EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

A DISSERTATION PRESENTED TO THE NOVA MEDICAL SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (AREA OF GENETICS, ONCOLOGY, AND HUMAN TOXICOLOGY)

TESE PARA OBTENÇÃO DO GRAU DE DOUTOR EM MEDICINA, NA ESPECIALIDADE EM BIOMEDICINA (ÁREA DE GENÉTICA, ONCOLOGIA E TOXICOLOGIA HUMANA), NA FACULDADE DE CIÊNCIAS MÉDICAS

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SEPTEMBER 2016

NOVA MEDICAL SCHOOL FACULDADE DE CIÊNCIAS MÉDICAS | UNIVERSIDADE NOVA DE LISBOA

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Dissertation supervised by David Lyden MD, PhD,
And co-supervised by Jacqueline Bromberg, MD, PhD, and Jose Rueff, MD, PhD

A Dissertation Presented to the NOVA Medical School
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Area of Genetics, Oncology, and Human Toxicology)

Tese para obtenção do grau de Doutor em Medicina, na Especialidade em Biomedicina (Área de Genética, Oncologia e Toxicologia Humana), na Faculdade de Ciência Médicas

To my mother, the reason of it all.

BIOGRAPHICAL SKETCH

MARIA DO ROSÁRIO ANDRÉ

Maria do Rosário Gomes André was born in April 7, 1981 in Marinha Grande, Portugal. Rosário grew up in a small village with her parents, her two older sisters and two older brothers, who all had a hand in shaping her interests and her desire to know the unknown. Rosário's elementary and secondary education took place in Leiria, where she attended the Colégio Nossa Senhora do Rosário de Fátima and the Escola Secundária de Domingos Sequeira. After concluding high school in 1999, Rosário moved to Lisbon to attend the NOVA Medical School of NOVA University of Lisbon for her Medical Degree. During this time, Rosário demonstrated a special interest towards science, and joined the Genetics Department during her second year. Rosário concluded her Medical Degree in 2005, and started her Medical Oncology Residency in 2007 at Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisbon, Portugal. She was determined to do a Research Fellowship in Basic Science, and in 2009 she joined Dr. David Lyden's laboratory at Weill Cornell Medical College in New York, NY, USA, initially for a 12-months period. During this time, Rosário decided to enter a PhD program. She enrolled at the NOVA Medical School of NOVA University of Lisbon in October 2009, and decided to continue her doctoral work in Dr. David Lyden's laboratory. She returned to Portugal in the end of 2010 to proceed her Medical Oncology Residency alongside with her PhD. She completed her Medical Oncology Residency in the spring of 2013, and joined Fundação Champalimaud, Lisbon, Portugal, in August 2013. In November 2014 she joined Novartis as Medical Advisor for breast cancer and melanoma and recently, she accepted a new challenge and moved to Italy as the new Region Europe Medical Affairs Medical Director for breast cancer. After graduating, Rosario plans on continuing developing her research interests in breast cancer and the biological processes underlying metastatic development, contributing as much as possible to a better understanding of cancer and a better treatment of cancer patients.

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ABBREVIATIONS

EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

ABC	Advanced Breast Cancer
APC	Antigen-Presenting Cell
ASCO	American Society of Clinical Oncology
BC	Breast Cancer
Bm	Basement membrane
BM	Bone-marrow
BMDC	Bone marrow-derived cell
BRCA	BReast CAncer gene
BSA	Bovine Serum Albumine
CA	Cancer Antigen
CAF	Cancer Associated Fibroblast
CEA	Cancer Embryonic Antigen
CM	Cyclophosphamide and Methotrexate
CNS	Central Nervous System
CT	Computed Tomography
CTC	Circulating Tumor Cell
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition
EORTC	European Organization for Research and Treatment of Cancer
EPC	Endothelial Progenitor Cell
ER	Estrogen Receptor
ESMO	European Society for Medical Oncology
ET	Endocrine Therapy
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDG	Fluorodeoxyglucose
FGFR	Fibroblast Growth Factor Receptor
FN	Fibronectin
GFP	Green Fluorescent Protein
HER-2	Human Epidermal growth factor Receptor 2
HGFR	Hepatocyte Growth Factor Receptor
HR	Hormone Receptor
IACUC	Institutional Animal Care and Use Committee
ID1	Inhibitor of Differentiation 1
IGFR	Insulin-like Growth Factor Receptor

IL	Interleukin
INF	Interferon
Irf8	Interferon regulatory factor 8
LHRH	Luteinizing Hormone-Releasing Hormone
LLC	Lewis Lung Carcinoma
Luc	Luciferase
M-CSF	Macrophage Colony Forming Factor
MDSC	Myeloid Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MMP	Metalloproteinases
MRI	Magnetic Resonance Imaging
NCCN	National Comprehensive Cancer Network
NO	Nitric Oxid
OS	Overall Survival
PBS	Phosphate-Buffered Saline
pCR	Pathologic Complete Response
PDGFR	Platelet-Derived Growth Factor Receptor
PET	Positron Emission Tomography
PFS	Progression Free Survival
PIGF	Placental Growth Factor
PR	Progesterone Receptor
PyMT	Polyomavirus Middle T antigen
qPCR	quantitative Polymerase Chain Reaction
RARC	Research Animal Resource Center
Rb	Retinoblastoma
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
STAT	Signal Transducers and Activators of Transcription
TAM	Tumor Associated Macropahges
TCM	Tumor Conditioned Media
TDM-1	Trastuzumab-Emtansin
TGF-β	Transforming Growth Factor-β
TNBC	Triple Negative Breast Cancer
TNF-α	Tumor Necrosis Factor-α
Treg	Regulatory T-cell
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor Receptor 1
WT	Wild-Type

SUMMARY

EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

Traditionally, cancer research has had its centre of attention in tumor cells and alterations in their genes, but recently, there has been a shift in focus beyond the tumor cells themselves to the surrounding tumor microenvironment. Specifically, it has become clear that bone-marrow derived cells (BMDCs) play a critical role in metastases development.

We demonstrate that IL-6 knockout mice bearing breast or melanoma tumors had a reduction in the number of metastatic foci and metastatic burden as compared to wild-type mice. Analysis of pre-metastatic lungs and blood showed an IL-6 dependent increase in Stat3 activation with CD11b+Gr1+ MDSCs mobilization and recruitment to these sites during metastatic progression. Inducible-ubiquitous overexpression of activated Stat3 increased hematopoietic progenitor cells (Sca1+c-Kit+) and MDSCs in the bone marrow and promoted their mobilization to the lungs, which was abrogated in IL-6 deficient mice. A requirement for bone marrow derived IL-6 for metastasis was determined, as restoration of metastatic growth was observed in IL-6 knockout mice transplanted with wild-type bone marrow.

We also demonstrate that in response to IL-6 and TGF-β, upregulation of the Inhibitor of Differentiation 1 (Id1) redirects BMDC differentiation towards Id1-high expressing MDSC with a reciprocal decrease in DC numbers. Genetic inactivation of Id1 largely corrects the myeloid imbalance, whereas Id1 overexpression in the absence of tumor-derived factors re-creates it. These results reveal a critical role for Id1 in suppressing the anti-tumor immune response during tumor progression.

We also explored the role of VEGFR1 expression in BMDCs, and our results clearly demonstrate an important function for VEGFR1-regulated CXCL4 expression by BMDCs in regulating angiogenesis at the primary tumor and metastatic microenvironments.

Taken together, our results reinforce the concept of the BMDCs and bone marrow microenvironment as necessary participants in solid-tumor metastasis development.

SUMÁRIO

EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

Historicamente, a investigação em cancro centrou-se nas células tumorais e nas alterações genéticas destas células, mas recentemente, tem ocorrido uma mudança de foco para o microambiente tumoral. Especificamente, vários estudos têm demonstrado um papel crítico das células derivadas de medula óssea (BMDCs) no processo de tumorigénese e metastização.

Os resultados do nosso trabalho demonstram redução significativa no número de lesões metastáticas pulmonares em ratinhos IL-6 KO com tumores da mama ou melanoma comparativamente com ratinhos wild-type. A análise de sangue e tecido pulmonar em fase pré-metastática mostrou ativação Stat3 dependente de IL-6, com mobilização e recrutamento de MDSCs CD11b + Gr1 + para os futuros órgãos metastáticos. A indução de sobreexpressão de Stat3 resultou num aumento de células hematopoiéticas progenitoras (Sca1 + c-Kit +) e MDSCs na medula óssea, e promoveu a sua mobilização para os pulmões, o que não se verificou em ratinhos IL-6 KO. Nos ratinhos IL-6 KO transplantados com medula óssea wild -type houve recuperação do padrão metastático, o que demonstra o papel importante da IL-6 no processo de metastização.

O nosso trabalho também demonstrou que a sobreexpressão de Id1, em resposta a IL-6 ou TGF- β, redireciona a diferenciação de BMDCs no sentido de MDSC com elevada expressão de Id1, com uma diminuição recíproca no número de DCs. Demonstramos ainda que a inactivação genética de Id1 corrige o desequilíbrio mieloide, e que a sobreexpressao de Id1 na ausência de factores tumorais, recria este desequilíbrio. Estes resultados revelam um papel crítico de Id1 na supressão da resposta imune anti-tumoral durante o desenvolvimento tumoral.

O nosso trabalho explorou ainda o papel da expressão de VEGFR1 em BMDCs, e os nossos resultados demonstram que a expressão de CXCL4 dependente de VEGFR1 pelas BMDCs desempenha uma importante função na regulação da angiogênese do tumor primário e do microambiente metastatico.

Globalmente, os nossos resultados reforçam o conceito das BMDCs e do microambiente da medula óssea como participantes necessários no desenvolvimento de metástases de tumores sólidos.

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1. CHAPTER ONE
INTRODUCTION TO CANCER AND METASTATIC DISEASE
A CLINICAL OVERVIEW OF ADVANCED BREAST CANCER

EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

1. CHAPTER ONE
INTRODUCTION TO CANCER AND METASTATIC DISEASE
A CLINICAL OVERVIEW OF ADVANCED BREAST CANCER[1]

1.1 SUMMARY

Advanced breast cancer (ABC), also called metastatic or secondary breast cancer, is a treatable but still incurable condition, although important advances have been made in our understanding and management of this disease. The treatment of ABC is complex and dependent not only on tumor related factors, but also on patient characteristics. The increased knowledge of breast cancer biology and gain of new therapeutic options have changed the way we treat ABC patients nowadays.

In this chapter, epidemiology and general recommendations for the management of patients with advanced breast cancer will be reviewed.

1.2 INTRODUCTION

Breast cancer (BC) is the most prevalent malignancy in women and the second leading cause of cancer -related death in developed countries. Worldwide, the estimated incidence of 1.7 million cases and 522,000 deaths per year correspond to 11.9% of total new cases and 6.4% of total cancer deaths, respectively (Ferlay et al., 2015). In 2015, about 231,840 new cases of invasive BC will be diagnosed in women in

[1] Based on: Rosario Andre, Simona Ruxandra Volovat, Fatima Cardoso. Chapter: Treatment of advanced disease - guidelines. Textbook: Breast Cancer – Innovation in research and management (Submitted for publication)

the United States and 40,290 women will die from this disease (Siegel et al., 2015). In Europe, there were an estimated 464,000 new BC cases and 131,000 BC-related deaths in 2012 (Ferlay et al., 2013), and in Portugal about 6,088 new BC cases were diagnosed and around 1,570 women died from this disease in the same year.

The majority of breast cancer-related deaths are associated with metastatic disease. Over the past decades, we have observed a stage migration, with a greater proportion of patients being diagnosed with early BC. Nonetheless, 6–10% of women diagnosed with BC present with metastatic disease ab initio in developed countries. The percentage reaches 50-60% in developing countries (El Saghir et al., 2011). Additionally, 20–50% of patients first diagnosed with early BC will eventually develop metastatic disease (Lu et al., 2009). In Portugal, 6% of patients present metastatic disease at initial diagnosis, while 32% present with Stage I disease, 36% with Stage II and 17% with Stage III (Andre et al., 2014). Nomenclature wise the words advanced, metastatic and secondary are all used to define stage IV (cancer that has spread to other organs or parts of the body) BC. For consistency we will mostly use ABC throughout this chapter.

Significant advances in the treatment of patients with ABC have been observed in the last 30 years. More therapeutic options are available nowadays, contributing to an improvement in the overall outcome of this disease and in the quality of life of patients with ABC. Nevertheless, very few of these treatments have provided a survival benefit, and the prognosis remains poor with a median overall survival (OS) of only 2 to 3 years (Wilcken et al., 2008; Giordano et al., 2003). In a population-based retrospective study of female BC cases from the Southern Portugal Cancer Registry, which included 2821 cases, we observed a 5-year OS of 20% for patients presenting with stage IV disease (Andre et al., 2014) (Figure 1.1).

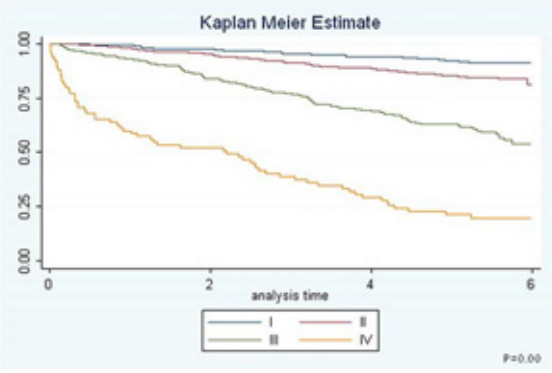

Figure 1.1. BC survival by stage, Kaplan-Meier. There is an association between survival and staging (p=0.00), with a 5-year survival of 92% for patients with stage I disease, 86% for stage II, 60% for stage III and 20% for stage IV.

ABC continues to be generally incurable, but it is a treatable disease. The main treatment goals in this setting are to control symptoms and to extend survival, without compromising quality of life. These treatment goals should be discussed with the patient, in an accessible and comprehensive way, always respecting individual characteristics, beliefs and cultural differences.

BC is a heterogeneous disease and various molecular alterations, activation/inhibition of different cellular pathways separates it into different subtypes, characterized by different clinical behavior and response to various treatments options. As more information regarding the biology of BC has emerged, various tailored treatments are currently available according to the specific BC subtype, resulting in improved outcomes, specifically in the Human Epidermal growth factor Receptor 2 (HER-2) positive ABC and, more recently, in luminal ABC.

According to the German Tumor Registry Breast Cancer study (Marschner et al., 2013) that included 1409 ABC patients, the proportional distribution of the BC subtypes in the advanced setting does not differ much from the distribution in the early setting. These researchers have found that 58% of cases were hormone-receptor (HR)-positive/HER-2 negative, 19% were triple positive (HR-positive/HER-2 positive), 13% had triple negative disease (HR-negative/HER-2-negative) and 10% of the patients had HR-negative/HER-2 positive BC. This study also confirmed that ABC patients usually receive a substantial number of lines of therapy, with 60% of HR-positive population and 40% of the HR-negative receiving at least three lines of treatment.

The management of ABC not only depends on various tumor related factors, but also on patient characteristics and previous drug exposure. As a general idea, patient's age should not be the only factor when deciding to withhold treatment (in elderly patients), or to over-treat (in young patients) (Cardoso et al., 2014; Paluch-Shimon et al., 2016; Cardoso et al., 2012). Taking into account the complexity of the disease and also the management, ABC patients should be treated in specialized units, where all appropriate specialties (including but, not restricted to, medical, radiation, surgical oncologists, imaging experts, pathologists, nurses, psycho-oncologists) forming a multidisciplinary team could be involved. Although routinely applied in the early BC setting, a multidisciplinary expert approach is not always offered to ABC patients. The development of the international ABC consensus guidelines has reinforced this need, and contributed to the development of international standards and to the improvement in ABC care.

There are few proven standards of care with high level of evidence in ABC management. Clinical trials addressing important unanswered clinical questions in this setting are urgently needed. Whenever clinical trials are available, patients should be offered inclusion in well-designed, prospective, independent trials (Cardoso et al., 2014).

1.3. **DIAGNOSIS AND STAGING RECOMMENDATIONS**

The staging evaluation of women who present with metastatic or recurrent BC should always include a history and physical examination, complete hematology and biochemistry tests including liver function tests, renal function, electrolytes, calcium, total proteins and albumin, and imaging of the chest, abdomen and bone (Lin et al., 2013). Although patients with HER-2 positive or triple negative ABC have a higher probability of central nervous system (CNS) metastatic disease, current recommendations do not support routine brain imaging in asymptomatic patients (Cardoso et al., 2012). Positron emission tomography scan (PET-scan) should not be routinely part of the staging workup but should be used selectively, namely following equivocal findings on conventional imaging techniques when a relapse is suspected (Pennant et al., 2010), or to confirm the diagnosis of oligo-metastatic disease. The usefulness of serum tumor markers in BC is not well established for diagnosis or follow-up after adjuvant therapy. However, if initially elevated, tumor markers such as Cancer Antigen (CA) 15-3, Cancer Embryonic Antigen (CEA) or CA-27.29 may aid in evaluating response to therapy, particularly in patients with non-measurable metastatic disease (Lin et al., 2013). Tumor markers should not be used alone for treatment change decisions, in particular in the beginning of treatment. An early rise in tumor marker levels during the first 4 to 6 weeks of a new therapy can be a result of a tumor flare (Harris et al., 2007).

Biopsy of metastatic disease at presentation or at first recurrence of disease should be performed, if easily accessible, in order to confirm the diagnosis of metastatic disease and to test for HR [estrogen receptor (ER) and progesterone receptor (PR)] and HER-2 expression (Lin et al., 2013; Gradishar et al., 2015). Depending on the metastatic site (e.g. bone tissue), technical considerations need to be discussed with the pathologist. Adequate characterization of the BC phenotype will allow better definition and selection of treatment strategy. Several reports have demonstrated discordance between ER/PR and HER-2 status of primary tumor and corresponding metastases (Bogina et al., 2011; Pusztai et al., 2010; Sari et al.,

2010). The reasons for this discordance may be related to clonal selection during tumor progression, intra-tumoral heterogeneity, selective pressure from therapy, independent evolution of a clone in both sites, or false shifts related to evaluation including tissue processing and scoring interpretation (Bogina et al., 2011). If the results of tumor biology in the metastatic lesion differ from the primary tumor, it is currently unknown which result should be used for treatment-decision making. Following ABC recommendations, targeted therapy [endocrine therapy (ET) and/or anti-HER-2 therapy] should be considered when receptors are positive in at least one biopsy, regardless of timing (Lin et al., 2013).

1.4 **TREATMENT RECOMMENDATIONS**

BC is a heterogeneous disease with diverse clinicopathological features, deregulation of distinct signaling pathways, and different drug sensitivity. Selecting therapies in ABC must therefore take into account both the biology as well as disease extent and patient characteristics. Many factors must be considered for tailoring the decision in this setting, always giving priority to the patient's preferences; these factors are described in the table below (Table 1.1).

Disease-related factors	HR status HER-2 status BRCA 1/2 mutation Disease-Free interval Tumor burden (defined as number and site of metastasis) Need for a rapid disease/symptom control
Patient-related factors	Patient's choice Biological age Performance status Co-morbidities Liver, renal, heart function Menopausal status Socio-economic and psychological factors
Treatment-related factors	Previous therapies received Response to previous therapies Toxicity of previous therapies Availability of therapies

Table 1.1. Factors to consider in treatment-decision making for ABC patients (Adapted from Cardoso et al., 2014)

A. HR-POSITIVE/ HER-2 NEGATIVE ABC

HR-positive BC is the most common subtype among ABC patients, representing approximately 75% of cases (Rugo et al., 2007). In these patients, ET is an effective treatment (Wilcken et al., 2003) and should be used as first option, even in the presence of visceral metastasis, unless there is immediate life-threatening disease or visceral crisis, in which case chemotherapy should be considered (Cardoso et al., 2013). According to the ABC2 Consensus Guidelines, "visceral crisis" is defined as "severe organ dysfunction as assessed by signs and symptoms, laboratory studies and rapid progression of disease, implying important visceral compromise, not only the presence of visceral disease" (Cardoso et al., 2014). The use of ET in HR-positive ABC is supported by its mechanism of action, low toxicity profile, and lower response of this type of tumors to chemotherapy (Rugo et al., 2007).

All international guidelines (Cardoso et al., 2014; Partridge et al., 2014; Gradishar et al., 2016) recommend ovarian suppression/ablation combined with additional ET as the first choice for premenopausal women. The additional endocrine agent can be an aromatase inhibitor or tamoxifen, according to the type and duration of prior adjuvant ET. Fulvestrant is also a valuable option, but fewer data exists regarding its use in premenopausal patients and for the moment it requires concomitant ovarian suppression/ablation (Cardoso et al., 2012; Cardoso et al., 2014).

For postmenopausal women, the preferred first line ET depends on the type and duration of adjuvant ET, as well as the time elapsed from the end of adjuvant ET. An aromatase inhibitor, tamoxifen or fulvestrant are all acceptable options (Cardoso et al., 2012; Cardoso et al., 2014; Gradishar et al., 2016).

The addition of everolimus to an aromatase inhibitor is an option for some postmenopausal patients with disease progression after a non-steroidal aromatase inhibitor, since it significantly prolongs progression free survival (PFS) (Cardoso et al., 2012; Beaver et al., 2012). Due to its toxicity profile and lack of OS benefit, the decision must be taken on a case-by-case basis, after careful discussion with the patient and with adequate education on preventive measures for the most common toxicities.

In the phase 2 trial PALOMA-1/TRIO-18 (Finn et al., 2015), the addition of palbociclib (a CDK4/6 inhibitor) to an aromatase inhibitor yielded a substantial PFS benefit, as first line therapy for postmenopausal women. The results from the phase 3 PALOMA-2 trial presented at the 2016 ASCO Annual Meeting con-

firmed these previous results, demonstrating a PFS improvement by > 10 months with the addition of palbociclib to letrozole (Finn et al., 2016).

The addition of palbociclib to fulvestrant, mostly in 2nd line therapy for post, peri and premenopausal patients, has provided a PFS benefit in the PALOMA 3 trial (Cristofanilli et al., 2016). Due to its favorable toxicity profile and improvement of quality of life, despite not yet providing OS benefit, it can be considered as a treatment option in this setting. For pre/peri-menopausal patients, the addition of a luteinizing hormone-releasing hormone (LHRH)-agonist is needed.

