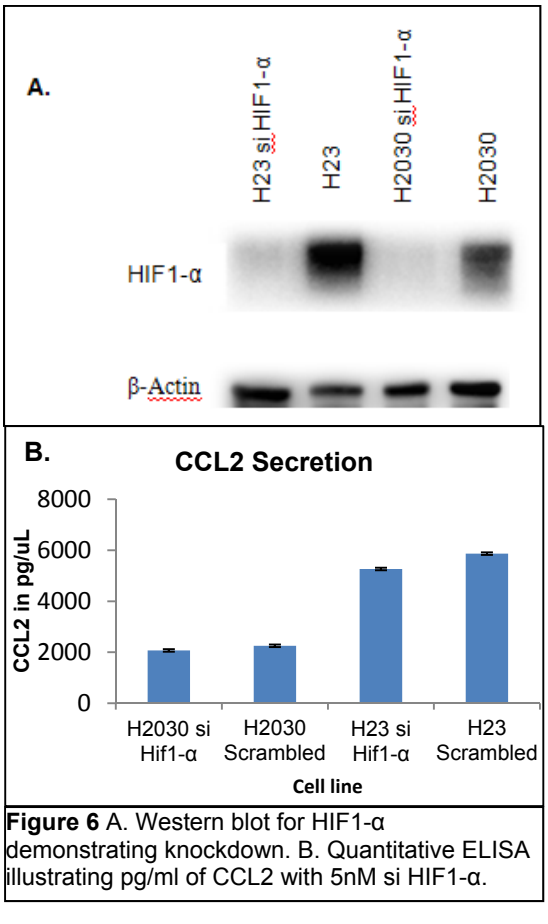


Figure 6 A. Western blot for HIF1-α demonstrating knockdown. B. Quantitative ELISA illustrating pg/ml of CCL2 with 5nM si HIF1-α.

We then interrogated the role of NF-κB signaling on CCL2 secretion. NF-κB signaling mediated by IL-1β and TNFα has also been shown to regulate CCL2 in rat astrocytes (16). FLIP1 (an-LKB1 interacting cytoplasmic protein) has been shown to negatively regulate TNF-α induced NF-κB activation (17). We demonstrated increased NF-κB activity in LKB1 null lines (H23,H2030,A549 and H838) compared to LKB1 wild-type lines (H2009 andH1975) (p=0.018) (Figure 7). Furthermore, in LKB1 deficient lines A549, H838, H23 and

H2030 cells treated with the NF-κB inhibitor, Bay 11-7082 5μM, CCL2 secretion was reduced by an average 91% with minimal effects on cell viability (Figure 8).

Function

CCL2 is an inflammatory chemokine known to recruit monocytes to infected or injured tissue. To test the chemotaxic effects of LKB1 deficiency, conditioned H23 media was utilized in a chemotaxis assay using human peripheral blood mononuclear cells (PBMCs). Monocytes preferentially migrated toward H23 conditioned media (8.2% of the total PBMC population was $CD11c^{+}$ and $CD14^{+}$ (monocyte markers), but only 1.7% for H2009 conditioned media (Figure 9). The necessity of the CCL2/CCR2 signaling axis on monocyte recruitment in H23 conditioned media was evaluated using a neutralizing CCL2 antibody, CCR2 or CCR4 antagonist with peripheral blood mononuclear cells in a migration assay. All treatments reduced monocyte migration toward H23 conditioned media as evaluated by flow cytometry (Figure 10).

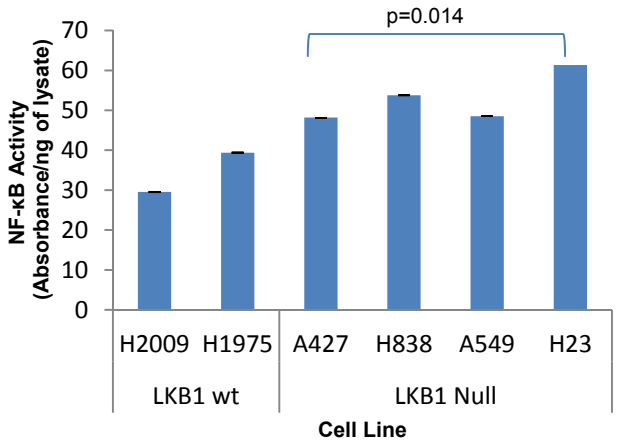


Figure 7 NF-κB activity ELISA demonstrates significantly higher activity in $LKB1^{-/-}$ cell lines compared to LKB1 wild-type (p=0.014).