Concomitant chemotherapy and ET has not shown a survival benefit and should not be performed outside a clinical trial (Cardoso et al., 2014).

B. TRIPLE NEGATIVE ABC

Triple negative breast cancer (TNBC) identifies invasive BCs that lack the expression of ER, PR and HER-2, and accounts for 15% of all BC (Chacón et al., 2010). Generally, patients with metastatic TNBC have a poorer prognosis compared with women with other ABC subtypes, with a median survival of only 13 months (Kassam et al., 2009; Foulkes et al., 2010). There is no specific systemic regimen for sporadic (non-BRCA associated) TNBC, and little data to support treatment selection (Cleator et al., 2007; Gluz et al., 2009). Platinum agents, including carboplatin and cisplatin, may be of special interest in cells that are deficient in homologous recombination repair mechanisms such as BRCA-mutated cells. Evidence from preclinical and some clinical studies seem to confirm the efficacy of this strategy (Leong et al., 2007; Rocca et al., 2007; Silver et al., 2010). In particular, recent phase II randomized trials demonstrated improved pathologic complete response (pCR) rates in patients treated with neoadjuvant treatment that included a platinum compound (Sikov et al., 2013; von Minckwitz et al., 2014). In metastatic TNBC patients previously treated with anthracyclines with or without taxanes in the (neo)adjuvant setting, carboplatin demonstrated comparable efficacy and a more favorable toxicity profile, compared to docetaxel, in the TNT UK trial, and is therefore considered an important treatment option (Koshy et al., 2010; Cardoso et al., 2014; Tutt et al., 2015).

C. HER-2-POSITIVE ABC

The amplification of HER-2 occurs in approximately 20% of all BC and was associated with a more aggressive disease phenotype, with a poorer prognosis and shorter overall survival (Slamon et al., 1987). The development and approval of trastuzumab, the first HER-2-targeted therapy, has changed significantly the natural history of HER-2 positive ABC patients. Since then, several other HER-2-targeted therapies have been approved for the treatment of this BC subtype. In fact, HER-2 positive ABC is probably the subtype for which highest level of evidence exists for the largest number of management strategies. Level 1 evidence supports the recommendations for early (as 1st line) administration of anti-HER-2 therapy to all patients with HER-2 positive ABC, except in the presence of contra-indications, and for continuing anti -HER-2 therapy with subsequent treatment in patients progressing on an anti-HER-2 agent combined with chemotherapy or ET (Cardoso et al., 2012; Cardoso et al., 2014; Gradishar et al., 2015).

For patients with ER-positive/HER-2-positive ABC for whom ET was chosen over chemotherapy, the use of anti-HER-2 therapy (either trastuzumab or lapatinib) in combination with ET for highly selected patients can be considered (Cardoso et al., 2016). Moreover, if chemotherapy plus anti-HER-2 therapy was chosen as 1st line therapy and provided a benefit, it is reasonable to use ET plus anti-HER-2 therapy as maintenance therapy, after stopping chemotherapy, although this strategy has not been studied in clinical trials (Cardoso et al., 2016).

It has become a not so uncommon situation to have ABC patients with HER-2 positive tumors in complete remission for long periods of time. An important clinical question is when to stop the anti-HER-2 therapy in these cases. The ABC consensus (Cardoso et al., 2014) suggests that stopping the anti-HER-2 therapy, after several years of sustained complete remission, may be considered in some patients, particularly if treatment re-challenge is available in case of progression.

The current preferred first-line therapy, for patients previously untreated with anti-HER2 therapy, is the triplet trastuzumab + pertuzumab + chemotherapy, which has been shown to improve PFS and OS in the CLEOPATRA trial (Swain et al., 2013). For patients previously treated in the (neo)adjuvant setting with anti -HER-2 therapy, the combination of chemotherapy + trastuzumab + pertuzumab is an important option for first line therapy, but not the only standard of care since very few (88) of these patients were treated in the CLEOPATRA trial and all with trastuzumab-free interval > 12 months. In addition, in the MARIANNE

trial (Ellis et al., 2011), dual-blockade with trastuzumab-emtansin (T-DM1) and pertuzumab was not superior to T-DM1 alone nor to trastuzumab plus chemotherapy (taxanes) in the first line setting.

For patients who relapse either on or within 12 months of adjuvant trastuzumab there are currently no data regarding the best treatment strategy, since these patients were excluded from the CLEOPATRA (Swain et al., 2013) and MARIANNE (Ellis et al., 2011) trials. This is therefore a research priority, in view also of their bad prognosis.

Results from the EMILIA (Verma et al., 2012) and TH3RESA (Wildiers et al., 2013) trials support the use of T-DM1 as the standard of care for patients with disease progression after treatment with at least one line of trastuzumab-based therapy. However, there are no data on the use of T-DM1 after dual blockade with trastuzumab and pertuzumab. In case of progression on trastuzumab-based therapy, the combination trastuzumab and lapatinib is a reasonable treatment option for some patients.

In view of these new compounds recently approved in HER-2-positive ABC, optimization of sequencing and combining strategies and better predictive markers of response are of paramount importance.

D. CYTOTOXIC THERAPY

Classic chemotherapy still plays an important role in the treatment of ABC. Unlike the adjuvant setting, in which the goal of therapy is cure, the aim of therapy in the setting of ABC is essentially palliation. Therefore, besides efficacy, tolerability and quality of life are major factors that need to be taken into account when evaluating potential gains in disease response and survival.

In recent years, the patterns of use of chemotherapy in ABC patients have changed, and in the majority of patients sequential single-agent therapies are preferred over aggressive multidrug regimens. Several randomized trials as well as a Cochrane meta-analysis provide level I evidence for the recommendation to preferably use sequential monotherapy, since the overall efficacy is similar to combinations and the toxicity and quality of life are better (Dear et al., 2013; Cardoso et al., 2009; Tomova et al., 2009; Soto et al., 2006; Sledge et al., 2003; Sjostrom et al., 1999; Koroleva et al., 1999; Conte et al., 2004; Beslija et al.,

2006; Alba et al., 2004). Therefore, all international guidelines recommend that sequential single-agent therapy should be the preferred choice for most ABC patients, except in cases of rapid progression, visceral crisis, or highly symptomatic disease (Cardoso et al., 2014; Gradishar et al., 2015). This strategy will allow, in patients not requiring rapid tumor shrinkage, significantly lower toxicity without compromising efficacy and disease control.

Metronomic chemotherapy is also a good treatment option for patients not requiring rapid tumor response, and has a very favorable toxicity profile. The better-studied regimen is CM (low dose oral cyclophosphamide and methotrexate), but others, such as capecitabine and oral vinorelbine are being evaluated (Montagna et al., 2014; Munzone et al., 2015).

E. SPECIFIC SITES OF METASTASIS

In recent years, the role of local treatment of metastatic lesions in patients with ABC has been growing. Besides surgery, alternative modalities such as stereotactic radiotherapy or tumor embolization with isotope-loaded microspheres may be considered for the local treatment of metastatic lesions.

According to some retrospective data, oligo-metastatic disease in the liver or lung can be treated with "curative-intent" surgery, providing long-term complete remissions (Pockaj et al., 2010). However, the reported outcomes were in a highly selected patient population and, although encouraging, this approach can only be considered in selected cases with good performance status, limited liver/lung involvement, no extra-hepatic or extra-pulmonary lesions, and after adequate disease control by systemic treatment (Cardoso et al., 2014). Further prospective studies evaluating the impact of local treatment on survival are needed. Moreover, a multi-disciplinary team involving medical oncologists, surgeons, radiation oncologists and radiologists is crucial to define the best therapeutic strategy for each individual patient.

In patients with bone metastasis, further radiological assessments that could indicate signs of pathological fractures are recommended in case of persistent and localized pain. In case of fracture of a long bone, an orthopedic evaluation is required in order to establish the indication for surgery, which is generally followed by radiotherapy. In the absence of a clear fracture risk, radiotherapy is recommended (Cardoso

et al., 2014). In cases of neurological symptoms and signs suggestive of spinal cord compression, further investigations should be urgently recommended in order to identify one or multiple concomitant lesions. Due to increased sensitivity, magnetic resonance imaging (MRI) is preferred over computed tomography (CT) scans. If immediate decompressive surgery is not optional, emergency radiation therapy should be performed. The early use of a bone-modifying agent (bisphosphonate or denosumab) in combination with other systemic therapy is supported by different international recommendations (Cardoso et al., 2012; Wong et al., 2012; Van Poznak et al., 2011). In cases of oligo-metastatic disease, with an isolated bone lesion, it is not clear to date which is the optimal regimen and duration of the bone modifying treatment, but there is no strong reason to stop after 2 years as it was initially recommended. Radiotherapy should be offered to patients with painful bone metastasis and for the management of spinal cord compression (George et al., 2015). In cases of isolated bone lesions, stereotactic body radiotherapy or vertebroplasty should be considered, with the goal of delaying morbidity associated with the lesion and maintaining/improving quality of life.

Brain metastases are relatively frequent in patients with HER-2 positive and triple negative ABC. In HER-2-positive ABC, brain involvement occurs later and the outcome is dependent on the response to anti -HER-2 therapy and control of extracranial disease. In patients with triple-negative ABC, brain metastases appear earlier in the course of the disease and are associated with a poorer outcome. In recent years, the role of local management has increased for brain metastases. Neurosurgery development has been associated with a decrease in perioperative mortality and the introduction of non-invasive techniques such as stereotactic radiosurgery has allowed for the use of less toxic approaches in selected patients. In patients with a single or a small number of brain lesions, surgery or radiosurgery should be used (Patchell et al., 1990). If surgery or radiosurgery is performed, it may be followed by whole brain radiotherapy but this should be discussed in detail with the patient, balancing the longer duration of intracranial disease control and the risk of neurocognitive effects (Cardoso et al., 2014). In fact, whole brain radiotherapy is now delayed as much as possible, especially in HER-2 positive ABC since these patients can now live several years. For other cases, where these less toxic options are not feasible, whole brain radiotherapy is the treatment of choice (Cardoso et al., 2012).

F. LOCO-REGIONAL TREATMENT OF THE PRIMARY TUMOR IN DE NOVO STAGE IV PATIENTS

Several retrospective series and a meta-analysis of these retrospective data have suggested a survival benefit associated with the removal of the primary tumor in patients with de novo stage IV breast cancer. To achieve that survival benefit, the surgery must be performed with the same quality as in early breast cancer, i.e., complete removal of the tumor and management of the axilla. There is no evidence to perform the so-called "palliative mastectomy" except in cases of need to control local symptoms such as bleeding or ulceration and where the surgical approach could improve the quality of life. Even in those cases, palliative radiotherapy must also be discussed as an alternative option.

More recently, two small but prospective randomized studies were presented (Soran et al., 2013; Badwe et al., 2015) and did not confirm the survival benefit. These were small studies, with different timing and patient selection for surgery, and do not yet provide a definite answer to this important question.

Further clinical trials evaluating this approach concerning the timing, patient population and methods are currently ongoing.

1.5. FOLLOW-UP OF PATIENTS WITH ADVANCED BREAST CANCER

In order to effectively manage patients with metastatic disease, serial evaluation is a key component of the care that clinical oncologists must provide. There is currently a lack of evidence from prospective randomized clinical trials comparing surveillance strategies in patients with ABC. Thus, information must be drawn from current available guidelines (ABC, ASCO, ESMO, NCCN) and from common clinical practice.

The goals for evaluation and follow-up of patients with ABC are to manage symptoms associated with the disease and treatment and to direct therapy, in an effort to maximize both length and quality of life (Cardoso et al., 2012). Therefore, assessment should include review of toxicities, symptoms and quality of life evaluation, physical examination, imaging and blood tests. Strong consideration should be given to the use of validated patient-reported outcome measures for patients to record the symptoms of disease

and side effects of treatment experienced as a regular part of clinical care. These outcome measures should be simple, and user-friendly to facilitate their use in clinical practice. Systematic monitoring would facilitate communication between patients and their treatment teams by better characterizing the toxicities of all anticancer therapies. This would permit early intervention of supportive care services enhancing quality of life. As an important prognostic factor, performance status should be assessed at each visit, as it may have a major impact on treatment decisions and overall goals of care. In cases where patients are being treated with oral regimens, adherence to treatment should also be assessed. Initial radiological evaluation of response to treatment should be performed 2 to 4 months after beginning each line of treatment for endocrine treatment or after 2 to 4 cycles for chemotherapy, depending on the dynamics of the disease and type of treatment (Cardoso et al., 2012). The timing and interval between subsequent evaluations should be adapted to the clinical situation and disease aggressiveness. Most commonly, imaging studies will include CT scans of the chest/abdomen and bone scans. Routine pelvis CT, although performed in some countries, has a very low yield, adds cost, and appears unnecessary in most cases. 18F-Fluorodeoxyglucose (FDG)-PET and PET-CT can define extent of disease and demonstrate alterations in tumor size and metabolic activity over time; however, robust data demonstrating cost-effectiveness relative to CT/bone scan-based approaches are lacking. In this context, PET-CT is not recommended for routine staging of ABC patients, but it can be used to confirm oligo-metastatic disease, and relapse or progression in case of doubt (Cardoso et al., 2014). In patients where cord compression is suspected, MRI is the modality of choice.

In patients with bone metastases, bone scans remain the mainstay of evaluation, since data from a meta-analysis has failed to demonstrate significant benefits of PET over bone scan in this context. Interpretation of bone scans must be cautious during the first months of treatment, since a possible “flare” may be observed.

If progression of disease is suspected, additional testing should be performed irrespective of the interval from the last set of staging evaluations.

Therapy for ABC should be continued as long as the therapeutic index remains positive. There is no evidence that changing to an alternative therapy (endocrine or chemotherapeutic) regimen before progression is beneficial.

Although currently not in clinical practice, detection of circulating tumor cells (CTCs) might be useful for the follow-up of ABC patients. Several studies have shown that the dynamics of CTCs after treatment initiation are a useful predictor of treatment efficacy in ABC, being associated with PFS (Liu et al., 2009; Hartkopf et al., 2011). In the SWOG 0500 study, patients with metastatic disease and elevated CTCs under systemic treatment are randomly assigned to either continue current therapy (until evidence of disease progression, evaluated by traditional evaluation) or to make an early switch to an alternative treatment (Cardoso et al., 2012). CTCs also reflect tumor biology. Their unique phenotypic characteristics and the possibility of collecting sequential blood samples may potentially allow for real-time monitoring of treatment efficacy and for better defining the adequate treatment strategy. For example, in the NCT01185509 trial trastuzumab-based chemotherapy will be offered to patients with HER-2-negative BC according to biopsy and with HER-2-positive CTCs. In the EORTC TREAT CTC trial, patients with HER-2 positive CTCs are also randomized to receive trastuzumab.

1.6. **CONCLUSION**

The management of ABC patients has changed significantly over the past decades. The appearance of new therapeutic options has had a positive impact on the outcome of this disease and on the quality of life of patients with ABC. Nevertheless, the prognosis of metastatic BC patients remains poor with a median OS of only 2 to 3 years.

Metastatic disease continues to be the main cause of breast cancer-related deaths and overall of all cancer-related deaths. In fact, cancers figure among the leading causes of morbidity and mortality, with approximately 8.2 million cancer-related deaths worldwide in 2012 (Ferlay et al., 2015). The three most common causes of cancer deaths are lung cancer, colorectal cancer and breast cancer (American Cancer Society, 2015), and transversely metastases are the main driver of this outcome. The goal of treatment in stage IV cancer patients is, as stated before for ABC, to prolong survival and maintain quality of life. Treatments that may be used for metastatic disease include chemotherapy, targeted therapies, immunotherapy, radiation therapy or surgery. The choice of treatment depends on several factors, including the primary tumor, symptoms, burden of metastatic disease, previous treatments, performance status and

patient's preferences. Although in the past years, several advances have been made regarding treatment options for metastatic disease, the outcome for these patients is still very poor with 5-year survival rates of 4,3% for stage IV lung cancer and 13,5% for stage IV colorectal cancer (Howlader et al., 2015).

The majority of significant advances achieved in cancer treatment have been based on our increased knowledge of the underlying biology of the cancer cell. Therefore, it seems logical that in order to develop better treatment options and improve care for metastatic patients, a deeper understanding of the biology of metastatic disease is crucial.

1.7. **REFERENCES**

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2. CHAPTER TWO
THE BIOLOGY OF METASTATIC DISEASE

EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

2. CHAPTER TWO
THE BIOLOGY OF METASTATIC DISEASE [2]

2.1 SUMMARY

Metastatic disease is the primary cause of cancer-related mortality, being responsible for more than 90% of cancer deaths (Weigelt et al., 2005). Although in recent years the world has testified significant advances in the diagnosis and treatment of cancer, the overall prognosis of a patient with metastases remains very low (Riihimaki et al., 2013; Steeg, 2016). Over the past decades, results of randomized clinical trials have failed to report benefits in survival of patients with metastatic breast, gastric or pancreatic cancers (Tevaarwerk et al., 2013; Bernards et al., 2013; Worni et el., 2013). Moreover, in breast cancer patients, five-year relative survival remains reduced at 24% for distant-stage disease while 99% for localized disease (Howlader et al., 2013). Improvements in cancer survival are needed and will only be tangible with a deeper knowledge and a better management of metastatic disease.

The process of metastasis is generally considered to follow a stochastic, sequential cascade that involves local invasion by the primary tumor cells, intravasation into the blood or lymphatic system, dissemination through blood and/or lymphatic vessels, extravasation into a secondary organ, angiogenesis, and, finally, secondary tumor growth (Massagué et al., 2016). In the last few years, studies have proposed new and interesting perspectives on the nature of metastatic disease, propelling a conceptual shift in the canonical metastatic theory. Areas of progress include not only the nature of the tumor cells initiating metastasis, how and when they migrate from the primary tumor mass, how they survive and proliferate at secondary sites but also the secondary sites they preferentially migrate to and why. In addition, recent studies

[2] Based on: Maria do Rosario Andre, Bruno Costa da Silva, Maria Rita Dionísio, David Lyden. Chapter: Metastasis; Tratado de Oncologia. 1st Edition. Editora Atheneu. 2013

have provided increasing evidence regarding not only the degree to which tumor cells are dependent on normal cells in the immediate microenvironment but also the importance of the immune system in disease progression.

In this chapter some of the most important findings of these and other topics in the context of metastatic development will be explored and the current state of metastasis research will be discussed.

2.2 TUMORS AS COMPLEX ORGANS

Cancer has been long viewed as a cell-autonomous process, in which gain of successive alterations in the cell's genetic material drives tumorigenesis (Foulds, 1954; Nowell, 1976). In fact, the field of cancer research has had its centre of attention in cancer cells and alterations in their genes, and many researchers have strived to understand and identify the molecular changes that transform a normal cell into a cancer cell. In 1960, the first chromosomal abnormality associated with cancer was identified with the detection of an abnormal minute chromosome in chronic myeloid leukaemia cells (Nowell and Hungerford, 1960). Nowell and Hungerford named this variation, the "Philadelphia chromosome". Since then, thousands of other chromosomal alterations such as deletions and duplications were identified in several malignancies, an indication that cancers originate from single cells that start proliferating and expanding due to these genetic modifications. Additionally, identification of gain-of-function mutations of proto-oncogenes and loss-of-function tumor suppressor genes during tumor progression have reinforced the concept that sequentially acquired genetic alterations are needed and are responsible not only for cancer development but also for the transitions between progressive tumor stages (Weinberg, 2007; Gupta et al., 2006).

In the past years, however, our understanding of cancer biology has changed and it is now well established that tumors are not simply clones of cancer cells. Clinical evidence that the microenvironment plays an important role in tumorigenesis came from the association between chronic inflammation and higher cancer incidence (Grivennikov et al., 2010). An example, is the association between hepatocellular carcinoma and liver cirrhosis (Sangiovanni et al., 2004)) or the increased risk of colorectal cancer in patients with inflammatory bowel disease and colitis (Beaugerie et al., 2013). In fact, nowadays tumors are seen

as complex organs, composed not only of genetically altered malignant cells, but also of many other cell types, namely endothelial cells, fibroblasts, immune cells, and bone marrow (BM)-derived stem and progenitor cells (Quail et al., 2013) (Figure 2.1). Furthermore, it has been proposed that the construction of a 'cancer niche' is an early and necessary step that is required for neoplastic cells to evolve towards a clinically relevant cancer (Barcellos-Hoff et al., 2013).

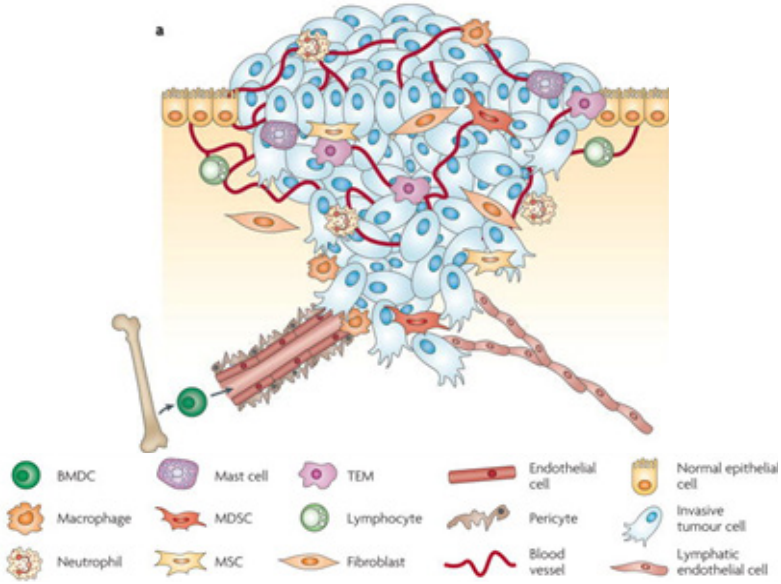

Figure 2.1. Tumors as complex organs. The tumor microenvironment is composed of a multitude of cell types, namely endothelial cells, immune cells (macrophages, neutrophils, lymphocytes, and myeloid-derived suppressor cells), BMDC and fibroblasts. This mixture of cells and ECM resembles an organized organ, although functionally abnormal. (Adapted from Joyce and Pollard, 2009)

More than innocent bystanders, these stromal cells provide a great advantage to the tumor. Through the production of chemokines, growth factors, and matrix-degrading enzymes, they support blood vessel formation, break down basement membrane barriers, and increase tumor cell dissemination (Wels et al., 2008). As an example, studies in breast cancer and glioma have demonstrated that the EGF-CSF1 paracrine loop between tumor cells and macrophages create a chemotactic relay system that facilitates tumor cell migration and invasion (Wyckoff et al, 2004; Coniglio et al., 2012). In addition, it has been demonstrated that exosomes secreted by cancer associated fibroblasts (CAFs) are taken by tumor cells, mobilizing autocrine Wnt-PCP signaling, leading to cell migration and increased invasiveness (Luga et al., 2012).