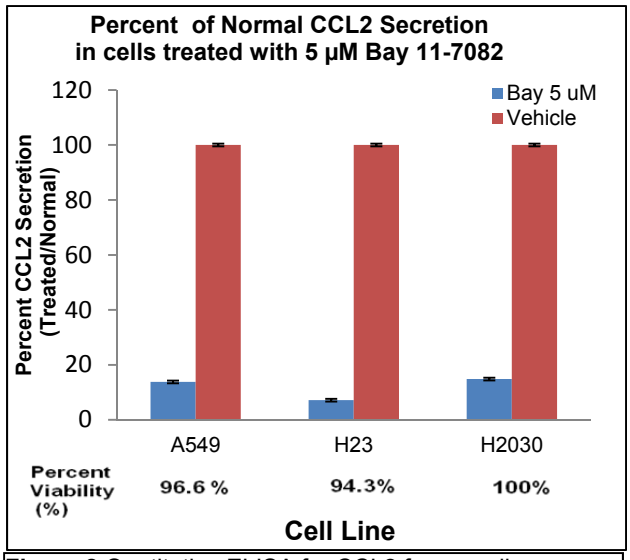


Figure 8 Quatitative ELISA for CCL2 from media collected from A549, H838, H23, H2030 cells treated with 5μM Bay 11-7082 for 4 hours. Viability assessed by cell titer glo (Promega G9241) luminescence kit measuring ATP levels.

In vivo

To assess the effects of CCL2/CCR2 signaling in LKB1- NSCLC *in vivo* we infected KRas/LKB1/Luciferase mice with Cre recombinase adenovirus via intra-tracheal injections. To mimic patient staging at diagnosis, mice were imaged six weeks post infection, using a Xenogen to assess tumor burden. Mice were then randomized to treatment groups of: Vehicle (PBS),

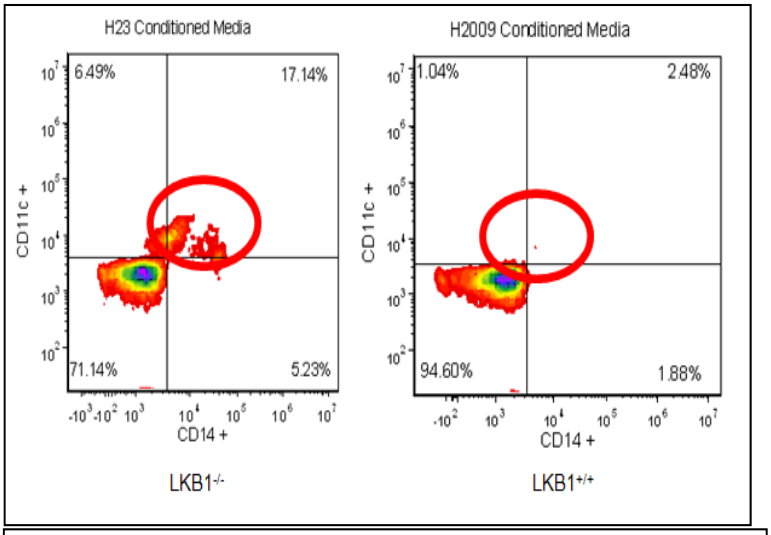


Figure 9 Flow cytometric analysis of peripheral blood mononuclear cell migration towards conditioned media. 17.14% of migrated cells stained with CD14 and CD 11c were double positive (monocytes) with H23 conditioned media and 2.48% were double positive with H2009 conditioned media.