Stromal cells, through production of enzymes, can also remodel the extracellular matrix, contributing therefore in a crucial way to tumor cell invasion and dissemination (Gao et al., 2010). Furthermore, TGFβ signalling at the primary site has been shown to prime breast tumor cells specifically for lung metastasis seeding, through Smad signalling and angiopoietin-like 4 synthesis by cancer cells (Padua et al., 2008).

Taken together, these data suggest that the tumor-stroma crosstalk occurring at the primary site not only enhances growth of the primary tumor but also influences subsequent metastatic traits of cancer cells and overall metastatic disease.

2.3 THE MULTI-STEP PROCESS OF METASTASIS DEVELOPMENT

One of the first references to the spread of cancer was made in 1595 by Nicolas Abraham de la Framboisiere, who described that a tumor can develop "delitescence" that is, spreading to the internal organs. Later, in 1757, Henri Ledran reinforced this concept. He defined cancer in its beginning as a local disease that, in later stages, spreads to the local lymph nodes and into the blood-stream, where it may involve the lungs. In an attempt to describe the mechanisms involved in metastasis, Rudolf Ludwig Carl Virchow (1821-1902) did not describe metastasis as a disease generated by the dissemination of cancer cells. In direct contradiction to what might have been expected, he stated that metastasis is caused by infectious agents or poisons from primary cancers. These agents are transported through the blood or lymph to distant sites of the body where, following interaction with connective tissues, metastases are formed.

At present, it is believed that metastases are derived from cancer cells that have escaped from the primary tumor mass. This process, also known as the metastatic cascade (Figure 2.2), is generally considered to follow a stochastic, sequential cascade that involves changes in migration and cell-cell adhesion properties, degradation and invasiveness to the basement membrane and extracellular matrix, entrance (also known as intravasation) to and survival in the blood stream, dissemination through blood and/or lymphatic vessels, identification of a suitable organ in which to settle, and extravasation followed by the invasion of the metastatic organ (Valastyan et al., 2011; Kang and Pantel, 2013; Massagué and Obenauf, 2016).

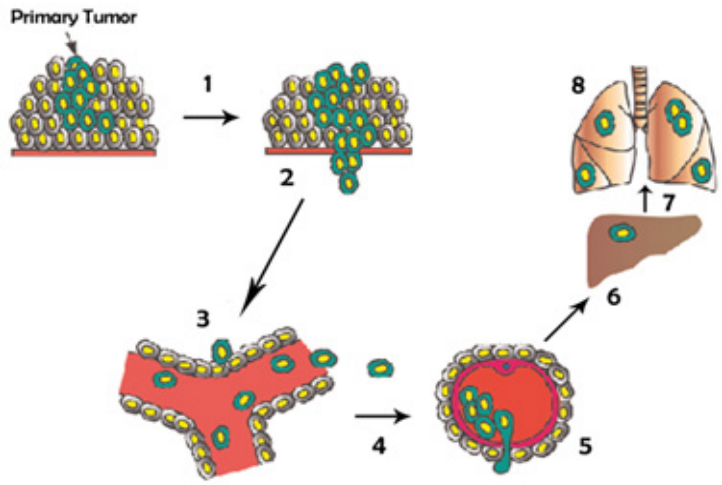

Figure 2.2. The metastatic cascade. The metastatic process is composed of a number of steps that a tumor cell must complete in order to reach its metastatic niche. These steps include: 1. Changes in migration and cell-cell adhesion (Epitelial-Mesenquimal Transition); 2. Degradation and invasiveness into the basement membrane; 3. Intravasation; 4. Survival in the blood stream; 5. Extravasation; 6. Micrometastasis formation; 7. Angiogenesis and 8. Secondary tumor growth (Adapted from Hunter et al., 2008)

CHANGES IN MIGRATION AND CELL-CELL ADHESION (EPITHELIAL-MESENCHYMAL TRANSITION)

Physiological tissue architecture prevents the migration required for tumor cell invasion and metastasis. As an example, in the mammary gland, myoepithelial cells oppose carcinoma cell invasion by contributing to maintain the basement membrane integrity. Experiments involving coimplantation with myoepithelial cells reversed the invasiveness of breast cancer xenografts (Hu et al., 2008). A critical component of the epithelial tissue organization is the E-cadherin-mediated intercellular junctions that prevent the dissociation of cells within epithelial cell sheets into individual cells. In order to overcome this obstacle, tumor cells may enter a cell program known as epithelial-mesenchymal transition (EMT). Originally described as one of the most important embryological mechanisms for tissue remodeling such as gastrulation and segment formation, the process of EMT has also been associated with cancer progression and metastasis (Thiery et al., 2009). This process consists of multiple steps: disintegration of cell–cell adhesion (with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as vimentin), loss of basoapical polarization and acquisition of front-rear polarization, and remodeling of the cytoskeleton with

changes in cortical actin and actin stress fibers. Closely resembling that observed in embryonic development, the process of EMT in cancer progression involves the activation of EMT-related signal pathways observed during development such as TGF-β and transcriptional repressors of E-cadherin as zinc finger proteins (ZEB1, ZEB2), bHLH protein (Twist), and the snail family of zinc finger proteins (Snail, Slug) (Thiery et al., 2009). Besides enhanced motility, cells induced to undergo EMT can exhibit resistance to apoptosis, another key requirement for successful metastasis. Nevertheless, recent studies in breast and pancreatic cancer models have suggested that EMT, although contributing to the aggressiveness of cancer cells by inducing chemoresistance, could be unessential for the establishment of metastasis (Fischer et al., 2015; Zheng et al., 2015). This suggests that the impact of EMT might be less pronounced than thought before.

REMODELING OF THE BASEMENT MEMBRANE AND EXTRACELLULAR MATRIX

Another important step in the formation of locally invasive cancers and its subsequent metastasis is the invasion and disruption of the basement membrane (Bm) and the barrier of extracellular matrix (ECM) that surrounds tumor cells. It may occur either by mechanical forces or by enzymatic degradation of the ECM, a process that happens when collagen, laminin, fibronectin (FN) and vitronectin, metalloproteinases (MMP), cysteine cathepsins and/or serine proteases are carried out by tumor cells and especially tumor-recruited host cells, e.g. macrophages, endothelial cells, and fibroblasts (Quail and Joyce, 2013). Additionally, the ECM that surrounds tumor cells functions as a repository for growth factors essential to malignant progression (Kessenbrock et al., 2010). The loss of balance between MMPs and their inhibitors is one of the main characteristics of invasive and metastatic tumors. This process, together with EMT, contributes to the migration of cancer cells from the tumor mass into the surrounding tissue stroma where they finally undergo dissemination by passing through the endothelial wall of vessels into lymphatic and systemic circulation.

INTRAVASATION, RESISTANCE TO APOPTOSIS, EXTRAVASATION, AND SECONDARY INVASION

Once tumor cells have invaded through the epithelial Bm and ECM and have migrated through the local stroma, they may come into contact with tumor-associated microvasculature. Although lymphatic disse-

mination of carcinoma cells is frequently observed and represents an important prognostic marker for disease recurrence, hematogenous dissemination is the main mechanism by which metastatic carcinoma cells diffuse (Gupta et al., 2006). Through integrin-mediated processes, tumor cells may interact and traverse (especially by proteolytic enzyme-mediated dissolution) endothelial cell Bm, adhere and pass among the vascular endothelial cells, and then enter into systemic circulation. Recent technology has allowed the detection of CTCs in the blood of cancer patients (Nagrath et al., 2007; Pantel et al., 2008, Stott et al., 2010). These CTCs represent tumor cells that are travelling between the primary tumor sites and sites of dissemination. During this journey, cancer cells must survive a variety of stress factors while in circulation. They must evade immune effectors (specifically natural killer cells), oxidative stress, and resist hydrostatic sheer forces (i.e., turbulence within vessels). Since tumor cells are more likely to be destroyed than cells travelling in clumps, it is believed that contacts with platelets, leukocytes, and vascular endothelium may shield and protect the tumor cells from immune and mechanical destruction during the metastatic process (Labelle et al., 2011; Joyce et al., 2009).

After all these obstacles are overcome, tumor cells may then adhere, stimulate cell reaction and pass through to the microvessel endothelium, degrading the underlying Bm through the secretion of proteases, and then establishing a secondary tumor in a new site. It is important to bear in mind, however, that not all cells within metastatic tumors are capable of metastasizing. In order to overcome homeostatic growth controls, immune response, and environmental restraints, characteristics such as genetic and phenotypic instability, coupled with a Darwinian type of selection – survival of the fittest – are crucial to the development of resistant tumor cells.

2.4 GENETIC DETERMINANTS OF METASTASIS

Regarding the metastatic competence of the malignant cell *per se*, and the molecular changes that confer acquired abilities that promote cell proliferation and survival, only recently the identity of certain genes that specifically mediate and induce metastases has been determined. The first evidence that metastases development was dependent on intrinsic characteristics of tumor cells came from a series of experiments where clonal murine melanoma cells were repeatedly injected intravenously into mice. Metastatic

colonies were harvested from their lungs, resulting in clones with different metastatic capability (Fidler and Kripke, 1977). This study also showed that the initial cell-line was heterogeneous with highly metastatic clones already present in this parental population. These results contributed to the theory that metastatic progression is a consequence of sequential somatic mutations producing variant cell populations, coupled with a selection of aggressive and highly-metastatic subpopulations of cells within the tumor – the "somatic progression model". Recently, gene-expression profiling of tumors has shown that molecular "signatures" predictive of metastasis are already present in primary tumor samples (Ramaswamy et al., 2003; Mittempergher et al., 2013). This appears to contradict the model mentioned above, which advocates that metastatic signatures should only be evident later in tumor progression, because time is critical for accumulation of somatic mutations and for the production of highly metastatic clones. However, a model that bases itself only in pre-determined genetic characteristics is also insufficient. It fails to provide not only an explanation of why dormant cells eventually give rise to full-blown metastasis but also the existence of genes that are expressed in metastatic cells but not in primary tumors. To overcome these gaps, an integrative model has been proposed postulating that metastatic capability is acquired as the primary tumor is growing and becoming locally invasive, whereas the growth of tumor cells in distant organs implies further selection from subsequent genetic heterogenic sub-populations (Weiss, 1990).

Genes whose altered activities participate in metastasis pathogenesis, the metastasis genes, can be grouped into three classes: initiation, progression, and virulence (Chiang and Massague, 2008; Table 2.1). The metastasis initiation genes confer an advantage in the primary tumor by facilitating tumor cells to invade the surrounding tissue, enter the circulation and to reach their metastatic sites. Genes that are involved in cell motility, invasion or angiogenesis are included in this class. TWIST1, SNAI1 and SLUG genes promote EMT and are therefore examples of this class of metastasis genes (Yang and Weinberg, 2008). The metastasis progression genes are defined as genes that are involved in functions in the primary tumor and are also fundamental in metastatic colonization (Nguyen et al., 2009). VEGF is a good representative of this group because it has angiogenic properties and can also promote the recruitment of VEGFR1-positive bone-marrow-derived cells to pre-metastatic niches, influencing the colonization of distant sites. The metastasis virulence genes are involved in metastatic colonization but not in primary tumor development. They accentuate the metastatic capability of cancer cells that have successfully achieved the stages of metastatic initiation and progression. This means that their altered expression becomes evident only in tumor cells at distant sites (Nguyen and Massague, 2007).

CLASSES OF METASTASIS GENES	FUNCTIONS	GENES
Metastasis Initiation Genes	Detachment Motility Invasion Bone-marrow progenitor cells recruitment Angiogenesis EMT transition	LOX CSF-1 ID1 TWIST1 MMP-9 NEDD9
Metastasis Progression Genes	Vascular remodelling Immune evasion Extravasation	VEGF EREG COX-2 MMP-1 ANGPTL4
Metastasis Virulence Genes	Extravasation Organ-specific colonization Emergence from dormancy	CXCR4 RANKL IL-11

Table 2.1. Classes of metastasis genes

The exact mechanisms that transform specific genes into mediators of metastasis are also becoming clarified. Any alteration that results in the activation of pro-metastatic genes or in the suppression of genes that interfere with metastasis can be involved, as long as it confers a selective advantage to the cancer cell. This means that chromosomal rearrangements, copy-number aberrations or mutations, as well as epigenetic changes, microRNAs or altered translational or post-translational mechanisms may be involved. At the moment, there are few clinically validated examples for most of these processes. NEDD9, a gene that encodes a protein that enhances focal contact formation and invasion, was found to be amplified in a mouse melanoma model and in metastatic human melanomas (Kim et al., 2006). CDH1 is a tumor suppressor gene that encodes a cell-adhesion receptor, E-cadherin. The loss of function of this receptor is characteristic of EMT, a phenotype that is fundamental for the invasive behavior of cancer cells (Thiery et al., 2002). Inactivating mutations have been described in breast and gastric cancer (Richards et al., 1999), although the main mechanism of E-cadherin loss is epigenetic silencing through DNA hypermethylation. Another example of epigenetic regulation of metastasis is hypomethylation of S100A4, a calcium-

binding protein that is involved in the regulation of cell cycle progression and differentiation and that also is implicated in metastasis. This epigenetic modification is associated with gene activation in a variety of cancers, like medulloblastoma (Lindsey et al., 2007 and pancreatic adenocarcinoma (Rosty et al., 2002). Also, several metastasis-promoting microRNAs have been described. miR-10b promotes cell migration and invasion in BC (Ma, 2010) and miR-373 and miR-520c modulate metastasis through the suppression of CD44 (Huang et al., 2008). The miR-200 family and miR-205 inhibit EMT, also modulating metastasis development (Gregory et al., 2008).

There is growing evidence that genetic alterations of the cancer cell alone cannot explain the complex metastatic process, and host genetic factors may also play a role. For breast and ovarian cancer, epidemiological studies have clearly shown the role of family history as an important risk factor for the development of these tumors. In 1988, it was shown for the first time that breast cancer segregated as an autosomal dominant trait in some families (Newman et al., 1988). Population-genetic studies have given evidence of the importance of inherited factors in the development of BC, and have led to the identification of several susceptibility genes, including BRCA1, BRCA2, TP53 and PTEN. Mutations in BRCA1 and BRCA2 appear to be responsible for disease in 45% of families with multiple cases of breast cancer and in 90% of families with both breast and ovarian cancer (Easton et al., 1993). Host genetic factors may influence and modulate not only cancer initiation, but also metastatic dissemination. The first evidence that supported this hypothesis came from a set of experiments using the transgenic mouse mammary tumor model induced by expression of polyomavirus middle T antigen (PyMT) oncogene (Lifsted et al., 1998). FVB/NJ-TgN(MMTV-PyMT)634Mul mice develop palpable tumors with a 100% penetrance, and 85-95% of the mice develop pulmonary metastasis at 100 days. When male FVB/NJ-TgN(MMTV-PyMT)634Mul mice were bred to females from various different inbred strains, varying the genetic background, metastatic progression was significantly modulated, with some of the animals developing less pulmonary metastasis, while others had a 2-3 fold increase in pulmonary metastasis (Figure 2.3). These results suggest that polymorphic loci present in the germline can modulate metastatic efficiency. Furthermore, it was demonstrated by subsequent studies that the expression of metastasis signature genes was different between normal mammary tissues derived from different PyMT strains, suggesting that this hereditary genetic variation that modifies and modulates metastasis is apparent even before the onset of disease (Yang et al., 2005). Translating these results into humans is very difficult because of the highly genetic heterogeneity in the human population. Nevertheless, there are already some promising results that show an association between Sipa1, identified in the PyMT model as a metastasis efficiency modifier gene, and metastasis in breast cancer patients (Park et al., 2005).

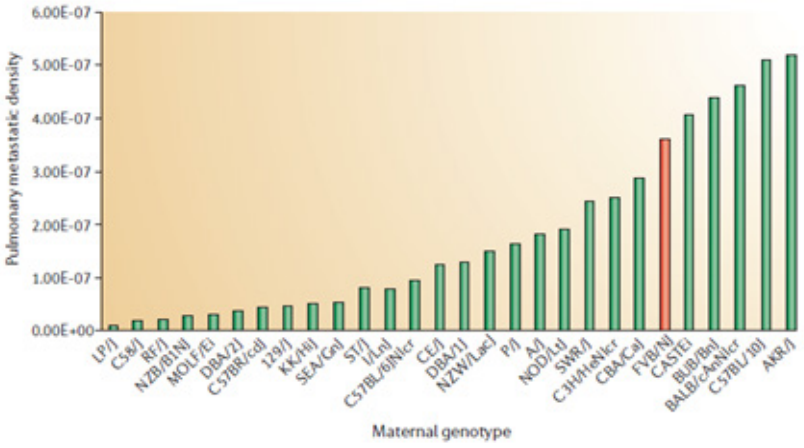

Figure 2.3. The effect of host genetic factors on metastatic development. Male PyMT expressing mice were bred to female from various strains (x axis) and pulmonary metastasis in the progeny were quantified (y axis). The red bar represents the metastatic quantification in the original FVB/NJ background. (Adapted from Hunter, 2006)

An important implication for host genetic factors is that they impact not only the primary tumor, but also all tissues in the body, including the future metastatic organs. The importance of the microenvironment in metastasis formation and development has been emphasized over the past several years, and it will be discussed further in this chapter.

2.5 MICROENVIRONMENTAL REGULATION OF METASTATIC PROGRESSION

In order for metastasis to occur, cancer cells must successfully detach from the primary tumor, intravasate into blood or lymphatic vessels, survive in circulation, spread to capillary vessels of distant organs, extravasate into the parenchyma, and proliferate in the target organ. Tumor cell-autonomous changes alone are not sufficient for this process to be efficient; the microenvironment also plays a crucial role. In fact, during cancer development, an active crosstalk between tumor cells and stroma cells occurs, not only mediated by cell-cell interaction but also by paracrine cytokine and by growth factor signaling. Although the microenvironment can exert inhibitory effects on malignant cells, cancer cells can overcome these inhibitory signals during tumor progression and instead, will exploit and modify these surrounding cells, resulting not

only in an enhancement of primary tumor growth but also in the invasion and metastatic dissemination process. The importance of interactions between metastatic cells and the microenvironment was clearly stated by Paget in 1889 in his "seed and soil" hypothesis. Paget concluded from his studies of ABC cases that certain organs seemed to be more prone to metastasis than others, and that this could not be explained by mechanical factors such as blood flow alone. He suggested that the microenvironment (soil) of these organs was more receptive, thus enabling the tumor cells (seeds) to engraft and develop to macrometastasis. Or in a more modern version, in order to metastasize, cancer cells need to acquire mutations that confer the capability to detach from the primary tumor, to survive in the hematogenous or lymphatic system, and to form metastasis in a distant organ. This target organ must have characteristics that allow cancer cells to engraft and proliferate, whereas other organs may remain non-receptive. Indeed, modern studies have supported this concept, in that circulatory patterns alone cannot fully explain the preferred sites of metastasis (Fidler, 2003). Attention to the metastatic soil has grown again in recent years, and several groups have been exploring and characterizing the local microenvironment and stromal cells both in the primary tumor and in the metastatic sites.

The presence of bone marrow-derived cells within primary tumors was first observed in the nineteenth century and, for many years, was considered as a simple consequence of a failed immune response to tumor cells. However, it became clear that tumors not only are able to evade immune response, but that they also actively recruit and modify bone marrow-derived cells, turning them tumor promotive instead of tumor suppressive (Gabrilovich et al., 2012). Several recent studies have shown an association between certain types of inflammatory cells in the primary tumor and patient outcome. Increased number of tumor -associated macrophages (TAMs) is associated with shortened survival in patients with classic Hodgkin's lymphoma (Steidl et al., 2010) and in several types of solid tumors, including breast, bladder cancer and thyroid cancer (Zhang et al., 2012). On the other hand, decreased number and defective functionality of mature dendritic cells (DCs) have been demonstrated in patients with several different malignancies, including breast (Pinzon-Charry et al., 2007), pancreatic (Bellone et al., 2006), and non-small cell lung (Perrot et al., 2007) cancers. Moreover, the degree of infiltration of primary tumors by mature DCs has been shown to be associated with significantly longer survival in BC (Iwamoto et al., 2003), cutaneous melanoma (Ladányi et al., 2007) and non-small-cell lung cancer (Dieu-Nosjean et al., 2008).

TAMs are the most frequently found immune-cells within the tumor microenvironment. Macrophages are functionally plastic, and can alter their polarization state to accommodate different conditions. At the

extremes of their phenotypic continuum, macrophages can be classified into M1 and M2 types. M1 or "classically activated" macrophages express high-levels of Interleukin-12 (IL-12) and major histocompatibility complex (MHC) molecules and are capable of priming anti-tumor responses (Biswas et al., 2010). On the other hand, M2 or "alternatively activated" macrophages express higher levels of arginase and IL-10, low levels of IL-12 and facilitate tumor progression. TAMs have an M2 phenotype and are involved in multiple steps of tumor development, namely angiogenesis (Lin et al., 2006), protection of tumor cells from chemotherapy-induced apoptosis (Zheng et al., 2009), invasion and metastasis (Qian et al., 2009). In metastatic development, cancer cell intravasation requires a disruption of endothelial cell contacts and a degradation of vascular basement membrane, which is, in part, mediated by proteases secreted by macrophages (Gocheva and Joyce, 2007). TAMs also have a role driving invasive cellular phenotypes. Studies in BC and glioma have shown that TAMs facilitate tumor invasion via a paracrine signaling loop involving tumor-derived CSF-1 and macrophage-derived EGF (Coniglio et al., 2012). Besides these non-immune mechanisms, TAMs also affect tumor progression via immune mechanisms. TAMs eliminate M1 macrophage-mediated innate responses and impair T cell activation by several mechanisms (Gabrilovich et al., 2012).