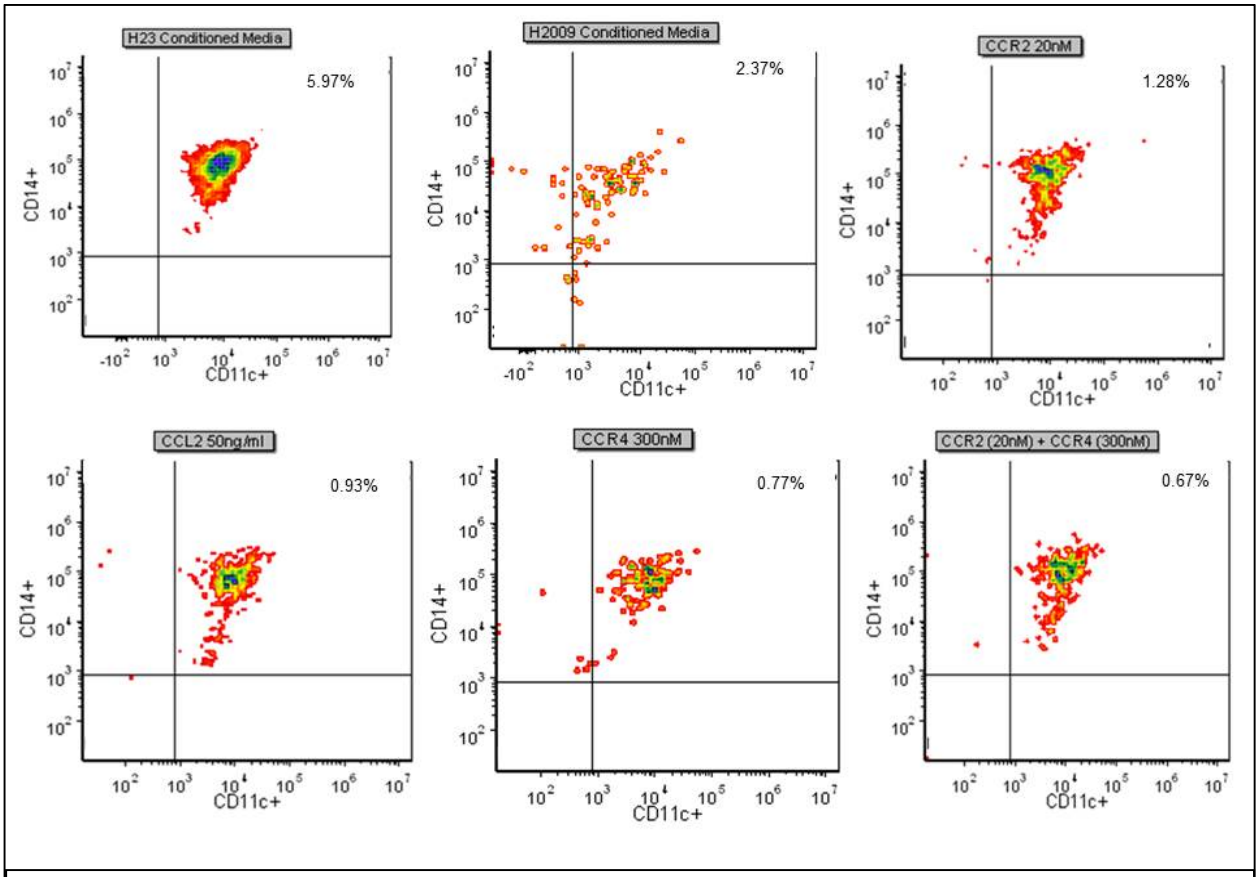


Figure 10 Flow cytometry analysis of PBMC migration toward H23 conditioned media +/- CCL2 (50ng/ml), CCR2 (20nM), and CCR4 (300nM). Percentage indicates CD14+ and CD11c+ positive cells out of the total collected population of PBMCs.