A critical step in tumor progression and metastatic development is evasion and suppression of the immune system (Motz et al., 2013). This can be accomplished by inhibiting effector immune cells or stimulating immunosuppressive cells. DCs are the most potent antigen-presenting cells, able to recognize, acquire, process, and present antigens to naïve T cells for the induction of an antigen-specific immune response (Steinman and Banchereau, 2007). Activation of DCs in response to stimuli associated with bacteria, viruses or damaged tissues, lead to profound changes in their gene expression, resulting in increased expression of co-stimulatory molecules and upregulation of chemokine receptors (Shortman and Heath, 2010). In cancer, tumor infiltrating DCs can lack CD80 and CD86 (Chaux et al., 1996) and abnormal myelopoiesis results in decreased production of mature functionally competent DCs and increased production of immature myeloid cells (Gabrilovich et al., 2004). These alterations result in the inability of DCs of stimulating an adequate immune response, with a consequent evasion of tumor cells to immune recognition. Several tumor-derived soluble factors have been shown to affect and impair DC differentiation, namely vascular endothelial growth factor (VEGF), macrophage colony forming factor (M-CSF) and Interleukin-6 (IL-6) (Gabrilovich et al., 2012).

Another bone marrow-derived cell type associated with tumor progression is the myeloid derived suppressor cell (MDSC). MDSCs are a heterogeneous group of immunosuppressive, immature myeloid cells, gene-

rically defined as CD11b+GR1+ (Talmadge and Gabrilovich, 2013). These cells are elevated in the blood, spleen, and bone-marrow of tumor-bearing mice, and their levels increase with tumor progression (Youn et al., 2008). MDSCs inhibit immune response by blocking the function of CD4+ and CD8+ cells, by increasing regulatory T-cells, and by inhibiting NK cell activation (Kusmartsev et al., 2004; Huang et al., 2006; Sica and Bronte, 2007). The pathways implicated in the accumulation of these cells with tumor progression are not completely understood. However, inflammation seems to play a role, and studies have shown that pro-inflammatory S100 proteins may be involved in this process (Sinha et al, 2008).

The importance of the tumor microenvironment in tumor progression and metastasis development has been further underlined by the identification of the pre-metastatic niche.

THE PRE-METASTATIC NICHE

After tumor cells successfully intravasate into blood vessels, they need to survive, travel through the circulatory system, and reach their secondary site. Circulating cancer cells that are able to survive are faced with a new challenge: they need to find a receptive environment where they can establish themselves, and proliferate.

The pre-metastatic niche model suggests that in order for tumor cells to engraft and form metastatic lesions at secondary sites, a suitable microenvironment must evolve in these pre-metastatic organs (Figure 1.3). This theory advocates that metastatic proliferation does not depend solely on the characteristics and genetic alterations of the cancer cell itself, but that the formation of the pre-metastatic niche is also essential for metastasis to occur. These niches form as a consequence of growth factors, e.g., VEGF and placental growth factor (PIGF) (Kaplan et al., 2005), transforming growth factor-β (TGF-β) (Hiratsuka et al., 2006), or microvesicles (Grange at al., 2011; Peinado et al., 2012) secreted by the primary tumor.

Of note, the patterns of metastatic spreading seem to depend on the nature of the soluble factors secreted by the primary tumor. This was demonstrated by a series of experiments where conditioned media from cell cultures of B16 melanoma cells were injected intraperitoneally into mice bearing Lewis Lung Carcinoma (LLC). After this procedure, an alteration of the metastasis pattern with secondary lesions in

organs characteristic of B16 melanomas was found (Kaplan et al., 2005). In response to these soluble factors, tumor-associated cells such as hematopoietic progenitor cells or myeloid cells are mobilized to the pre-metastatic niches, and together with other resident cells produce chemokines, growth factors, and matrix degrading proteins (e.g. MMP9) that alter the surrounding microenvironment, making it more suitable for the engraftment of tumor cells and the formation of metastatic lesions (Psaila and Lyden, 2009).

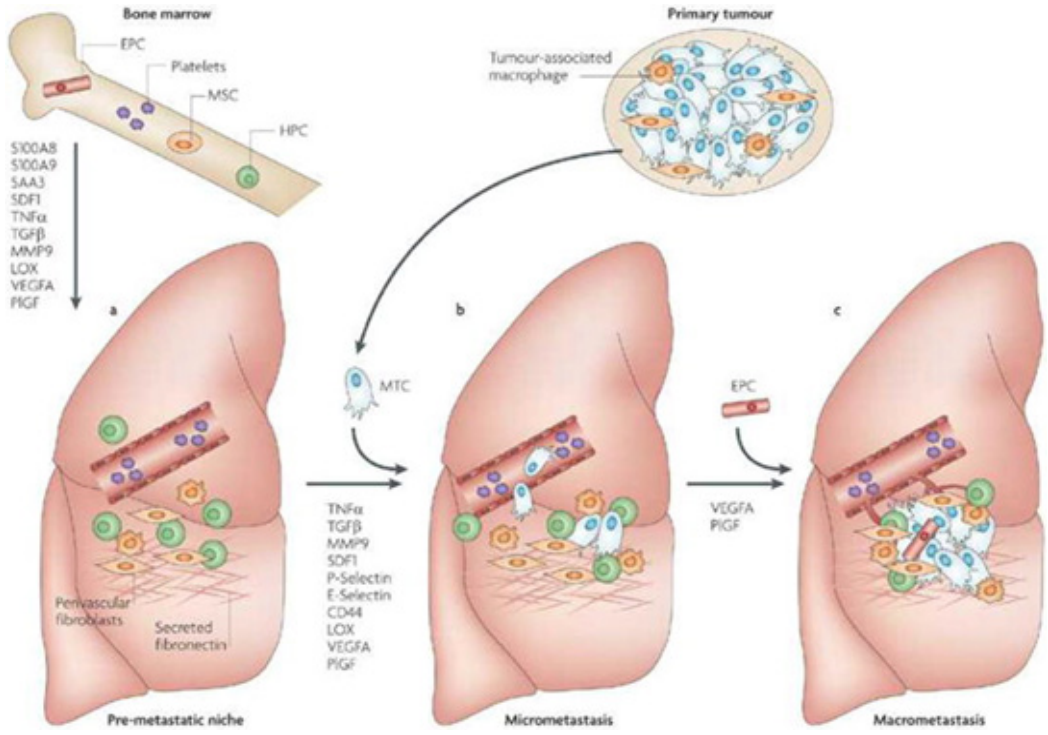

Figure 2.4. The metastatic niche. In response to factors secreted by the primary tumor, bone marrow-derived cells are mobilized from the bone marrow to the peripheral blood and from there to future sites of metastasis. Here, they form the pre-metastatic niche, interacting with residents cells and altering the microenvironment, enhancing tumor cell survival capacity. Metastatic tumor cells invade the niche and form micrometastasis, which evolves to macrometastasis through the recruitment of EPCs and activation of angiogenesis. (Adapted from Psaila and Lyden, 2009)

In fact, vascular endothelial growth factor receptor 1 (VEGFR1) positive bone marrow cells are seen in the pre-metastatic sites prior to the arrival of tumor cells. These cells are of myeloid lineage and preserve the expression of immature markers, such as KIT and SCA-1 (Kaplan et al., 2005). Furthermore, they express the FN receptor VLA-4. FN is a glycoprotein involved in different cellular processes, such as embryonic cell migration and vascular development, and it is expressed in pre-metastatic lungs near the terminal bronchioles and bronchiolar veins, common sites for tumor cell engraftment. This leads to the hypothesis that

clusters of myeloid immature cells and FN serve as docking sites for tumor cells in pre-metastatic organs. The recruitment of immature myeloid cells to the pre-metastatic niche is not only induced by soluble factors secreted by the primary tumor, but also by inflammatory cytokines, including S100A8 and S100A9 or serum amyloid A3 (SAA3) upregulated in the pre-metastatic organs in response to growth factors secreted by tumor cells, including VEGF-A, TGF-β, and tumor necrosis factor-α (TNFα) (Hiratsuka et al., 2008). These data reinforces the concept that inflammation is not only associated with tumor initiation, but also plays a crucial role in tumor progression, and in particular in metastatic development.

Besides immature myeloid cells, other cells are also involved actively in the formation of the pre-metastatic niche. Platelets, resident fibroblasts, and endothelial cells are also important in this process. At the pre-metastatic niche, the mobilized bone marrow-derived cells together with resident cells produce chemokines, growth factors, and matrix degrading proteins (e.g. MMP9). These alter the surrounding microenvironment, making it more suitable for the engraftment of tumor cells and the formation of metastatic lesions. As an example, TNF-α is secreted by myeloid cells in response to tumor-derived factors, and recent studies using LLC cells in a tail vein metastasis model have shown that the absence of this cytokine leads to a significant decrease of metastasis in the lungs. Tumor cells arrive at these destination sites of future metastasis, extravasate into local tissues, engraft in the pre-metastatic niche, and grow progressively into micrometastasis and, possibly, macrometastasis. As mentioned above, tumor cells preferentially localize in areas of FN deposition and in clusters of myeloid cells. However, the exact engraftment mechanism is not yet completely understood. Previous studies have shown an association between the genetic signatures of the tumor cells and their propensity to metastasize to certain organs. The majority of these genes are involved in the interaction of tumor cells with their microenvironment, reinforcing once again the importance of successful interactions with the soil of the future metastatic organs. After engraftment, cells start to proliferate and to form micrometastases. In order for these lesions to progress to macrometastasis, a good and functional vasculature supply is required. Bone marrow-derived endothelial progenitor cells (EPCs), as well as hematopoietic and mesenchymal cells, are crucial regulators in the activation of the angiogenic switch. These cells are recruited to metastasis by VEGF-A signalling, and EPCs themselves express a variety of angiogenic molecules which suggests that their recruitment further potentiates angiogenesis and metastasis growth.

Recent findings on tumor-derived microvesicles, released from the cells when multivesicular bodies fuse with the plasma membrane (Lakkaraju et al., 2008), have changed the way we view communication bet-

ween tumor cells and the surrounding host macro- and microenvironment. Increasing evidence suggests that tumor-derived exosomes have a crucial role in the regulation of angiogenesis (Wysoczynski et al., 2009), activation of fibroblasts (Webber et al., 2010), and modulation of the immune response and the hematopoietic system in general, including lineage-specific differentiation of bone marrow precursors, dendritic cell function and transference of molecules (Yu, 2007; Valadi, 2007) . Additionally, exosomes enable crosstalk between the primary tumor and bone marrow-derived cells, "educate" the latter towards a pro-metastatic phenotype and eventually leading to the homing of both cell types to sites of future metastasis for the creation of a pre-metastatic niche (Peinado et al., 2012).

2.6 **CONCLUSION**

Metastatic disease is the primary cause of cancer-related mortality. In spite of the advances in the diagnosis and treatment of cancer, the overall prognosis of a patient with metastasis remains very low. Improvements in cancer survival will only be possible based on a deeper knowledge of the metastatic process and on the better management of metastatic dissemination. Currently, the use of adjuvant treatment such as chemotherapy or hormonotherapy may reduce the risk of distant metastasis. However, a vast number of patients receiving adjuvant treatment would still have survived without these therapies. Because we cannot identify accurately which patients are at risk to metastasize, some of them will be overtreated unnecessarily. In the future, molecular assays will be used to divide cancer patients into three groups: those that have a very low risk of metastasis requiring no further treatment, those at high risk, with no detectable metastatic disease, and those with established metastasis. It is easily understood that the second group will be the one to benefit the most from those therapeutics that specifically target metastasis development. National Cancer Institute Surveillance Epidemiology and End Result (SEER) data indicate that an important group of patients can be included in this category at the time of initial diagnosis: more than 20% of patients with breast, kidney, and pancreas cancers, more than 30% of patients with colon, cervix, lung, and stomach cancers, and more than 40% of patients with oral cancers. It is important to understand that, because of the metastatic process, not all component steps may be of comparable therapeutic benefit. At the time of diagnosis, the metastatic cascade has already started and, at this point, it is too late to stop certain aspects of metastasis, such as invasion. It is the growth of distant micrometastasis

to clinically detectable, large, life-threatening metastasis that remains to be completed at this stage and, thus, may hold the most therapeutic promise.

During the last decades, mounting evidence has been collected for the involvement of the microenvironment not only in tumor progression but also in metastatic development. The pre-metastatic niche model suggests that a suitable microenvironment must evolve in the future metastatic sites in order for tumor cells to engraft and constitute large metastatic lesions at these secondary sites. Understanding and identifying which factors are involved in the formation of the pre-metastatic niche may constitute an opportunity to abrogate the growth of metastatic disease.

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3. CHAPTER THREE
THE ROLE OF HOST-DERIVED INTERLEUKIN-6 IN METASTATIC PROGRESSION

EXPLORING THE ROLE OF **INTERLEUKIN-6** AND **OTHER HOST-ASSOCIATED FACTORS** IN **TUMOR PROGRESSION** AND **METASTASIS DEVELOPMENT**

3. CHAPTER THREE
THE ROLE OF HOST-DERIVED INTERLEUKIN-6 IN METASTATIC PROGRESSION [3]

3.1 SUMMARY

Determining the molecular mechanisms of metastasis will lead to innovative therapeutic approaches resulting in improvements in patient survival. It is increasingly apparent that bone marrow-derived cells (BMDCs) play a critical role in metastatic progression. Thus, identifying/blocking the positive regulators of BMDC mobilization, recruitment to metastatic sites is hypothesized to abrogate metastasis. Elevated levels of the pro-inflammatory cytokine IL-6 correlates with advanced disease in numerous cancer types, suggesting it may participate in the development of metastasis. Identifying the phenotypic, cellular and molecular consequences of this factor in regulating metastatic progression is the aim of this study. We demonstrated that, compared to wild-type mice, IL-6 knockout mice bearing breast or melanoma tumors, had a reduction in the number of metastatic foci and metastatic burden. Analysis of pre-metastatic lungs and blood showed an IL-6 dependent increase in Stat3 activation with CD11b+Gr1+ MDSCs mobilization and recruitment to these sites during metastatic progression. Inducible-ubiquitous overexpression of activated Stat3 increased hematopoietic progenitor cells (Sca1+c-Kit+) and MDSCs in the bone marrow and promoted their mobilization to the lungs, which was abrogated in IL-6 deficient mice. A requirement for bone marrow derived IL-6 for metastasis was determined, as restoration of metastatic growth was observed in IL-6 knockout mice transplanted with wild-type bone marrow. Our results demonstrate a requirement of bone marrow derived IL-6 in mediating metastatic disease, reinforcing the concept of the bone marrow microenvironment as a necessary participant in solid-tumor metastasis development.

[3] Based on: Maria do Rosario Andre, Hector Peinado, Min Zhang, et al. Interleukin-6 expression in bone marrow-derived cells at early stages of tumor progression regulates metastatic disease. Manuscript in preparation for submission.

3.2 INTRODUCTION

Responsible for more than 90% of cancer deaths, metastatic disease is the primary cause of cancer-related mortality (Weigelt et al., 2005). Despite the recent advances in cancer diagnosis and treatment, the overall prognosis for patients with metastatic disease remains dismal (Riihimali et al, 2013). Thus, understanding of the metastatic process is imperative to devise more effective treatment approaches and improvements in patient survival. In addition to the cancer cell itself, the metastatic soil or local microenvironment and related mechanistic pathways that drive tumor progression have been studied and characterized by several groups (Hiratsuka et al., 2002; Kaplan et al., 2005; Erler et al., 2009; Peinado et al., 2012; Costa-Silva et al., 2015). The pre-metastatic niche model suggests that in order for tumor cells to engraft and constitute metastatic lesions at secondary sites, a suitable microenvironment must evolve in these pre-metastatic organs. The niches form as a consequence of tumor-derived growth factors (Kaplan et al., 2005; Erler et al., 2009; Peinado et al., 2012; Hiratsuka et al., 2006) or microvesicles (Peinado et al., 2012; Grange et al., 2011; Costa-Silva et al., 2015) secreted by the primary tumor. In response to these mediators, tumor-associated cells such as hematopoietic progenitor cells or myeloid cells, are mobilized to pre-metastatic niches and, together with resident cells, create a suitable microenvironment for the engraftment of tumor cells and the formation of metastatic lesions (Kaplan et al., 2005; Psaila and Lyden, 2009).

IL-6 is a potent, multifunctional inflammatory cytokine that mediates several physiological functions, including differentiation of lymphocytes, induction of acute-phase proteins, cell proliferation, cell survival and anti-apoptotic signals (Kishimoto, 1989; Sehgal et al., 1995; Hong et al., 2007). It was first described in early 1980s as a T-cell derived lymphokine that induces final maturation of B-cells into antibody producing cells (Muraguchi et al., 1981; Teranishi et al., 1985). In 1986, IL-6 was molecularly cloned and its structure revealed (Hirano et al., 1986). Human IL-6 consists of 184 amino acids with two potential N-glycosilation sites and four cysteine residues. Comparison of this structure to other known proteins showed a significantly homology with G-CSF, which suggests that the genes for IL-6 and G-CSF might be evolutionarily derived from a common ancestor gene. Further studies showed that the function of this cytokine is not specific to a certain lineage of cells, but it affects a wide variety of tissues and almost all stromal cells and cells of the immune system can produce IL-6, including T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangium cells, and several tumoral cells (Kishimoto, 1989; Hunter and Jones, 2015).

IL-6 gene transcription in normal tissues is induced by a multitude of factors, including virus infection, bacterial endotoxin, lipopolysaccharide, serum, IL-1, TNF, and Interferons (IFNs) (Sehgal et al., 1995; Hirano et al., 1986). Normal physiological concentrations of IL-6 in human serum are relatively low (1-5 pg/ml), but in the context of infection, autoimmunity or cancer these levels are rapidly increased and can reach concentrations in the μg/ml range (Fraunberger et al., 2006; Mroczko et al., 2010). The IL-6 promoter is inhibited by p53 and the retinoblastoma (Rb) gene product, which means that the overexpression of IL-6 in many tumors occurs as a consequence of the loss of one of these negative regulators of transcription (Hong et al., 2007). IL-6 functions are mediated by binding to a specific receptor, IL-6R, consisting of two distinct components. The ligand-binding portion is an 80-kDa molecule named IL-6Rα that associates directly with IL-6 (Kishimoto et al., 1992). The second component is glycoprotein 130 (gp130; also known as CD130) which is the signal-transducing component of the IL-6R complex, also called the IL-6Rβ chain. IL-6 binding to IL-6Rα induces homodimerization of the receptor gp130, present in a great variety of cells. The Janus family kinases JAK1, JAK2 and TYK2 are constitutively associated with the cytoplasmatic tail of gp130. JAKs phosphorylate and activate the signal transducers and activators of transcription (STATs) that are associated with the gp130, and this phosphorylation induces STAT dimerization, translocation to the nucleus and regulation of genes with IL-6-responsive elements (Figure 3.1). IL-6 signaling can also occur through a soluble form of the IL-6Rα (sIL-6Rα). This soluble form was first purified from human urine (Novick et al., 1989) but has been shown to be also present in human blood. IL-6 binds directly to circulating sIL-6Rα, and this soluble complex binds to gp130, inducing signal transduction and gene expression. This process, named trans-signaling, enables cells that do not express the IL-6Rα to respond to IL-6.

The main STAT activated by IL-6 is STAT3. STAT3 was first described as a DNA-binding activity from IL-6 stimulated hepatocytes (Lutticken et al., 1994). Structurally, STAT3 is similar to other STAT proteins, with a conserved amino-terminus involved in tetramerization, a DNA-binding domain, an SH2 domain involved in receptor recruitment as well as dimerization, and a carboxy-terminal transactivation domain. STAT3 is usually inactive in non-stimulated cells, but it becomes rapidly activated by various cytokines and growth factors, such as IL-6 and EGF family members (Hirano et al., 2000). The growth factor receptors that are known to activate STAT3 include the epidermal growth factor receptors EGFR and HER-2, fibroblast growth factor receptor (FGFR), insulin-like growth factor receptor (IGFR), hepatocyte growth factor receptor (HGFR), platelet-derived growth factor receptor (PDGFR) and VEGFR.

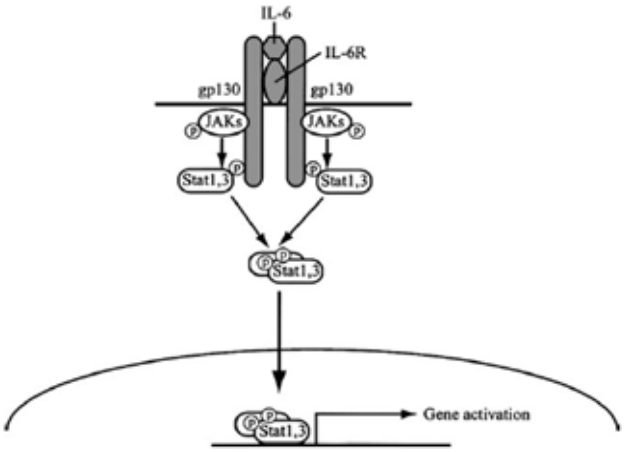


Figure 3.1. IL-6 signal transduction. IL-6 induces homodimerization of gp130, activating JAKs, and Stat1 and Stat3. Activated Stat1 and Stat3 form homodimers or heterodimers, which induce activation of various genes. (Adapted from Kishimoto, 2010)

As with other STAT proteins, STAT3 activation requires phosphorylation of a critical tyrosine residue which mediates its dimerization and allows its nucleus entry and DNA binding (Yoshimura et al., 2007). Under physiological conditions, STAT3 activation turns on strong negative feedback loops that ensure that cytokine-induced STAT3 activation is a transient event. However, in cancer this tight regulation is lost and STAT3 is often found to be constitutively activated (Al Zaid Siddiquee and Turkson, 2008).

Unlike all other members of the STAT family, germline ablation of STAT3 results in early embryonic lethality (Takeda et al., 1997). In fact, loss of STAT3 is lethal even to embryonic stem cells, highlighting the key role of STAT3 in cell growth and survival (Raz et al., 1999). STAT3 has also been shown to be involved in the control of acute-phase responses (Alonzi et al., 2001), wound healing (Sano et al., 1999), granulopoiesis (McLemore et al., 2011) and in cancer (Bromberg J, 2002). Following the discovery that STAT3 is constitutively phosphorylated in v-Src-transformed cells (Yu et al., 1995), considerable evidence has accumulated suggesting a critical role for STAT3 in tumorigenesis. Activated STAT3 has been shown in a variety of malignancies, and its abrogation has been shown to result in the reversal of the malignant phenotype (Bromberg et al., 1999; Catlett-Falcone et al., 1999; Kortylewski et al., 2005; Niu et al., 1999).