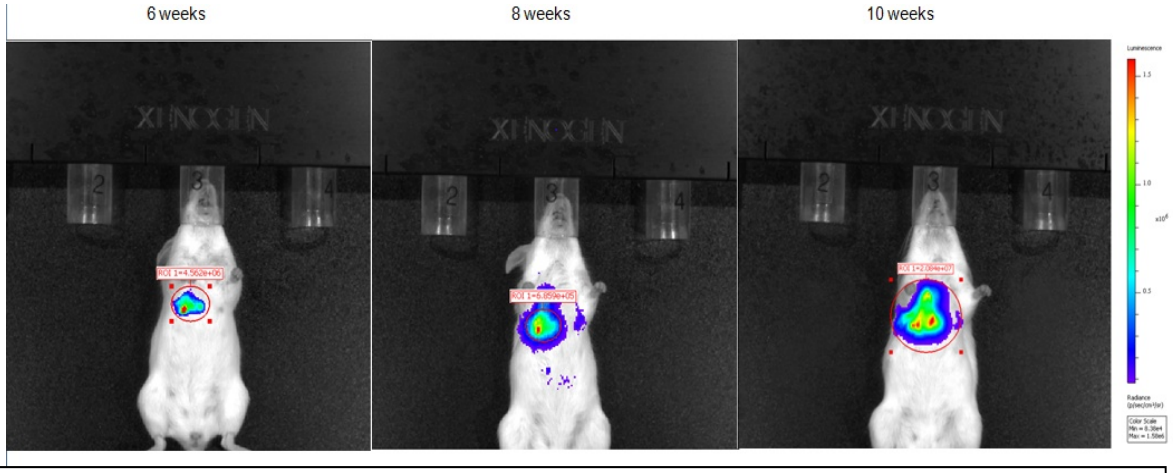


Figure 11 Transgenic LKB1/KRas/Luciferase mouse at 6 weeks, 8 weeks and 10 weeks post infection with Cre recombinase adenovirus via intra-tracheal injections. Treatment with CCR2 antagonist via oral gavage began at 6 weeks. See supplemental data for other treatment groups.

CCL2 neutralizing antibody at 5mg/ml twice per week (R&D; AB-479-NA) or a CCR2 antagonist (Sigma; SML0711-25mg) at 2mg/kg twice per day for a total of four weeks. Whole lung samples were digested at 10 weeks and analyzed via flow cytometry. Cells were stained with CD206, F4/80, Cd11b and GR-1. Although our sample size (3n) makes it difficult to definitively determine if inhibition of the CCL2-CCR2 signaling axis has effects upon LKB1- NSCLC progression, we did not see significant change in tumor progress via Xenogen imaging (Figure 11). However, reductions in the M2 (Cd11b+, F4/80+, CD206+):M1 (Cd11b+, F4/80+,CD206-) ratio was observed (Figure 12). Although there are distinct functions between N1 and N2 neutrophils, no definitive receptor markers differentiating the population have been identified. Therefore, TAN infiltrate was evaluated by measuring mature neutrophil (Cd11b+, Ly6G+) percentages to total lung population (Figure 13). Treatment with CCR2 antagonist reduced the M2:M1 ratio by an

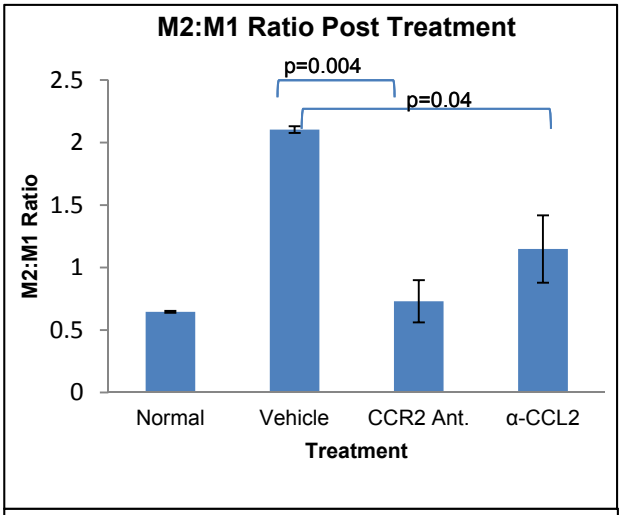


Figure 12 The ratio of M2 (Cd11b+, F4/80+, CD206+): M1(Cd11b+, F4/80+,CD206-) macrophages analyzed by flow cytometry. In mice treated with a CCR2 antagonist or α-CCL2, a significant reduction (p=0.004 and 0.04, respectively) was seen in the M2:M1 ratio.