IL-6 KNOCKOUT MICE

In an attempt to define the role of IL-6 during development, a knockout murine model was generated almost 20 years ago (Kopf et al., 1995). IL-6 knockout (-/-) mice were generated by replacing the first coding exon (exon 2) of the targeted gene with a neomycin resistance cassette. These mice are viable and fertile, presenting some phenotypic alterations including defects in responses to various viruses and in inflammatory responses to tissue damage or infection (Nishimura et el., 1999; von der Poll et al., 1997), abnormal glucose metabolism (Wallenius et al., 2002), and abnormal emotion, behaviour and cognitive function (Armario et al., 1998; Braida et al., 2004).

THE ROLE OF IL-6 IN CANCER

The role of IL-6 in several human diseases has been determined. It has been shown in the past that IL-6 is an important player in different pathologic states, including rheumatoid arthritis, systemic lupus erythematosus, Crohn disease, sepsis and osteoporosis (Srirangan and Choy, 2010; Tackey et al., 2004; Hack et al., 1989). Over the past decades, interest has grown in the role of this inflammatory cytokine in the context of cancer. Several clinical studies have shown that cancer patients exhibit higher levels of circulating IL-6 compared to healthy controls and that elevated levels of plasmatic IL-6 are associated with advanced disease and poor prognosis in several different types of cancer, including prostate (Adler et al., 1999; Nakashima et al., 2000), breast (Salgado et al., 2003), gastric (Kim et al., 2009), colorectal (Yeh et al., 2010), and head and neck cancer (Zhang et al, 2013; Chang et al, 2013). Single-nucleotide polymorphisms in the IL-6 promoter, particularly the -174 G/C polymorphism, have been associated with an increased susceptibility to breast cancer and with a more aggressive breast cancer phenotype (Iacopetta et al., 2004). Moreover, a recent meta-analysis suggested that IL-6 -634C/G polymorphism is associated with increased lung cancer susceptibility (Nie et al, 2014). Furthermore, results from the PROSPER study indicate that high innate production of pro-inflammatory cytokines (IL-6, IL1-B and TNF-α) is associated with an increased risk for cancer mortality, probably because of increased tumor growth and metastasis (Trompet et al., 2009).

Several mechanisms have been proposed by which IL-6 can be associated with and modulate cancer disease. In myeloma cells, activation of STAT3 by IL-6 signaling leads to an increase in tumor cell survival

through upregulation of anti-apoptotic genes (Catlett-Falcone et al., 1999). Blockade of IL-6 synthesis in lung cancer-derived cells results in inhibition of STAT3 activation and a reduction of tumor growth (Gao et al., 2007) and overexpression of IL-6 in breast tumor cell lines induces metastasis through its effects on angiogenesis and mobilization of myeloid cells (Chang et al., 2013). Another mechanism by which IL-6 can promote tumor growth is by upregulating the expression of the pro-angiogenic factor VEGF, promoting therefore angiogenesis (Wei et al., 2003; Nilsson et al., 2005). IL-6 has been also implicated in the maintenance of stem cell-like cancer cells, defined as CD44+ CD24- cells, observed at the highest frequency in basal-like breast tumors, and associated with aggressiveness and resistance to treatment (Marotta, 2011). IL-6 also plays an important role in the interaction between tumor cells and the immunological microenvironment. This cytokine inhibits differentiation of DCs (Menetrier-Caux et al., 1998) compromising their ability to stimulate the anti-tumor effects of CD8 T cells and natural killer cells. Moreover, IL-6 controls monocyte differentiation towards macrophages at the expense of DCs (Chomarat et al., 2000) and increases the survival of myeloid monocytes recruited to the primary tumor microenvironment (Roca et al., 2009).

Although IL-6 has been implicated in tumor growth and metastasis, its involvement in the pre-metastatic niche formation has not yet been elucidated. The studies discussed above provide a foundation for further investigation into the role of this cytokine produced by host cells in the pre-metastatic niche formation, identifying the phenotypic, cellular and molecular consequences of this factor in regulating metastatic progression.

In this chapter, we show that IL-6, produced by bone marrow cells, also regulates metastasis by promoting the recruitment of immature myeloid cells to the pre-metastatic niches. We found that IL-6 is up-regulated in bone marrow derived cells at early phases of tumor progression, even before metastatic disease becomes evident, and this increased expression is associated with metastatic development. Our results indicate that IL-6 plays an important role in the maintenance of an immature state crucial for metastatic progression, with an increase of progenitor cells in the bone marrow microenvironment and an increase of immature BMDCs being mobilized and recruited to the pre-metastatic niches, creating an adequate microenvironment for tumor cells to engraft and proliferate in metastatic organs. Our results demonstrate that IL-6 expression in BMDCs promotes metastatic progression, supporting the concept of the bone marrow as a principal mediator in metastasis in solid tumors.

3.3 MATERIALS AND METHODS

MICE

C57BL/6J and C57BL/6-B6.129S2-IL-6tm1Kopf/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Research Animal Resource Center (RARC) of Weill Cornell Medical College. The original IL-6 deficient transgenic 129Sv x C57BL/6J mouse line was created by Kopf and Kohler (Kopf et al., 1995), and sent to The Jackson Laboratory where it was backcrossed to C57BL/6J mice for eleven generations.

The CMV-rtTAxTet-O-Stat3C double-transgenic mice were generated from cross-breeding of the CMV rtTA transgenic mice (Harold Varmus and F. Cong) and the TetO-CMV-Stat3C transgenic mouse line (Lian et al., 2005). Oral doxycyline induces expression of activated Stat3 in most tissues of the double transgenic progeny.

Animals used in all experiments were matched for sex, age (6-10 weeks old), and genetic background. All animal procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Weill Cornell Medical College, protocol (IACUC 0709-666A).

TUMOR MODELS

The EO771 breast cancer and B16 melanoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to standard cell culture techniques.

For the EO771 breast cancer model, 1x105 cells were resuspended in 50 µL of 50% Roswell Park Memorial Institute Medium (RPMI) and 50% Growth Factor-reduced (GF-reduced) Matrigel and were injected directly into the mammary fat pad of the wild-type (WT) and IL-6 knockout (IL-6 -/-) mouse, accessed through a small incision in the flank, which was closed with a wound clip that was removed seven days post-injection. EO771 tumors were allowed to grow up to 35 days.

For the B16 melanoma model, 1x106 tumor cells in 200 µL Dulbecco's Modified Eagle Medium (DMEM) were injected into the right flank of the mouse and allowed to grow up to 24 days.

EO771 and B16 tumor cell lines labeled via lentiviral transduction with either Green Fluorescent Protein (GFP), mCherry (mCh) or Luciferase (Luc) were used, depending on the experiment.

IL-6 ENZYME LINKED IMMUNOSORBENT ASSAY (IL-6 ELISA)

The plasma levels of IL-6 in tumor-bearing mice, control mice with no tumor and PyMT mice, were determined by an enzyme-linked immunosorbent assay using the Quantikine Mouse IL-6 Immunoassay from R&D Systems (Minneapolis, MN). Plasma was isolated from whole blood by centrifugation at 3400 rpm, 4ºC, for 10 minutes, in a microcentrifuge and the ELISA kit was used according to manufacturer's instructions.

PREPARATION OF LUNGS FOR QUANTIFICATION OF METASTASIS AND IMMUNOHISTOCHEMICAL STAINING

After the indicated time post-tumor implantation, mice were sacrificed and lungs were perfused with Phosphate-Buffered Saline (PBS) through the righ ventricule. Lung tissues were either fixed in 1.6% paraformaldehyde/20% sucrose in PBS for 24 hours and then embedded in Optimal Cutting Temperature Compound (OCT, Tissue-Tek), fixed in 4% paraformaldehyde and embedded in paraffin, or saved for mRNA extraction for qPCR analysis.

HEMATOXYLIN-EOSIN AND IMMUNOHISTOCHEMICAL STAINING

Hematoxylin-eosin staining was performed on paraffin embedding tissues. 5-µm-thick sections were stained with hematoxylin-eosin and examined under light microscopy.

Immunohistochemical staining was performed on paraffin tissue sections using pSTAT3 (Cell Signaling Technology, Boston MA) and CD45 antibodies (BD, San Jose, CA).

Standard bright field microscopy of stained sections was performed on a Nikon Eclipse E800 upright microscope. Images were acquired using a QImaging Retiga EXi cooled camera and IPLab 3.7 Software. Subsequent image processing was done either with Adobe Photoshop CS or ImageJ 1.43u.

FLOW CYTOMETRY

Lungs were collected from control and tumor-bearing mice after perfusion with PBS by injection in the right ventricule, were incubated shaking at 37ºC for 35 minutes in Collagenase D (Roche) and cut in small pieces. After enzymatic inactivation with EDTA, the tissue was grinded and filtered with a 40-um strainer, in order to form a single cell suspension. This single cell suspension was treated with ammonium chloride-potassium (ACK) buffer (Invitrogen, Carlsbad, CA) according to manufacturer's instructions in order to remove red blood cells. Cells were resuspended at a concentration of 1x 106 cells/100 uL in 1% fetal bovine serum (FBS) and were stained at 4ºC with the appropriate antibodies. The antibodies used were anti-Gr1 and anti-CD11b (eBioscience, San Diego, CA). Cells were then washed and resuspended in 500uL PBS. Flow cytometry analysis was performed using a BD FACSCalibur (BD Biosciences, San Jose, CA) and FlowJo v10 software was used for data analysis.

For blood analysis, total blood was collected from mice via the retro-orbital sinus, and for bone-marrow analysis BM cells were flushed from femurs and tibias. Red blood cells were lysed with ACK buffer and washed for a total of 3 washes. Cells were resuspended at a concentration of 1x 106 cells/100 uL in 1% bovine serum albumine (BSA) in PBS and were stained as described above. Before cells were stained with specific antibodies, nonspecific binding sites were blocked, when needed, with purified anti-FcgRII/III (eBioscience, San Diego, CA). All samples were stained at 4ºC, using the following antibodies: anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), anti-Sca-1 (D7), anti-CD117 (2B8; all from eBiosciences). Stained cells were then washed and resuspended in 500uL PBS. Flow cytometry analysis was performed on FACSCalibur (BD Biosciences) using CellQuest software (Bectin Dickinson). FlowJo software (Tree Star, Inc.) was used for data analysis.

ISOLATION OF LINEAGE NEGATIVE CELLS

Bone-marrow cells were harvested from the femurs and tibias of PyMT and WT mice, and enriched for hematopoietic progenitor cells by depletion of lineage-specific cells using the EasySep Hematopoietic Progenitor Enrichment Kit (StemCell Technologies) as per manufacturer's instructions.

QUANTITATIVE ANALYSIS OF MRNA EXPRESSION

Total RNA was extracted from cells of interest using the RNEasy Mini-Kit (Qiagen, Valencia, CA), according to manufacturer's protocol. cDNA was synthesized using Superscript III reverse transcription (Invitrogen) according to manufacturer's instructions. IL-6 expression was quantified by quantitative polymerase chain reaction (qPCR) performed on a 7500 Fast Real Time PCR System (Applied Biosystems,) using Taqman gene expression assays and relative expression was normalized for β-actin levels.

COLONY FORMING UNITS ASSAY

BM cells were flushed from femurs and tibias of WT and IL-6 -/- C57BL/6J mice with and without tumors and isolated as described above. 1 x 105 cells were plated in triplicate, each in 1 mL of methylcellulose -based media (Methocult GF, StemCell Technologies) in a 25 cm2 flask. For peripheral blood analysis, total blood was collected, lysed with ACK buffer and washed for a total of 3 washes. 1x 107 cells were resuspended in 1 mL of Methocult medium and plated in a 25 cm2 flask, in triplicate. Cultures were incubated at 37ºC for 14 days and colonies were classified based on their phenotypic characteristics as CFU-Gs, CFU-Ms or CFU-GMs, and scored. Averages were taken for the individual colony types, as well as the total colony forming capacity (total number of colonies per plate).

BONE-MARROW TRANSPLANTATION

Bone marrow cells were harvested by flushing femurs and tibias of seven week-old WT C57BL/6J mice with 1% BSA in PBS with 2mM Ethylenediaminetetraacetic acid (EDTA). Cells were counted with Trypan Blue to exclude dead cells, and 1x106 cells in 100µL RPMI were injected via retroorbital injection into seven week-old recipient WT and IL-6 -/- C57BL/6J mice lethally irradiated with a single dose of 950 rad of whole-body irradiation 24 hours before. After 4 weeks, mice were orthotopically injected either by mammary fat-pad injection with EO771 cells or intradermally in the right flank with B16 cells.

STATISTICAL ANALYSIS

Statistical and graphical analyses were performed using GraphPad Prism software for Windows (version 4.00) and Microsoft Excel. Data were analysed for significance using the Student's unpaired t-test, and results were considered statistically significant at p-values < 0.05. Results were representative of two or more independent experiments, and data were expressed as mean ± SEM.

3.4 RESULTS

HOST IL-6 IS ASSOCIATED WITH METASTATIC DEVELOPMENT.

The association between high levels of circulating IL-6 and advanced cancer has been demonstrated in numerous clinical studies (Nakashima et al., 2000; Salgado et al., 2003; Kim et al., 2009; Yeh et al., 2010; Chang et al., 2013).

In order to confirm this association in animal models, C57Bl/6 mice were orthotopically inoculated in the mammary fat pad and subcutaneously in the flank with EO771 mammary adenocarcinoma and B16-F10 melanoma cells, respectively. On week 3 (pre-metastatic stage) and 5 (advanced metastatic stage) post-in-

jection of EO771 cells, mice were sacrificed and plasma was collected. For the B16 melanoma model, plasma was collected from mice at week 2 (pre-metastatic stage) and week 3 (metastatic stage). Circulating IL-6 levels were measured by ELISA and we observed an increase in plasma IL-6 when metastatic disease was evident in both the EO771 model ($p=0.0072$, Fig. 3.2, upper left panel) and the B16-F10 model ($p=0.0056$, Fig. 3.2, upper right panel). Although not reaching statistical significance, IL-6 levels were increased in mice in the pre-metastatic setting for both cell lines (Fig. 3.2, upper panels).

In order to determine if the rise in IL-6 levels was simply due to an increase in the primary tumor burden or as a consequence of metastatic capacity, we used the MMTV-PyMT transgenic model of mammary tumorigenesis in two backgrounds with similar primary tumor kinetics but different metastatic behavior (Lifsted et al., 1998; Hunter, 2006). PyMT/AKR mice develop pulmonary metastasis by approximately 10 weeks and MMTV-PyMT/FVB mice by approximately 14 weeks. We measured IL-6 levels in the PyMT/AKR (highly metastatic) at 9 weeks (pre-metastatic niche stage) and 12 weeks (metastatic stage) and in the PyMT/FVB (moderately metastatic) at 12 weeks (pre-metastatic niche stage) and 14 weeks (metastatic stage). Circulating IL-6 levels were significantly elevated during the time that pre-metastatic niche formation was occurring in the AKR ($p=0.018$, Fig. 3.2 lower panels) but not in the PyMT/FVB strain, and elevated in both once metastasis had formed ($p<0.01$). These data demonstrate an association between circulating IL-6 levels during the pre-metastatic stage and metastatic capacity.

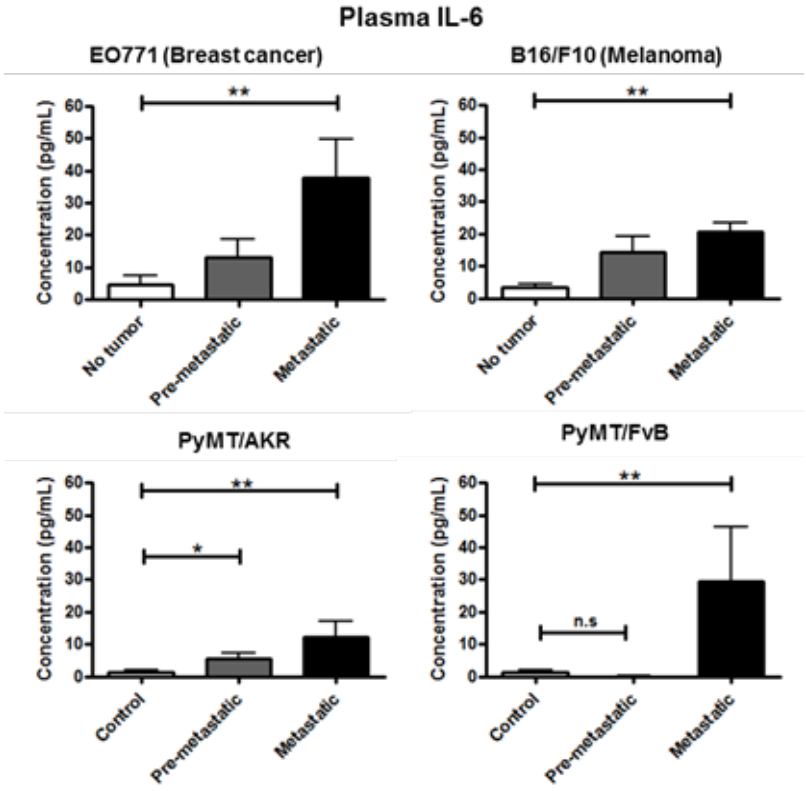
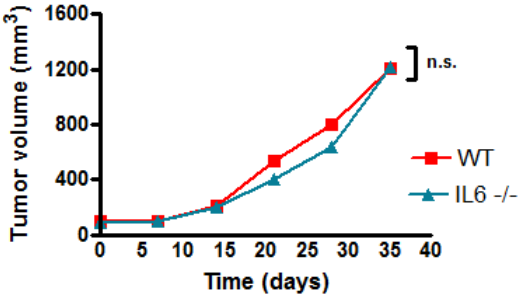


Figure 3.2. ELISA assay measuring levels of murine IL-6 in plasma of WT mice with no tumor, at 3 weeks (pre-metastatic stage) and 5 weeks (metastatic stage) after implantation of E0771 cells (n=4-7); WT mice with no tumor, at 2 weeks (pre-metastatic stage) and at 3 weeks (metastatic stage) after injection of B16-F10 cells (n=3-6); AKR WT and PyMT mice at 9 weeks (pre-metastatic stage) and 12 weeks (metastatic stage) (n=3-9). (Unpaired t-test, *p < 0.05, **p < 0.01)

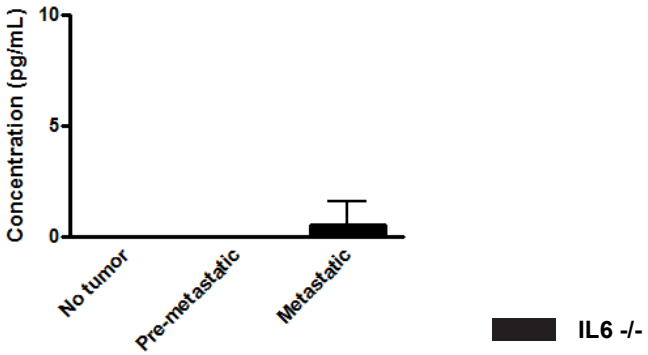
Given the positive association between circulating IL-6 levels and the development of metastatic disease, we hypothesized that host-derived IL-6 (rather than tumor) may be contributing to this phenomenon. In order to test this hypothesis, C57Bl/6 WT and C57Bl/6 IL-6 -/- mice were injected orthotopically with EO771 cells (high IL-6-producing tumor cells) and tumor volumes were determined weekly. No significant differences in primary tumor growth were observed (Fig 3.3 A). Interestingly, IL-6 plasma levels were undetectable in the EO771 tumor bearing IL-6-/- mice at 3 weeks and very low at 5 weeks following mammary fat pad injection, demonstrating that IL-6 is produced primarily by host cells (Fig. 3.3 B). In contrast to findings for primary tumor growth, a marked reduction in the number of pulmonary macrometastatic foci (p=0.04) and metastatic burden (p=0.009) was observed at 5 weeks in the IL-6-/- group compared to the WT group (Fig. 3.3 C). Notably, the number of micrometastases (<20 cells) was similar for the two groups (p=0.9, Fig. 3.3 D). Similar results were observed when WT and IL-6 -/- mice were challenged

with B16 tumors (Fig. 3.4 A and 3.4 B). Taken together, these results suggest that host-derived IL-6 is critical for the growth of "seeded" micrometastasis.