average 2.8 fold and reduced the TAN infiltrate by an average 1.2 fold. Treatment with anti-CCL2 reduced the M2:M1 ratio by 1.8 fold and had no significant effect on TAN infiltrate. An early stage study was also performed to evaluate the effects on early tumor progression. Mice were infected with Cre recombinase adenovirus via intra-tracheal injections then immediately randomized to treatment groups of: Vehicle (PBS), CCL2 neutralizing antibody at 5mg/kg twice per week or a CCR2 antagonist at 2mg/kg twice per day for a total of four weeks. No significant reduction in tumor progression was observed via Xenogen imaging after 4 weeks (data not shown), however, M2:M1 ratio and TAN infiltrate were reduced (S2 and S3).

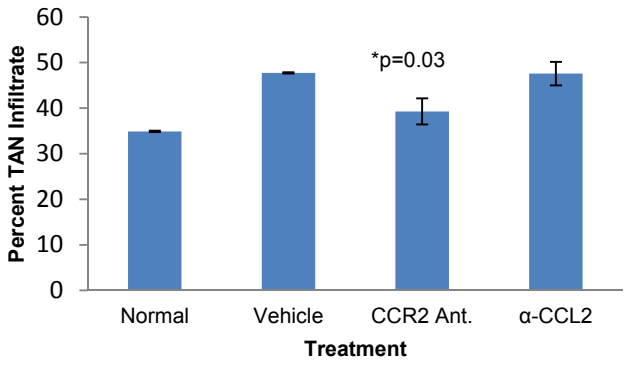


Figure 13 TAN infiltrate analyzed by flow cytometry. TAN (Cd11b+,Gr-1+) percentages assessed over total lung population.

Beyond CCL2

While CCL2 was the primary focus of this project, other cytokines and factors may also play a role in the alteration of the immune microenvironment. Based on our preliminary mRNA array we ran quantitative ELISAs for CCL5 and CCL20 in LKB1 wt and LKB1 null cell lines. LKB1 null lines secreted substantially higher of CCL5 and/or CCL20 compared to LKB1 wt (Table 3). A cytokine array was performed on H23 to assess potential factors leading to TAM and TAN polarization and tumor environment remodeling (Figure 14). Serpin E1 was 223 fold higher in KDLKB1 compared to

	Cell line	pg/ml CCL5	pg/ml CCL20
LKB1 +	H2009	29.14 +/- 0.13	37.1 +/- 0.05
	H358	0.36 +/- 0.01	0 +/- 0.01
	H441	32.75 +/- 0.06	2.30 +/- 0.05
LKB1-	H23	267.89 +/- 0.04	1221.78 +/- 0.05
	H838	58.71 +/- 0.02	29.06 +/- 0.05
	A549	104.56 +/- 0.05	2.83 +/- 0.07
	A427	8.29 +/- 0.04	6254 +/- 0.04
	H2030	0.083 +/- 0.01	81.2 +/- 0.07

Table 3 Quantitative ELISA for CCL5 and CCl20 from LKB1 $^{-/-}$ and LKB1 $^{+/+}$ NSCLC cell lines.

LKB1 wt (S4). Serpin E1 is a serine protease inhibitor that has been shown to be a biomarker for poor prognosis in various cancers. Serpin E1 promotes tumor progression likely by modulating matrix remodeling (18). I-309 (or CCL-1) is secreted by activated T-cells and also recruits monocytes. G-CSF, colony-stimulating factor, is a glycoprotein that stimulates the bone marrow to produce granulocytes. The inter-leukin family can have a wide effect on the immune cell population and activation.