A - EO771 (Breast cancer)



B - Plasma IL-6



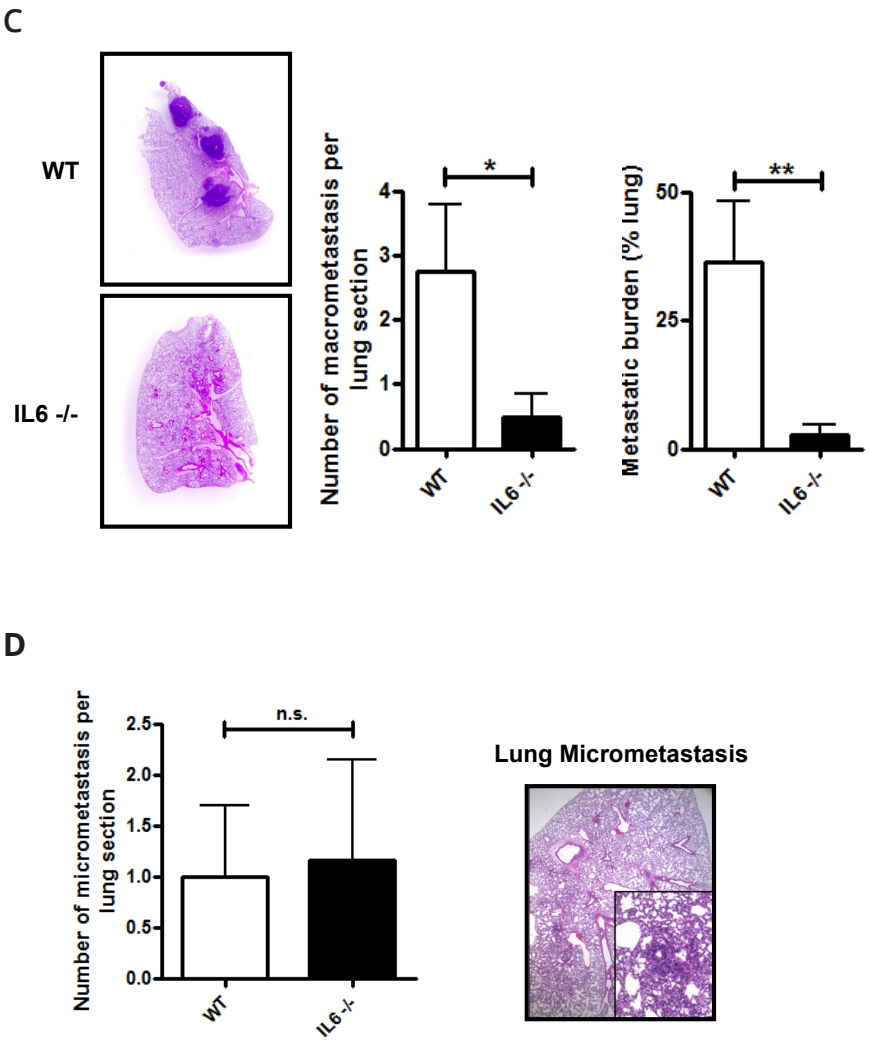


Figure 3.3. Host Interleukin-6 is associated with metastatic development. (A) Analysis of primary tumor volume from WT and IL-6 -/- mice following implantation of EO771 cells (n=5, Two-way ANOVA, n.s: non-significant). (B) ELISA assay measuring levels of circulating murine IL-6 in plasma of IL-6 -/- mice with no tumor, and at 3 weeks (pre-metastatic stage) and 5 weeks (metastatic stage) after injection with EO771 cells (n=3-4). (C) Lung metastasis determined by H&E in WT and IL-6 -/- mice at 5 weeks after implantation of EO771 cells, with quantification of number of macrometastatic (>20 cells) lesions per lung section, quantification of lung metastatic burden (defined as the area occupied by metastatic lesions divided by the total lung area) and (D) Quantification of lung micrometastatic (<20 cells) lesions. (n=4-6, Unpaired t-test, *p < 0.05, **p < 0.01)

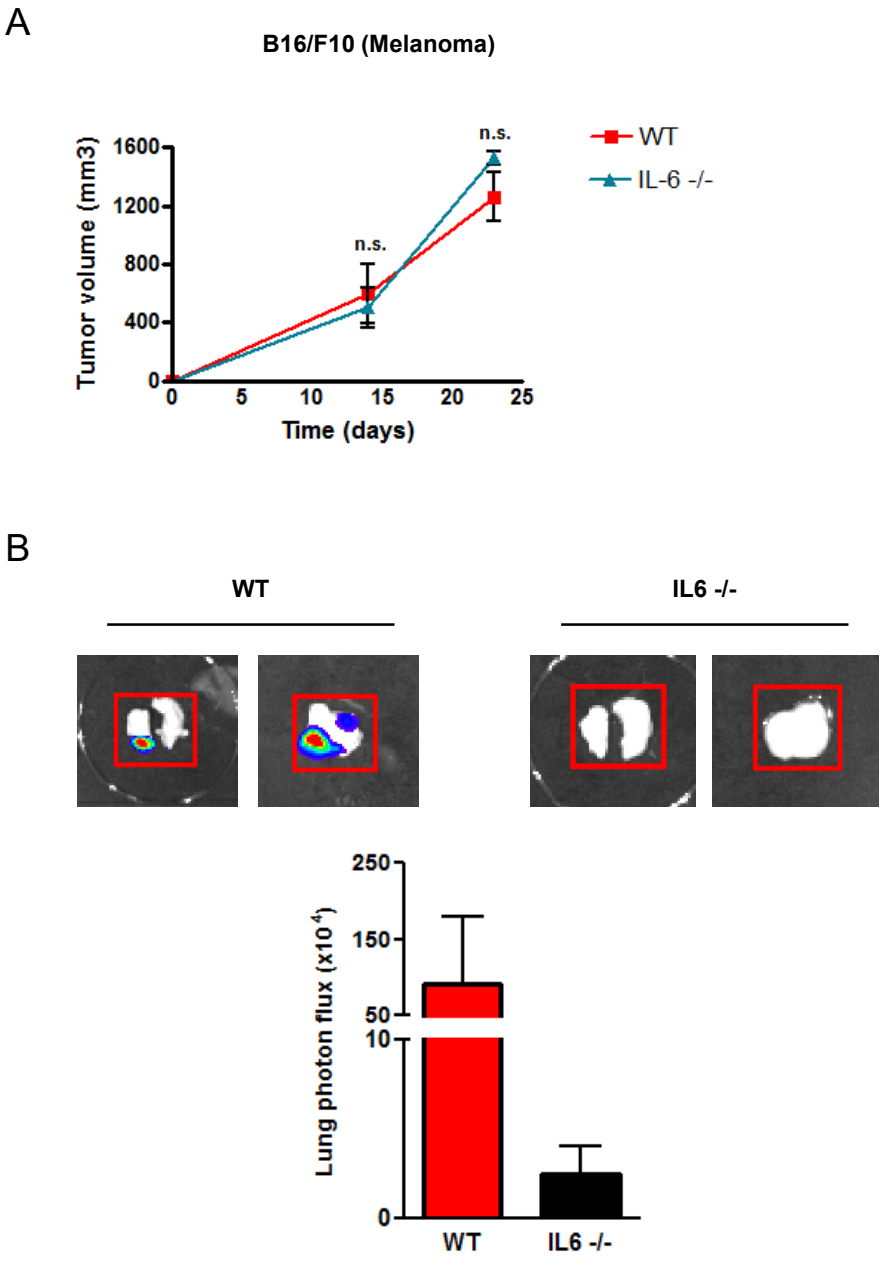


Figure 3.4. (A) Analysis of primary tumor volume from WT and IL-6 -/- mice at 14 and 23 days after implantation of B16-F10 cells. (n=6, Unpaired t-.test, n.s: non-significant) (B) Images illustrating lung metastasis in WT and IL-6 -/- mice at 3 weeks after implantation of B16-F10-luciferase+ cells, as evaluated by IVIS imaging, and quantification of lung metastatic burden per intensity of photon flux (n=3).

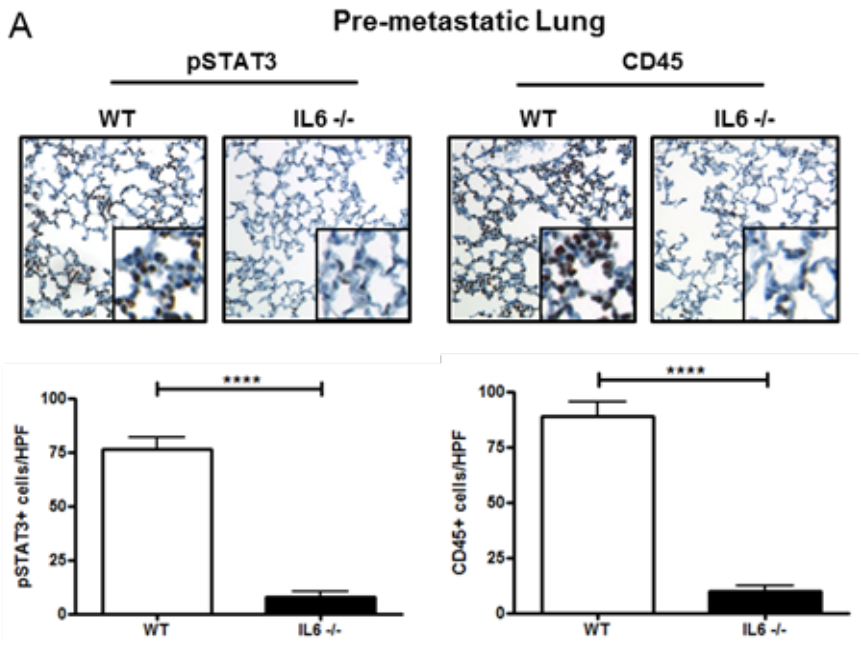
IL-6 ENHANCES THE MOBILIZATION AND RECRUITMENT OF BONE MARROW-DERIVED MYELOID CELLS TO PRE-METASTATIC NICHES.

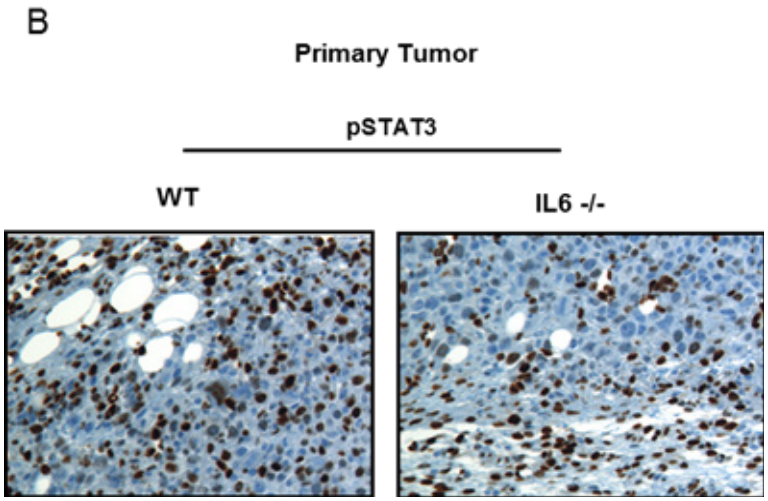
Given the observed differences in metastatic development and the apparent role of host-derived IL-6 in promoting metastasis, we sought to identify the putative mediators of this process. We hypothesized that IL-6 participates in the recruitment of BMDCs to the lungs during pre-metastatic niche formation. C57Bl/6 WT and IL-6 -/- mice were orthotopically injected with EO771 cells and the lungs and peripheral blood were examined in tumor-bearing mice during the pre-metastatic phase (3 weeks after injection). Although, as expected, there was no evidence of lung metastases in either WT and IL-6 -/- mice, we observed a significant increase ($p<0.0001$) in the number of pSTAT3 positive cells in the pre-metastatic lungs of WT tumor-bearing mice which was not evident in the pre-metastatic lungs of IL-6 -/- mice (Fig. 3.5 A, left panel). In contrast to the pre-metastatic niche, pSTAT3 levels in tumor cells were not significantly different in WT and IL-6-/- hosts (Fig. 3.5 B). We hypothesized that the pSTAT3+ cells in the pre-metastatic lungs were derived from the bone marrow. Indeed, a significant correlation (correlation co-efficient=0.9366) was apparent between the levels of pSTAT3+ and CD45+ cells (bone marrow derived leukocytes), with high numbers of CD45+ cells in the lungs from WT tumor bearing mice compared to the lungs of IL-6-/- tumor bearing mice expressing few CD45+ cells ($p< 0.0001$, Fig 3.5 A, right panel).

We next characterized the specific population of BMDCs recruited differentially to the lungs during the pre-metastatic phase in WT as compared to the IL-6 -/- mice. Recent reports have stressed the importance of the recruitment of MDSCs in the formation of the pre-metastatic niche (Kowanetz et al., 2010). Over-expression of IL-6 in breast tumors led to an increase in MDSC mobilization to pre-metastatic lungs (Chang et al., 2013). Additionally, S1PR1-STAT3 is activated in myeloid cells in pre-metastatic sites which is crucial for myeloid cell proliferation and evasion of apoptosis (Deng et al., 2012). We therefore analysed the pre-metastatic lungs for MDSCs (CD11b+Gr1+) populations by flow cytometry. Expectedly, high levels of CD11b+Gr1+ cells were found in the lungs of tumor-bearing WT mice as compared to tumor-naïve mice ($p<0.0001$, Fig. 3.5 C). In contrast, no significant differences in CD11b+Gr1+ numbers were detected in the lungs of IL-6 -/- mice without or with tumors ($p=0.051$, Fig. 3.5 C). Not surprisingly, CD11b+Gr1+ cells were significantly lower in the pre-metastatic lungs of IL-6 -/- mice versus WT tumor bearing mice ($p=0.029$, Fig. 3.5 C). Similarly, the number of CD11b+Gr1+ cells in the peripheral circulation of WT and IL-6-/- mice were similar in non-tumor expressing mice ($p=0.521$, Fig. 3.5 D). Notably,

MDSC levels increased approximately 7-fold in WT mice and much less in the IL-6-/- tumor-bearing mice (p=0.0036, Fig. 3.5 D).

Taken together, these results suggest that host IL-6 influences the mobilization and recruitment of MDSCs to pre-metastatic niches and that this may explain the reduction in metastasis development observed in the IL-6 -/- mice.





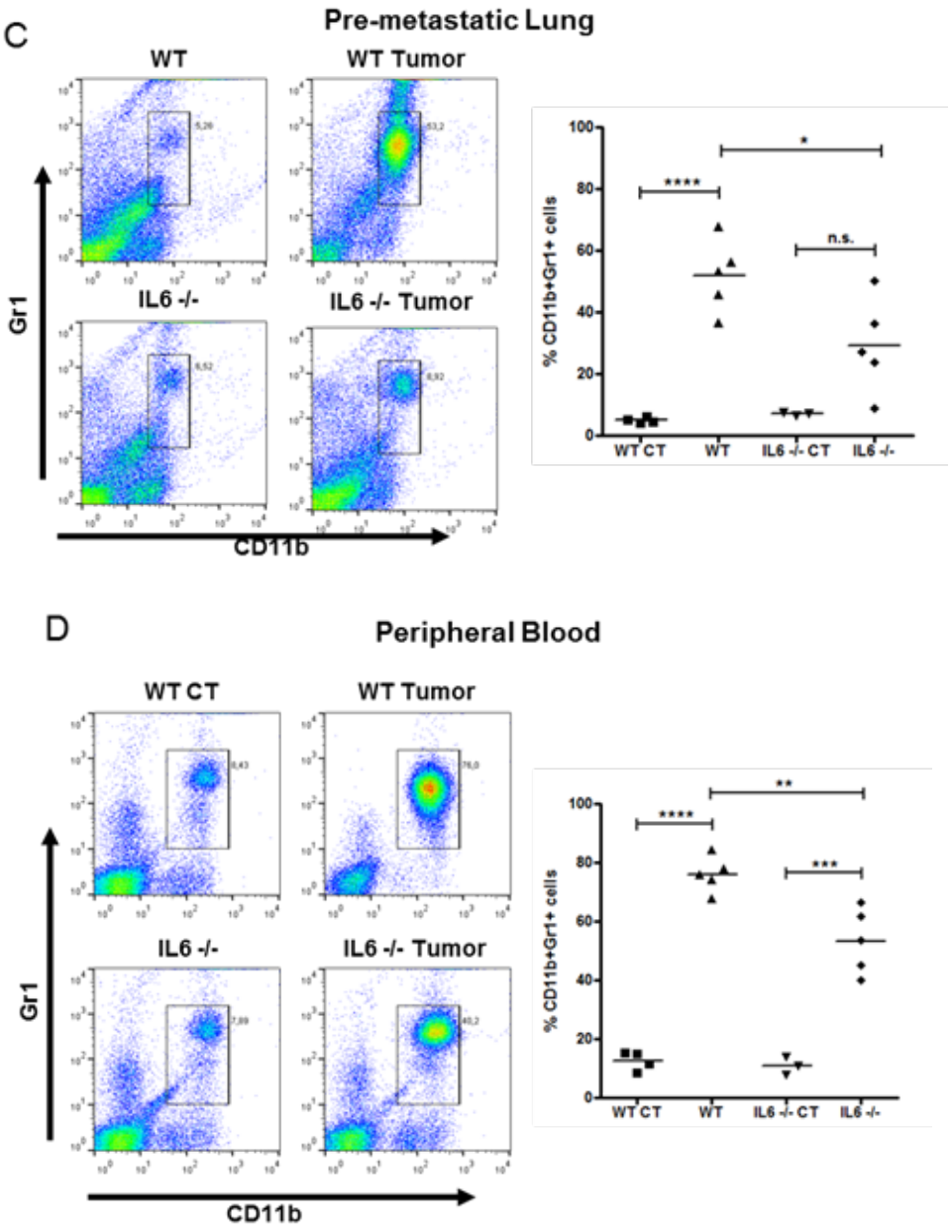


Figure 3.5. (A) pSTAT3 and CD45 immunostaining of cells in the lung of WT and IL-6 -/- mice at 3 weeks post EO771 injection (200x), with quantification of the number of stained cells per high power field (HPF) (n=4, Unpaired t-test, ****p < 0.0001). (B) pSTAT3 immunostaining in EO771 tumors from WT and IL-6 -/- mice at 5 weeks post- tumor cell implantation. (C) Flow cytometry analysis of lung tissue and (D) peripheral blood cells from WT and IL-6 -/- mice with no tumor or 3 weeks after EO771 tumor implantation for markers CD11b and Gr1, with quantification of percentage of CD11b+Gr1+ cells (n=3-5, Unpaired t-test, n.s: non-significant, *p < 0.05, **p < 0.01, ***p < 0.001)

ACTIVATED STAT3 DRIVES AN IL-6 DEPENDENT EXPANSION AND MOBILIZATION OF BONE MARROW-DERIVED MYELOID CELLS.

IL-6 signalling is mediated primarily through STAT3 who in turn participates in the "feed-forward" regulation of IL-6 (Fujitani et al., 1994; Guschin et al., 1995; Stahl et al., 1995; Nakajima et al., 1996; Chang et al., 2013). Additionally, given the recent published data showing that S1PR1-STAT3 signalling is crucial for myeloid cell colonization at future metastatic sites (Deng et al., 2012), we hypothesized that this pathway is involved in the phenotypic differences observed between WT and IL-6 -/- mice regarding metastasis development, mobilization and recruitment of BMDCs to the pre-metastatic niche. To determine the unique involvement of activated STAT3 to this process, we used an inducible transgenic model expressing constitutively activated STAT3 (Stat3C) ubiquitously. Specifically, CMVrtTAxTet-O-Stat3C (3crtTA) double-transgenic mice were generated by crossing CMV rtTA transgenic mice (Harold Varmus and F. Cong) to Tet-O-CMV-Stat3C transgenic mice (Lian et al., 2005; Wu et al., 2011), whereby constitutive STAT3 phosphorylation was induced throughout the host with doxycycline treatment (Fig. 3.6) (unpublished observations, J. B.). After 5 days of doxycycline, animals were sacrificed and lungs were harvested for analysis.

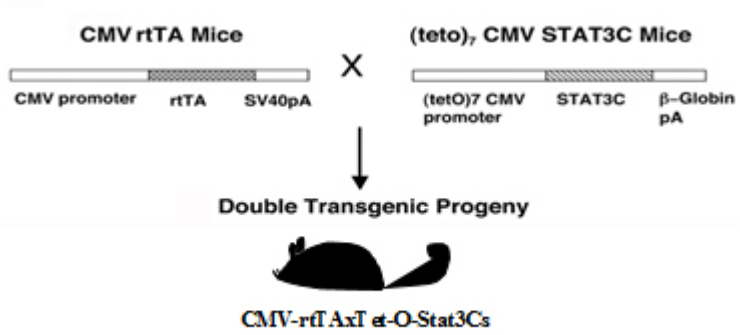


Figure 3.6. Schematic illustrating the generation of the CMV-rtTAxTet-O-Stat3C double-transgenic mice from cross-breeding of the CMV rtTA transgenic mice and the TetO-CMV-Stat3C transgenic mouse line.

In a similar pattern to what we observed in tumor-bearing mice, the lungs of 3crtTA mice showed an increase in the number of pSTAT3+ and CD45+ cells compared to those of the 3c- control mice (Fig. 3.7, top panels). This phenotype was completely abrogated in IL-6 deficient 3crtTA mice, as pSTAT3+ and CD45+ cells were not observed in the lungs (Fig. 3.7, lower panels).

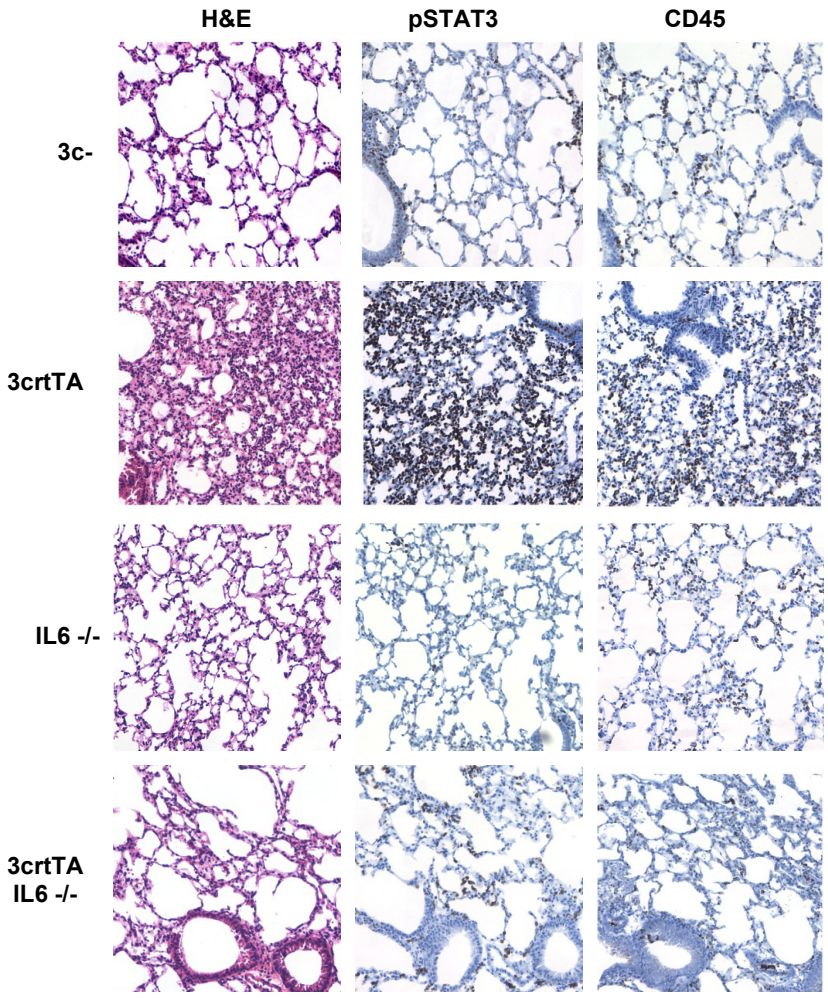


Figure 3.7. Lung histology determined by H&E and immunostaining for pStat3 and CD45 in lung sections from 3c-, 3crtTA, IL-6 -/- and 3crtTAIL-6 -/- mice (100x).

Similarly, an increase in MDSCs in the 3crtTA mice compared to control mice was observed in the lungs (p=0.023) and peripheral blood (p=0.007) which was largely reversed in the 3crtTA IL-6 -/- transgenic mice (lungs: p=0.352; blood: p=0.090, Fig. 3.8). Analysis of the bone marrow of these animals showed elevated IL-6 levels in the 3crtTA mice, but not in IL-6-/- transgenic animals (Fig. 3.9, left upper panel). Given these results, we hypothesized that the increased recruitment and mobilization of MDSCs could be due to an alteration in the bone marrow microenvironment itself, conditioned by the local expression of IL-6 in the bone marrow. In order to examine this, BMDCs were isolated and CD11b+Gr1+ MDSCs analyzed by flow cytometry. MDSCs were increased in the bone marrow of 3crtTA mice compared to control mice (p=0.049), with no differences observed between 3crtTA IL-6 -/- and IL-6 -/- mice (p=0.084) (Fig. 3.9, right upper

panel). Moreover, when we analysed the bone marrow for hematopoietic progenitor cells, we observed an increase in the number of Sca1+ckit+ cells in the bone marrow of 3crtTA mice compared to controls (p =0.010), which was abrogated in the IL-6 -/- transgenic mice (p=0.359, Fig. 3.9, lower panel).

The results from the 3crtTA and IL-6 -/- transgenic mice indicate that activation of STAT3 through IL-6 contributes to an increase in progenitor cells in the bone marrow as well as to the mobilization and recruitment of BMDCs to the pre-metastatic niche, which we hypothesize promotes metastasis.

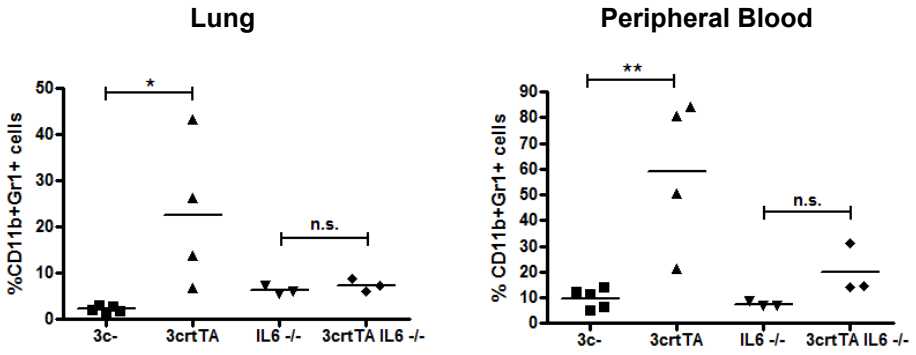


Figure 3.8. Flow cytometry analysis of lung tissue and of peripheral blood cells from 3c-, 3crtTA, IL-6 -/- and 3crtTA IL-6 -/- mice with quantification of percentage of CD11b+Gr1+ cells. (n=3-5, Unpaired t-test, n.s: non-significant, *p < 0.05, **p < 0.01);

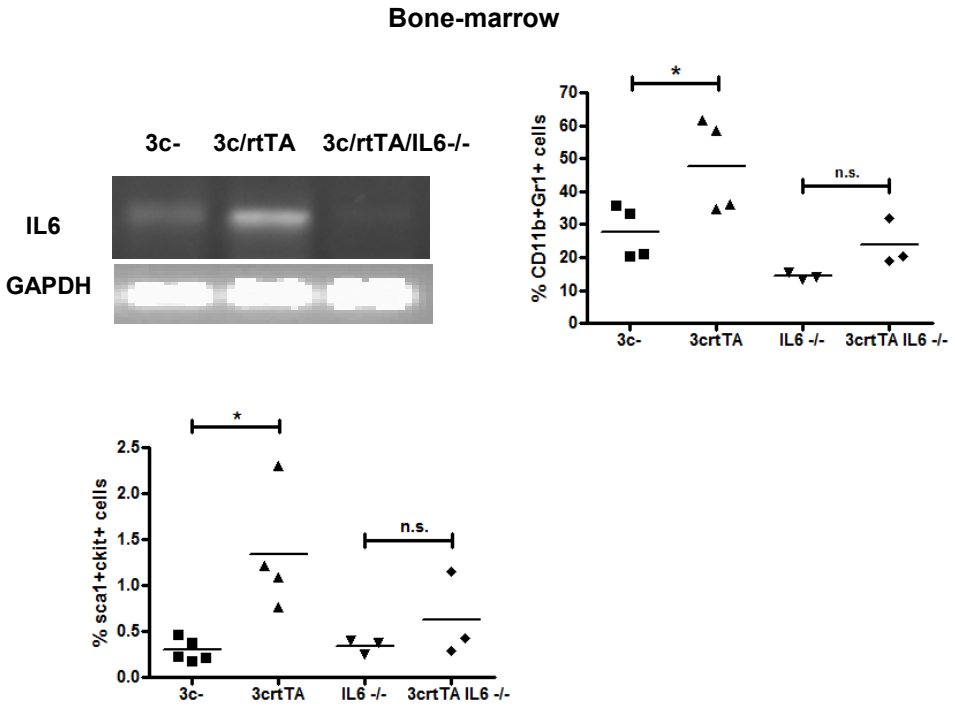


Figure 3.9. Analysis of IL-6 and GAPDH levels in bone marrow extracts from 3c-, 3crtTA and 3crtTaIL-6 -/- mice. Flow cytometry analysis of bone marrow cells from 3c-, 3crtTA, IL-6 -/- and 3crtTA IL-6 -/- mice with quantification of the percentage of CD11b+-Gr1+ cells and of sca1+ckit+ cells. (n=3-5, Unpaired t-test, n.s: non-significant, *p < 0.05)

BMDC IL-6 MEDIATES METASTATIC PROGRESSION

Given the observation that IL-6 is required for the expansion of HSCs and MDSCs through activated STAT3, we hypothesized that IL-6 expression would be induced in BMDCs during metastatic progression. We examined IL-6 expression in bone marrow progenitor cells in the PyMT spontaneous breast cancer model. Lineage-negative (Lin-) hematopoietic progenitor cells were isolated from the bone marrow of WT mice and PyMT mice with moderate and high metastatic potential (FVB and AKR backgrounds, respectively) at 6 weeks, 9 weeks, 12 weeks and 14 weeks of age. IL-6 mRNA expression levels were elevated in Lin- cells during tumor progression, with the highest values observed during the pre-metastatic (6 and 9 weeks) phases of tumor progression especially in the high metastatic AKR mice (Fig. 3.10).

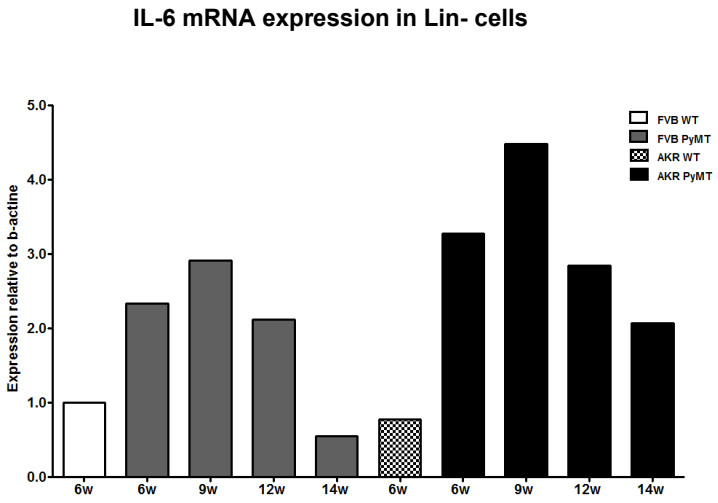
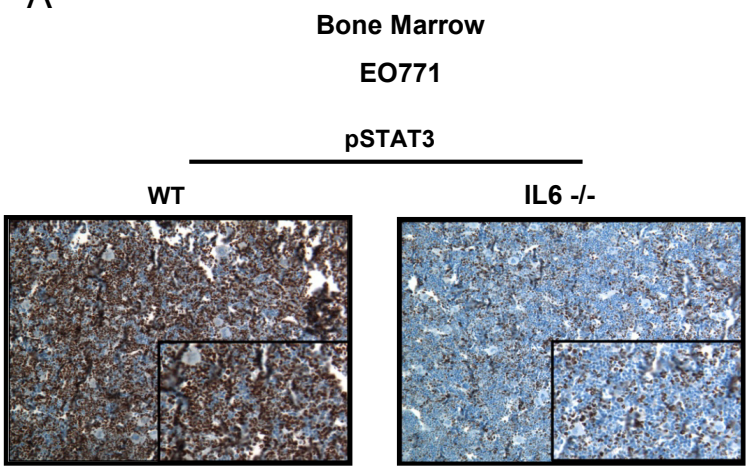


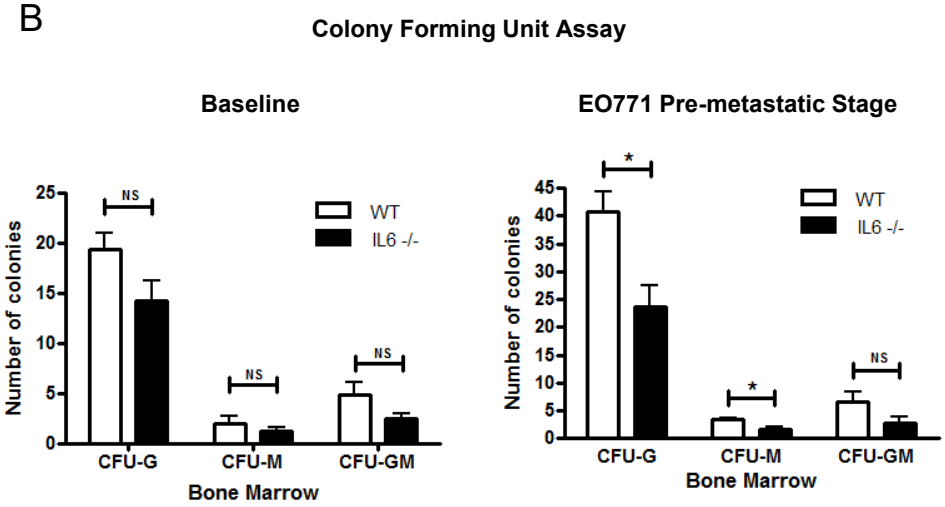
Figure 3.10. IL-6 mRNA relative expression levels of bone marrow Lin- cells isolated from FvB and AKR PyMT mice at 6, 9, 12 and 14 weeks compared to control mice, as determined by quantitative PCR analysis.

In order to determine if upregulation of IL-6 in BMDCs during tumor progression has an impact on haematopoiesis, we injected WT and IL-6 -/- mice orthotopically with EO771 and B16-F10 cells and analysed the bone marrow at different time-points of tumor progression. We first observed that, during pre-metastatic stages, there is an increase in pSTAT3+ cells in the bone marrow of EO771 tumor- bearing WT mice as compared to IL-6 -/- mice (Fig. 3.11 A). In the absence of tumors, no differences in colony-forming units (CFU) was observed from the bone marrow of WT and IL-6 -/- mice (CFU-G p=0.078, CFU-M p=0.341, CFU-GM p=0.119, Fig. 3.11 B, left panel). In contrast, there were fewer hematopoietic progenitor cells during pre-metastatic niche formation in the bone marrow and peripheral blood of IL-6 -/- mice compared to WT mice (bone marrow: CFU-G p=0.032, CFU-M p=0.024 ,CFU-GM p=0.124, Figure 3.11 B, right panel; peripheral blood: Fig 3.11 C). Similar results were obtained using the B16 model, with higher numbers of progenitor cells in the pre-metastatic bone marrow of WT mice compared to IL-6 -/- mice (CFU-G p= 0.002, CFU-M p<0.0001, CFU-GM p=0.002 , Fig. 3.11 D). These data suggest that increased expression of IL-6 in BMDCs during the early stages of tumor progression can promote the expansion of hematopoietic progenitor cells.

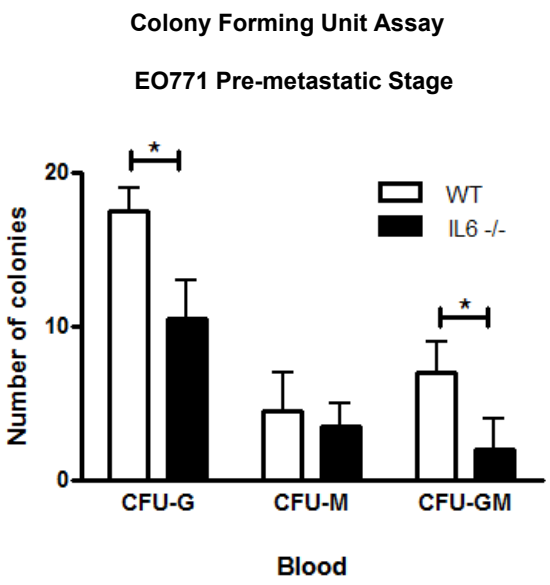
A


Bone Marrow
EO771
pSTAT3
WT
IL6 -/-

B


Colony Forming Unit Assay
Baseline
EO771 Pre-metastatic Stage
Number of colonies
WT
IL6 -/-
NS
*
CFU-G
CFU-M
CFU-GM
Bone Marrow

C



D

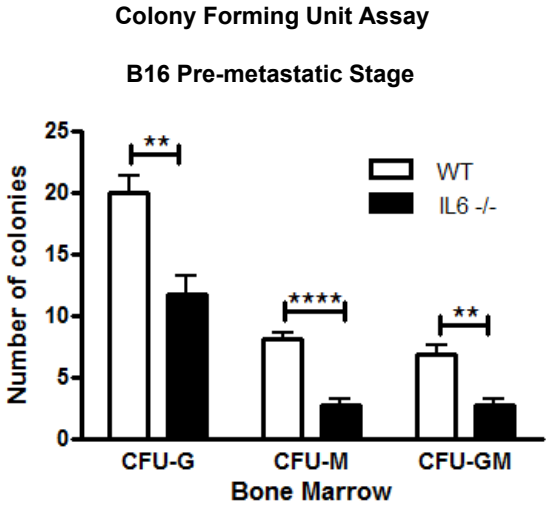


Figure 3.11. (A) pSTAT3 immunostaining of bone marrow from WT and IL-6 -/- mice at 3 weeks after EO771 tumor implantation (100x). (B) Myeloid colony forming unit assay with quantification of myeloid colonies (G: granulocyte, M: macrophage and GM: granulocyte/macrophage) formed (B) after culturing bone marrow cells isolated from WT or IL-6 -/- mice, at baseline and 3 weeks after implantation of EO771 tumor cells, in MethoCult ® media. (n=3, Unpaired t-test, NS: non-significant, *p < 0.05), (C) after culturing isolated cells from peripheral blood of WT or IL-6 -/- mice, at 3 weeks after implantation of EO771 tumor cells or (D) after culturing bone marrow cells isolated from WT or IL-6 -/- mice, at 2 weeks after implantation of B16-F10 tumor cells, in MethoCult ® media. (n=6, Unpaired t-test, *p < 0.05, **p<0.01, ****p < 0.0001)

In order to determine whether IL-6 expression in BMDCs regulates metastatic outcome, we transplanted WT and IL-6 -/- mice with bone marrow isolated from WT mice 4 weeks prior to the injection of tumor (EO771 or B16-F10) cells. Although we did not detect any statistically significant differences in primary tumor growth between the WT and IL-6 -/- groups, we observed a rescue of the WT metastatic phenotype in the IL-6 -/- mice (Fig. 3.12 A and Fig. 3.12 B).

These results demonstrate that high levels of IL-6 expression in BMDCs promote a pro-metastatic phenotype through the expansion of HSCs and MDSCs.

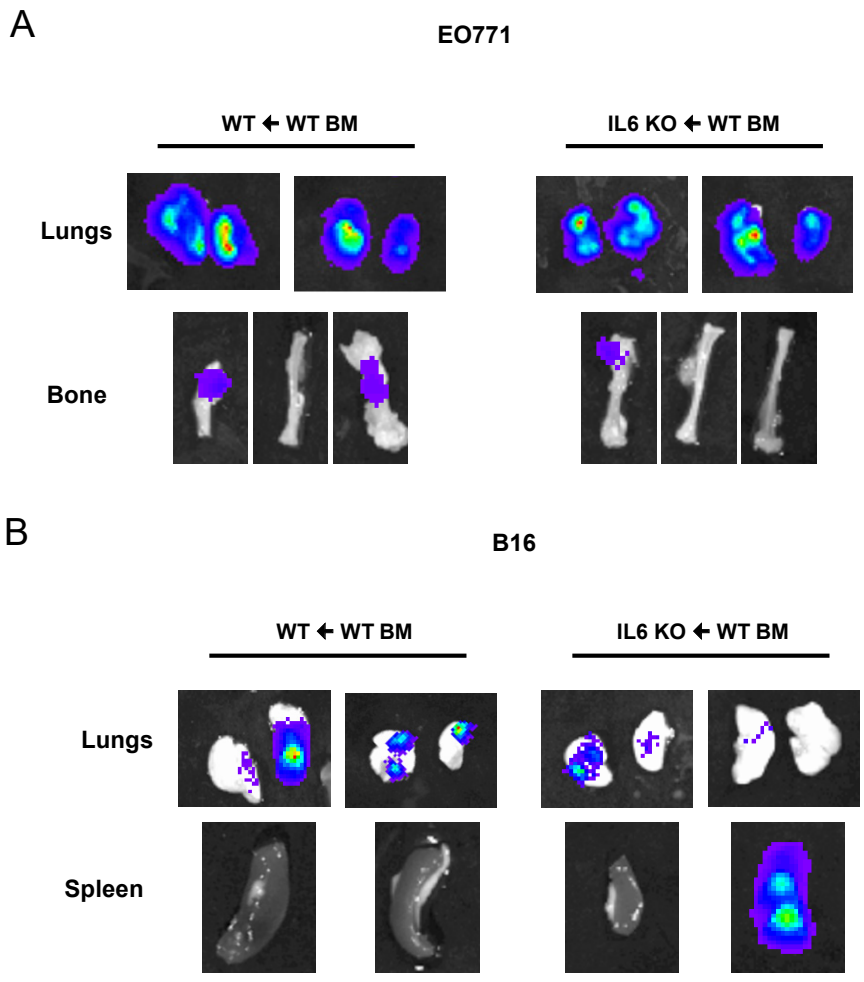


Figure 3.12. (A) IVIS imaging of lungs and bones 4 weeks after implantation of EO771 tumors in WT and IL-6 -/- mice transplanted with WT BM, illustrating metastatic foci (n=4). (B) IVIS imaging of lungs and spleens 3 weeks after implantation of B16-F10 tumors in WT and IL-6 -/- mice transplanted with WT BM, illustrating metastatic foci (n=4).

3.5 DISCUSSION

In this study, we demonstrate that upregulation of IL-6 in BMDCs during the early stages of tumor progression, is associated with increased levels of hematopoietic progenitor cells in the bone marrow microenvironment, with a subsequent mobilization and recruitment of immature myeloid cells to pre-metastatic niches. This increase in BMDCs in pre-metastatic organs renders this foreign microenvironment suitable for the proliferation of metastatic tumor cells. By transplanting IL-6-expressing BMDCs in IL-6 non-expressing mice and reverting the phenotype, we demonstrate that IL-6 expression in BMDCs is one of the driving events of metastatic development, reinforcing the requirement of the bone marrow microenvironment in the metastatic progression of solid tumors.

The role of IL-6 in regulating tumor progression and metastasis has been the focus of intense research over the past decade (Hong et al., 2007; Naugler et al., 2008). Evidence has accumulated that high circulating IL-6 levels in cancer patients are associated with advanced cancer stage and an independent prognostic factor for overall survival and disease-free survival (Shimazaki et al., 2013; Chang et al., 2013). However, most research has focused on the effects of IL-6 produced by tumor cells, and it remains unclear if plasmatic IL-6 detected in cancer patients is tumor-derived or is part of the host response to tumor development. Using two different tumor (EO771 breast cancer and B16-F10 melanoma) models, we demonstrate that plasmatic IL-6 levels are essentially undetectable in the IL-6 -/- mice, but markedly elevated in WT mice, during tumor progression, demonstrating the essential role of host/bone marrow derived IL-6 in response to tumor growth. These results reinforce previous observations that, in prostate cancer, IL-6 in plasma is largely host-derived (Kerr et al., 2010), and support the idea that the majority of plasmatic IL-6 detected in cancer patients is largely produced by cells that comprise the microenvironment.

EO771 breast cancer and B16-F10 melanoma cell lines are known to metastasize to the lungs (Kaplam et al., 2005; Kanda et al., 2009). We therefore evaluated metastatic development in this organ and observed striking differences between WT mice and IL-6 -/- mice, with fewer numbers and reduced volumes of lung macrometastatic lesions in the IL-6 -/- mice, but no differences in the number of micrometastases. These results suggest that, in the absence of IL-6, although tumor cells are arriving to future metastatic sites, the "soil" is neither prepared nor adequate for these cells to evolve into full macrometastases. MDSCs recruitment has been shown to parallel metastasizing capacity of cancer cells, through IL-6 trans-signaling,

facilitating metastasis development (Oh et al., 2013). Additionally, it has been demonstrated that pSTAT3 activation in MDSCs in the lungs is a crucial factor for MDSC proliferation and escape from apoptosis, during pre-metastatic niche formation (Deng et al., 2012). Our work not only supports these observations, but also demonstrates a requirement for host IL-6 for the activation of STAT3 and the mobilization of MDSCs to pre-metastatic niches.