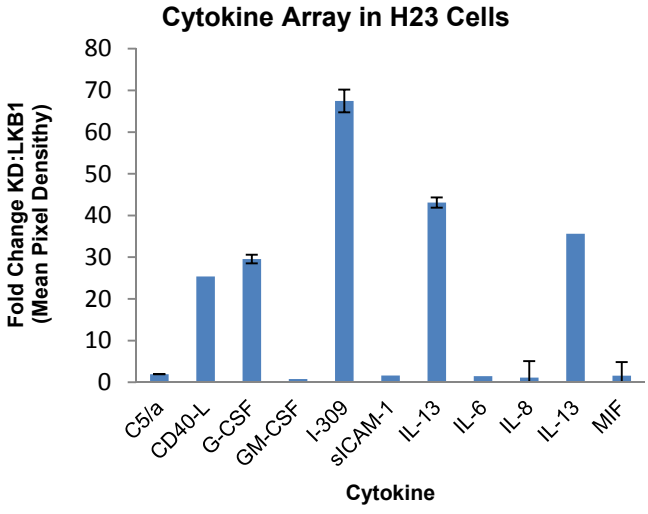


Figure 14 Cytokine Array in H23 cell indicating fold change in substrates with higher mean pixel density from kinase dead LKB1 to full length LKB1.

CHAPTER 4

DISCUSSION

Data Summary

We demonstrate that with deletion of LKB1, non-small cell lung cancer cells upregulate the chemokine, CCL2. Quantitative ELISA on H23, A549 and H838 NSCLC LKB1 deficient lines demonstrates significantly higher secretion of CCL2 than H2009, H358, and H441 NSCLC LKB1 wild-type lines. Full length LKB1 or Kinase Dead LKB1 was expressed in H23 cell lines; LKB1 expression in H23 cells considerably reduced CCL2 secretion. The reverse effect was observed in CMT64 with LKB1 knocked-down using the CRISPR system. With LKB1 deletion, CCL2 secretion is substantially increased compared to parental, LKB1 wild-type CMT 64 cells. This phenotype is also seen in transgenic KRas/LKB1 mice via immunohistochemistry for CCL2. Thus, we reveal that loss of LKB1 is correlated with up-regulation of CCL2. Inhibition of the CCL2/CCR2 signaling axis via anti-CCL2 antibody or a CCR2 antagonist significantly reduced monocyte migration from peripheral blood mononuclear cells *in vitro*. The molecular mechanism governing the increased expression needs to be further evaluated, however, we reveal a potential role for NF-κB mediated alteration of the immune microenvironment. Inhibition of NF-κB with the inhibitor, Bay 11-7082, substantially reduced CCL2 secretion in H23, H2030 and A549 cells. Our *in vivo* data demonstrate that treatment with a CCR2 antagonist reduces the M2:M1 ratio and TAN infiltrate in our transgenic LKB1/KRas/Luciferase mice. Anti-CCL2 and CCR2 antagonist treatment in the early stage group revealed a similar pattern in M2:M1 and TAN infiltrate reduction, however, a larger sample size is necessary to assess significance, as well as to ascertain what these changes have upon NSCLC progression. Loss of the LKB1 pathway causes up-regulation of the transcription factor, NF-κB which causes the expression of CCL2 and other chemokines, such as CCL5 and CCL20. These chemokines recruit monocytes and neutrophils to the tumor microenvironment where differentiation into M2 macrophages and TANs occurs. M2 macrophages and TANs have been shown to be correlated with poor prognosis in and increased tumor progression in many cancers, however, further research needs to be done to evaluate the

effects of M2 and TAN infiltrate in LKB1 deficient NSCLC (2). Taken together, these data indicate that loss of LKB1 in NSCLC leads to alterations of the immune cell environment within the tumor.

Significance and Therapeutic Opportunities

Despite aggressive therapy, non-small cell lung cancer (NSCLC) continues to be the primary contributor to cancer related deaths. Most patients present with distant metastases and often relapse after chemo-radiation. LKB1 is frequently mutated in conjunction with KRas in NSCLC yet little to no targeted therapeutics options are available. We sought to characterize the aggressive phenotype seen with LKB1 deletion and elucidate the role of the immune system in cancer progression. M2 macrophages enhance cancer progression by secreting proliferative factors (FGF), extra-cellular matrix remodeling enzymes (MMPs), metastatic capabilities (VEGF, Angiogenin) and adaptive immunity evasion (IL-10 and CCL17). N2 neutrophils further aid the progression by increasing proliferation (PDGFR signaling), extra-cellular matrix remodeling (MMPs), angiogenesis (Gro-α), cancer cell migration (HGF secretion), and immune cell evasion (CXCL9). Other studies suggest N2 neutrophils play a role in tumor initiation by reactive oxygen species mechanisms (13). We demonstrate the significance of the CCL2/CCR2 signaling axis in TAM and TAN infiltration. Antibodies against CCL2 and CCR2 have been investigated in pre-clinical trials in prostate cancer, breast cancer, ovarian cancer and have shown improved survival and reduced metastases (23). Trabectedin, derived from *Ecteinascidia turbinate*, is preferentially cytotoxic for both human and murine TAMs and inhibits production of CCL2 and IL-6 (24). Reparixin, a potent inhibitor of CXCR1 and CXCR2 has been demonstrated to selectively target human breast cancer cells and inhibit the recruitment and activation of neutrophils (25). The NF-κB pathway also shows promise for cancer therapy; bortezomib leads to lung tumor regression and increased survival KRas/P53$^{-/-}$ mice (26).

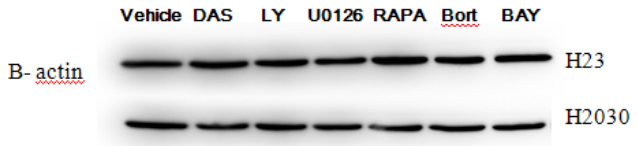
Future Studies

Further evaluation of the mechanism governing an inflammatory response and alteration of the immune system is critical to generating target therapeutics for LKB1 deficient NSCLC patients. While we show a correlation between NF-κB and CCL2, the exact mechanism governing the

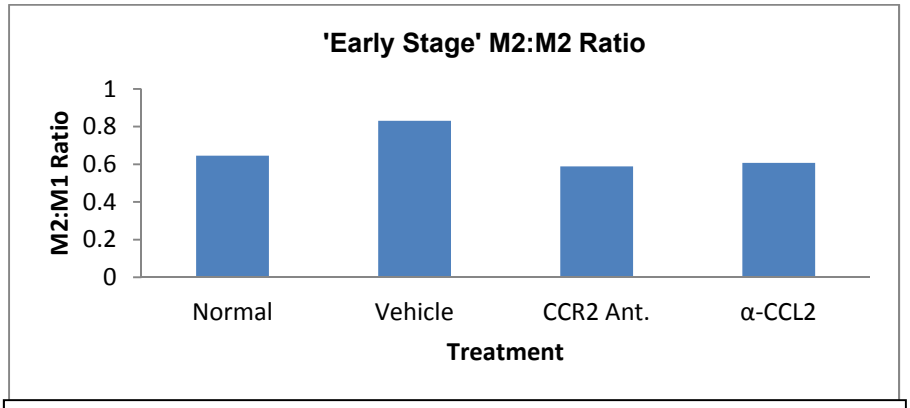
increased CCL2 expression with LKB1 loss need to be further examined. Bay-11-7082 is an inhibitor of NF-κB by selectively and irreversibly blocking TNF-α-induced phosphorylation of IκB-α. IkB-α binds to NF-κB and keeps it in the inactive form in the cytoplasm until phosphorylation of IκB causes release of NF-κB. However, to gain an accurate understanding of the function of NF-κB in regards to CCL2 secretion and LKB1 loss, NF-κB needs to be evaluated beyond the scope of a single drug. A dominant negative NF-κB or siNF-κB introduction could also be used in H23, H2030, H838 and A549 cells and CCL2 secretion evaluated. The pathway leading to the up-regulation of NF-κB should also be investigated. Discovering an upstream target of NF-κB may be a beneficial targeted therapeutic option for patients. However, an NF-κB inhibitor could be used in our transgenic KRas/LKB1/Luciferase model to evaluate the effects on tumor progression and pro-tumor immune cell infiltrate. Investigation of what factors are necessary for M2 and N2 polarization could provide a preventative avenue. Although we demonstrate an ability to reduce pro-tumor immune cell populations, blockading chemotaxic factors also reduces anti-tumor immune cell populations. Further investigation should be done to explore causative factors in anti-tumor immune cell differentiation such that pro-tumor cells can still function to reduce tumor growth. Studies have shown several factors cause M2 and N2 activation, but further exploration in an LKB1 deficient context could provide a better understanding of pro-tumor immune cell activation. Our *in vivo* study should be expanded to a larger treatment group, a longer time course allowed to evaluate metastases and CCR2/CCL2 treatment compared to combination therapy. The CCR2 antagonist could be combined with other receptor antagonists (such as CCR4) in addition to combination with cytotoxic drugs that would aid in reducing tumor proliferation and progression. Further analysis of the importance of CCL2/CCR2 signaling in the metastatic process is critical. CMT 64 CRISPR LKB1 knockdown cells could be injected into the flank of a CCR2 knockout mouse and metastatic seeding compared to CMT 64 empty vector flank injections.

CHAPTER 5

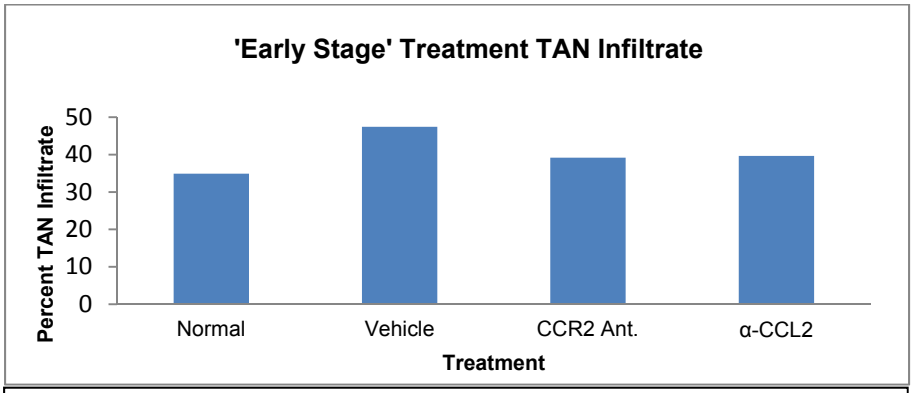
SUPPLEMENTAL DATA



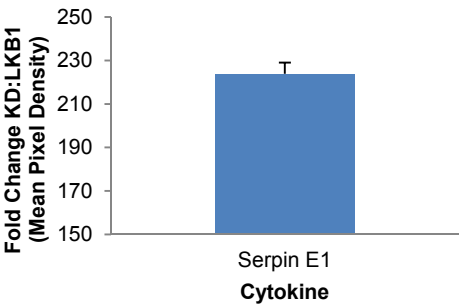
S1 Lysates from H23 and H2030 cell lines after indicated treatment were collected and blotted for ß-actin.



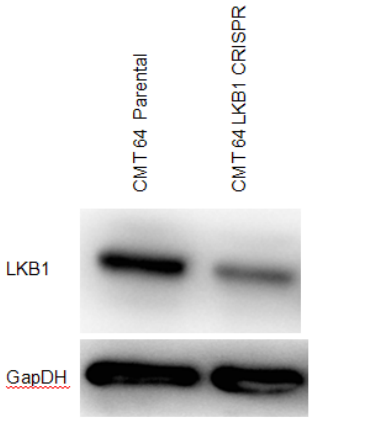
S2 The ratio of M2 (Cd11b+, F4/80+, CD206+): M1(Cd11b+, F4/80+,CD206-) macrophages analyzed by flow cytometry in mice treated with a CCR2 antagonist or α-CCL2 a for 4 weeks post infection, a reduction is seen in the M2:M1 ratio.



S3 TAN infiltrate analyzed by flow cytometry. TAN (Cd11b+,Gr-1+) percentages assessed over total lung population.



S3 Serpine E1 is 223 fold higher in H23 Kinase Dead versus LKB1 full length



S4 LKB1 knockdown in CMT CRISPR LKB1

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