IL-6 has been shown to be a critical activator of STAT3 in normal and cancer cells (Hirano et al., 2000; Sansone and Bromberg., 2012). In contrast to normal cells where STAT3 phosphorylation is tightly regulated, STAT3 is constitutively activated in tumors including both cancer and stromal cells (Sansone and Bromberg, 2012). Additionally, expression of a constitutively activated form of STAT3 (Stat3C) was shown to mediate tumorigenesis in a tumor intrinsic/extrinsic manner, in association with a robust inflammatory infiltrate (Bromberg et al., 1999; Li et al., 2007). Here we demonstrated that ubiquitous expression of Stat3C led to increases in HPCs (Sca1+ckit+), pSTAT3+ cells in the bone marrow and recruitment to pre-metastatic niches along with MDSCs. These observations are consistent with earlier studies demonstrating that transfection of Stat3C in bone marrow led to HSC expansion (Chung et al., 2006) and inducible Stat3C expression in pneumocytes, enhanced the mobilization of MDSCs to the lungs (Li et al., 2007; Wu et al., 2011). However, we further demonstrate that IL-6 plays an important role in mediating these phenotypes as HSC expansion and mobilization of MDSCs was largely (but not completely) abrogated in IL-6-/- mice. Thus, other growth factors are likely playing a role in this process. Tumor cells and tumor-associated stromal cells produce and release multiple soluble factors that can prime the myeloid cell population for its metastasis-promoting functions (Gabrilovich et al., 2012). VEGF, GM-CSF, IL-1β, IL-10 and TGFβ have been implicated in the switch of myeloid differentiation towards MDSCs at the expense of dendritic cells (Jayaraman et al., 2012; Sica and Bronte, 2007; Elkabets et al., 2010; Tanikawa et al., 2011; Fridlender et al., 2009). We have further explored this process, and validated ID1 as one of the main factors involved in this regulation (see Chapter Four – The role of ID1 in regulating the anti-tumor immune response).

Advances in our understanding of the molecular and cellular biology of cancer during the past decade underscore the importance of the bone marrow in solid-tumor progression and metastasis. In 2005, it was shown for the first time that BMDCs are crucial for the formation of the pre-metastatic niche and, consequently, for metastasis development (Kaplan et al., 2005). More recently, it was suggested that indolent tumors are incapable of systemically activating the bone marrow, while actively growing tumors are

capable of doing so (Albini and Sporn, 2007). In fact, emerging evidence suggests that tumor-derived soluble factors and exosomes are secreted into the peripheral circulation and may stimulate the bone marrow compartment to switch from a quiescent state into a pro-tumorigenic environment, inducing expansion of bone marrow progenitor cells and mobilization and recruitment of BMDCs to the tumors and to the pre-metastatic niches (Peinado et al., 2012; Gao and Mittal, 2009). Our study shows that, with STAT3 phosphorylation, IL-6 expression is increased in the bone marrow microenvironment and this in turn is associated with an increase not only in the number of MDSCs but also in the number of hematopoietic progenitor cells (Sca1+ckit+ cells). Using a spontaneous tumor model, we confirm that IL-6 expression is increased in bone marrow progenitor cells during tumor progression and that this alteration occurs at early stages of tumor progression, before metastatic disease forms. By transplanting IL-6 -/- mice with WT bone marrow and reverting the metastatic phenotype, we show that IL-6 expression in the bone marrow is crucial for metastatic development. These findings support the concept of a determinant role of the bone marrow in metastatic development and identify IL-6 as one of the principal factors mediating this process.

Our work reveals a previously unrecognized role for IL-6 in controlling pro-metastatic changes in the bone marrow microenvironment during tumor progression. Our findings support the evaluation of pharmacological strategies for targeting IL-6 at early stages of tumor progression. Anti-IL-6 therapies might offer benefit to patients in the adjuvant by reducing the numbers of hematopoietic progenitor cells in the bone marrow, as well as reducing myeloid-cell mobilization and recruitment to pre-metastatic niches, thereby impeding metastatic disease before it becomes clinically evident.

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4. CHAPTER FOUR
THE ROLE OF ID1 IN REGULATING BMDC DIFFERENTIATION

EXPLORING THE ROLE OF **INTERLEUKIN-6** AND **OTHER HOST-ASSOCIATED FACTORS** IN **TUMOR PROGRESSION** AND **METASTASIS DEVELOPMENT**

4. CHAPTER FOUR
THE ROLE OF ID1 IN REGULATING BMDC DIFFERENTIATION [4]

4.1 SUMMARY

In recent years, and as discussed in the previous chapters, it has become clear that bone-marrow derived myeloid cells play a crucial role in tumorigenesis and metastasis development. These cells affect cancer progression by interacting directly with tumor cells and indirectly by enabling a tumor stroma that promotes cancer growth. In fact, a central mechanism of tumor progression and metastasis involves the generation of an immunosuppressive 'macroenvironment' mediated through tumor and host-secreted factors. Our work demonstrates that upregulation of the Inhibitor of Differentiation 1 (Id1), in response to tumor-derived factors such as TGF-β, redirects BMDC differentiation towards Id1-high expressing MDSC with a reciprocal decrease in DC numbers. Genetic inactivation of Id1 largely corrects the myeloid imbalance, whereas Id1 overexpression in the absence of tumor-derived factors re-creates it. Id1 overexpression down-regulates key DC differentiation pathway molecules such as Interferon regulatory factor 8 (Irf8) and leads to regulatory T-cell (Treg) expansion, increased reactive oxygen species (ROS) production and suppression of CD8 T-cell proliferation which in turn promote primary tumor growth and metastatic progression. This study reveals a critical role for Id1 in suppressing the anti-tumor immune response during tumor progression.

[4] Based on: Marianna Papaspyridonos, Irina Matei, Yujie Huang, Maria do Rosário André, et al. Id1 suppresses anti-tumor immune responses and promotes tumor progression by impairing myeloid cell maturation. Nat Commun. 2015. 6:6840

4.2 INTRODUCTION

One of the key mechanisms of tumor outgrowth and progression to metastatic disease involves the ability of tumors to prevent the immune system from mounting an efficient anti-tumor response (Gabrilovich et al., 2012). At the core of this systemic tumor-induced immunosuppression lies the defective differentiation of bone marrow-derived myeloid cells occurring in response to circulating tumor and host derived factors (Serafini et al., 2006; Peinado et al., 2011; Engblom et al., 2016). Many tumor and host-derived factors, including IL-6, VEGF, IL-4, IL-13 and TGFβ, regulate redundant pathways likely related to myeloid cell differentiation (Fricke et al., 2007; Shojaei et al., 2007). In particular, these factors prevent the differentiation of BMDCs from giving rise to fully functional antigen-presenting cells (APCs), such as DCs and macrophages (Menetrier-Caux et al., 1998; Almand et al., 2000; Della Bella et al., 2003) and instead redirect the differentiation pathway towards the accumulation and expansion of a heterogeneous population of immature myeloid cells called MDSCs (Bronte et al., 2001; Cheng et al., 2008; Talmadge and Gabrilovich, 2013).

DCs are the most potent APCs, able to recognize, acquire, process, and present antigens to naive, resting T cells for the induction of an antigen-specific immune response (Steinman and Banchereau, 2007). Most DCs differentiate along the myeloid lineage pathway, with smaller percentages giving rise to CD8+ DC and plasmacytoid DC. Increasing evidence shows that the main DC pathway affected in cancer patients is the myeloid DC pathway (Gabrilovich, 2004). The consequences of decreased number of functionally competent DCs in tumor bearing hosts are clear: a decline in APCs renders immunostimulation less effective (Almand et al., 2000; Della Bella et al., 2003).

In contrast, numerous studies have confirmed an immunosuppressive role of MDSC accumulation through profound effects in T-cell suppression (Gabrilovich and Nagaraj, 2009; Serafini et al., 2006; Shojaei et al., 2007; Pages et al., 2010; Gabrilovich and Nagaraj, 2009). In mice, MDSCs are broadly characterized by the expression of the markers CD11b and Gr-1 and consist of two major subsets of Ly6G+Ly6Clow granulocytic and Ly6G–Ly6Chigh monocytic cells. Granulocytic MDSCs have increased level of ROS and undetectable level of nitric oxid (NO) whereas monocytic MDSC have increased level of NO but undetectable levels of reactive oxygen species. However, their suppressive activity per cell basis is comparable (Youn et al. 2011; Corzo et al., 2009). MDSCs use a variety of antigen-specific and non-specific immunosuppressive mechanisms to suppress T cell function, including increased arginase activity levels, as well as

NO and ROS production (Gabrilovich and Nagaraj, 2009; Kusmartsev et al., 2008; Kusmartsev et al., 2004; Rodriguez and Ochoa, 2008; Sica and Bronte, 2007). MDSCs have been found to accumulate in the circulation, lymphoid organs, primary and metastatic organs of most tumor models (Youn et al., 2008) and in patients with various types of cancers including breast, renal, and colorectal cancer (Almand et al., 2001; Fricke et al., 2007; Mandruzzato et al., 2009; Zea et al., 2005). MDSCs are thought to contribute, in large part, towards the limited effectiveness of cancer vaccines and other therapies, such as anti-VEGF treatment (Fricke et al., 2007; Kusmartsev et al., 2003; Shojaei et al., 2007). However, it currently remains unknown whether tumor-secreted factors drive an alternative developmental pathway that co-regulates the decline in DCs and expansion of MDSCs via the upregulation of common transcriptional regulators during tumor progression.

The Inhibitor of Differentiation 1 (Id1) is a member of a family of transcriptional regulators that inhibit basic helix-loop-helix transcription factors from binding DNA (Benezra et al., 1990; Chan et al., 2009). Increased Id1 protein expression in tumors has been shown to correlate with both cancer progression and poor prognosis (Fong et al., 2004, Perk et al., 2005). Furthermore, Id1 regulates endothelial cell differentiation and fosters tumor vasculogenesis (Lyden et al., 2001; Lyden et al., 1999), promotes progression from micro- to macrometastatic disease (Gao et al., 2008) via endothelial progenitor cell mobilization and has been involved in myeloid development (Buitenhuis et al., 2005; Geest et al., 2009; Jankovic et al., 2007; Suh et al., 2008). However, Id1 has not been previously involved in regulating the crosstalk between tumors and the host immune system at a systemic level and promoting tumor progression and metastasis via the suppression of myeloid cell differentiation.

In this chapter, we present Id1 as a novel pivotal regulator of the switch from DC differentiation to MDSC expansion during tumor progression. We demonstrate that inappropriate upregulation of Id1, primarily in response to tumor-derived TGF-β, redirects BMDC differentiation towards Id1-high expressing MDSCs with a reciprocal decrease in DC numbers. Id1 overexpression results in a systemic immunosuppressive phenotype that inhibits CD8 T-cell proliferation and increases primary tumor growth and metastatic progression. Our observations confirm and extend the promise of Id1 as a biomarker of cancer progression and as a therapeutic target in the management of advanced malignancies.

4.3 MATERIAL AND METHODS

MICE

C57BL/6 mice were purchased from Harlan Laboratories or the Jackson Laboratory (Bar Harbor, ME); OT-II mice were obtained from The Jackson Laboratory. Generation of Id1−/− mice has been previously reported (Chan et al., 2009). Animals used in all experiments were matched for sex, age (8-10 weeks old), and genetic background (C57BL/6/Sv129). All animal procedures were approved and performed under the guidelines of the IACUC at Weill Cornell Medical College, protocol (IACUC 0709-666A).

ISOLATION AND IN VITRO DIFFERENTIATION OF LINEAGE NEGATIVE CELLS

Bone-marrow cells were harvested from the femurs and tibias of 8-12 week-old mice and enriched for hematopoietic progenitor cells by depletion of lineage-specific cells using the EasySep Hematopoietic Progenitor Enrichment Kit (StemCell Technologies) as per manufacturer's recommendations. One million enriched HPCs were placed into each well of 6-well plates in 2-ml of RPMI supplemented with 10% fetal bovine serum and 20 ng/ml GM-CSF. Complete medium was replaced every 3 days and cells were collected for further analysis at indicated time points. To assess the effects of tumor-derived factors on DC differentiation, Lin- cells were treated with complete medium supplemented with 25% v/v serum-free medium conditioned overnight by subconfluent cultures of the B16F10 melanoma or control media.

PLASMIDS

PGEW-empty and PGEW-Id1 vectors were built from plasmid pCCL.sin.cPPT.PGK.GFP.WPRE as previously described (Chan et al., 2009).

VIRUS PRODUCTION AND TITRATION

Lentiviral vector stocks, pseudotyped with the vesicular stomatitis G protein, were produced by transient co-transfection of 293T cells and titred on HeLa cells, as previously described (Yuan, et al., 2011). Viral supernatants were concentrated to titres ≥ 108 transduction units per ml by ultracentrifugation.

TRANSDUCTION OF TUMOR AND BM LIN- CELLS

Lin- cells plated at a density of 1x106 cells per ml in StemSpan Serum Free Expansion Medium (StemCell Technologies) were transduced with concentrated virus for 12 h (multiplicities of infection = 50–60), washed and resuspended in PBS for transplantation in irradiated mice or subsequent in vitro studies.

IMMUNOFLUORESCENCE STAINING

B16F10 tumors and lung tissues were fixed in 4% paraformaldehyde before being embedded in Optimal Cutting Temperature compound. Immunofluorescence staining was performed using rat anti-mouse CD31 antibody (BD Biosciences), biotinylated anti-rat IgG as a secondary antibody and Texas Red Avidin DCS (Vector Laboratories, Inc.). Cryosections (Leica cryostat) were mounted with Vectashield containing DAPI and were visualized with an ultraviolet fluorescent microscope (Nikon Eclipse E800) with a Retiga camera (QImaging) through IP Lab version 3.65a imaging software (Scanalytics).

OT-I T-CELL ASSAYS

Equal number of GFP+ CD11b+ Gr1+ cells isolated by FACS from Id1-overexpressing and control vector splenocytes animals were co-cultured in the presence of OVA257-264 peptide with splenocytes isolated from OT-I transgenic mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J, JAX) and stained using CellTrace™ CFSE Cell

Proliferation Kit (Invitrogen). T-cell proliferation was measured by CFSE dilution following a 4-day incubation in 96-well tissue culture-treated plates (Corning).

OT-II T-CELL ASSAYS

Single-cell suspensions of splenocytes from Id1-overexpressing and control vector animals (105 cells) were co-cultured in the presence of OVA323-339 peptide with 105 CD4+ T-cells isolated from OT-II transgenic mice (C57BL/6-Tg(TcraTcrb)425Cbn/J, JAX) using the CD4+ negative selection kit (Miltenyi biotec) and stained using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). T-cell proliferation was measured by CFSE dye dilution and cytokine production by ELISA (R&D systems) following a 72-hour incubation in 96-well tissue culture-treated plates (Corning).

BONE MARROW TRANSPLANTATION

Recipient mice were lethally irradiated with a single dose of 9.5 Gy of whole-body irradiation. Twenty-four hours after irradiation, 2×106 donor lineage-depleted cells isolated from BM cells were injected via tail vein.

TUMOR IMPLANTATION

C57BL/6 mice were injected in the mammary fat pad with 2x105 EO771 cells or intradermally in the flank with 1x106 B16F10 cells. Both cell lines were obtained from the American Type Culture Collection. Tumor dimensions were calculated by caliper measurements and volume was calculated according to the equation: Volume = (length x width x depth)/ 2.

FLOW CYTOMETRY

Single-cell suspensions of splenocytes were stained at 4°C in PBS with 3% (vol/vol) FBS, following red blood cell lysis (Gibco, Invitrogen) and incubation with purified Fc-block (CD16/CD32, BD). The following antibodies were used for staining: Anti-mouse: anti-CD11c PE (HL3), anti-Gr1 PE (RB6-8C5), anti-CD11b FITC (M1/70), anti MHC Class II FITC(I-A/I-E; M5/114.15.2), anti MHC Class II FITC (I-Ek; 14-4-4S), anti-CD34 PE and anti-IFNγ, all obtained from BD Pharmingen; anti-Ly6G PE (1A8) and anti-Ly6C APC (HK1.4), both obtained from Biolegend; anti-CD115 APC (AFS98), abti-CD49b PE-Cy7 (DX5), anti-CD3 PE-Cy7 (145-2C11), anti-CD19 PE-Cy7 (1D3), anti-Ter119 PE-Cy7 (TER119), anti-Gr1 PE-Cy7 (RB6-8C5), anti-CD117 (c-kit) APC-eFluor780 (2B8), anti-CD16/CD32 Alexa700 (93), anti-Sca-1 PE-Cy5 (D7), anti-CD135 biotin (A2F10), Streptavidin PerCP-Cy5.5, anti-CD4 FITC (RM4-5), anti-CD4 Pacific Blue (RM4-5), anti-CD25 APC (PC61), anti-CD25 Alexa700 (PC61.5), anti-Foxp3 PE (FJK-16s), anti-CD11b PE-Cy5 (M1/70), anti-CD8a APC-eFluor780 (53-6.7), anti-V alpha 2 TCR PE (B20.1), anti-Gr1 APC (RB6-8C5), anti-CD11b APC (M1/70) and anti-Gr1 APC-eFluor780 (RB6-8C5), and anti-human anti-CD33 PE (WM53), CD11B PerCp-Cy5.5 (M1/70), anti-CD14 Alexa 700 or FITC (M5E2) and anti-HLA PE-Cy7 (L243), obtained from BD or eBioscience, and anti-VEGFR1 APC (49560) obtained from R&D Systems. Data were acquired on a FACSCalibur, a FACSCanto or an LSR II (BD Biosciences) and analyzed with FlowJo software (Treestar). FACS was performed on a Vantage cell sorter (BD Biosciences).

MEASUREMENT OF ROS

ROS was measured by labelling cells with the oxidation-sensitive dye dichlorodihydrofluorescein diacetate (DCFDA, Abcam) according to the manufacturer's instructions and analysis was carried out by flow cytometry on a FACSCalibur (BD Biosciences).

QPCR ANALYSIS

Total RNA was extracted from cells using the RNeasy Mini-Kit (Qiagen), according to manufacturer's protocol.

Genomic DNA was removed by treatment with DNase I (Qiagen). cDNA was synthesised using the Superscript III reverse transcription kit (Invitrogen). qPCR was performed on a 7500 Fast Real Time PCR System (Applied Biosystems), using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers for qPCR: Mouse Id1-forward primer: TTGTTCTCTTCCCACACTCTGTTC; Mouse Id1-reverse primer: CTGGCGACCTTCATGATCCT; Mouse Id1-probe: 5'FAM-CAGCCTCCTCCGCTCCCCTCC-3'TAMRA. All other sets were commercial proprietary Taqman assays purchased form Applied Biosystems. Relative expression was normalized to β-actin levels.

MICROARRAY PREPARATION AND ANALYSIS

Total RNA was isolated from Lin- BM cells transduced with Id1-overexpressing or control lentivirus for and cultured for 6 days as described above, using the RNeasy Mini Kit (Qiagen). The Affymetrix One-Round In Vitro Transcription RNA Amplification Kit was used to amplify 1.5 μg of total RNA. The cDNA was synthesized with a primer containing oligo(dT) and T7 RNA polymerase promoter sequences. Double-stranded cDNA was then purified and used as a template to generate biotinylated cRNA. The quantity and quality of the amplified cRNA was assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and an Agilent Bioanalyzer. The biotinylated cRNA was fragmented and hybridized to Affymetrix Mouse Genome 430A 2.0 arrays representing approximately 14000 well-characterised mouse genes. After hybridization, the GeneChip arrays were washed, stained and scanned using a GeneChip Scanner 3000 7G. Affymetrix GeneChip Operating Software was used for image acquisition. Analysis was performed using GeneSpring GX 15.11 software (Agilent Technologies Inc., USA). Robust Multichip Average (RMA) with Quantile normalization was used for background correction and normalization of CEL files. Genes differentially expressed were identified by using a fold-change cut-off of 1.4. Pathway analysis of differentially expressed genes was carried out using IPA to determine significant gene networks and canonical pathways in IPA version 8.6 (Ingenuity® Systems, www.ingenuity.com).

INFγ AND IL-10 ELISA

Plasma levels of IFNγ and IL-10 were determined using the Mouse IFNγ and IL-10 Quantikine ELISA Kits (R&D Systems,) according to manufacturer's instructions.

B16F10 EXOSOME PURIFICATION

To isolate exosomes, serum-free B16F10-conditioned media was centrifuged at 500g for 10 min. The supernatant was then removed and re-centrifuged at 12000g for 20 min. Exosomes were then harvested by centrifugation at 100000g for 70 min. The exosome pellet was resuspended and washed in 20 ml of 1xPBS and collected by centrifugation at 100000g for 70 min (Beckman Optima XE ultracentrifuge equipped with TY-70Ti rotor). Freshly isolated B16F10 exosomes were added to Lin- cell cultures at 10 μg ml -1.

WESTERN BLOT

Three million CD11b+ splenocytes isolated using CD11b+ micro-beads (Miltenyi Biotec) from naive or B16F10 tumor-bearing mice were lysed in 100 ml RIPA cell lysis buffer (Thermo Scientific) containing a cocktail of protease inhibitors (Roche). The supernatant of cell lysis was subjected to western blotting analysis with anti-mouse ID1 (Biocheck) and anti-b-actin antibodies (Santa Cruz). The western blot was carried out in three independent replicate experiments.

STATISTICAL ANALYSIS

Statistical and graphical analyses were performed using GraphPad Prism software (version 3.0). Data were analyzed using Student's unpaired t-test, one-way analysis of variance and Mann-Whitney test. Results were considered statistically significant at P values <0.05. Error bars depict standard error mean.

4.4 RESULTS

TUMOR-SECRETED FACTORS FAVOR BMDC DIFFERENTIATION TOWARDS ID1- HIGH MDSC

To assess differences in myeloid cell differentiation during tumor progression, we used the syngeneic EO771 breast cancer tumor model. Twenty one days following inoculation of C57BL/6 mice with mammary adenocarcinoma EO771 cells (at the advanced metastatic stage) spleens were harvested and splenocytes were analyzed by flow cytometry. We observed a decrease in the frequency and absolute numbers of DC, defined as CD11c+MHCII+ cells, in EO771 tumor-bearing mice compared to non-tumor-bearing mice (Figure 4.1 A). Conversely, we detected an increase in the frequency and absolute numbers of MDSC, defined as CD11b+Gr1+ cells, in tumor-bearing mice compared to controls (Figure 4.1B). Similar findings were observed in mouse spleens isolated 21 days after orthotopic implantation with the B16F10 melanoma cell line (Figure 4.2 A, B).

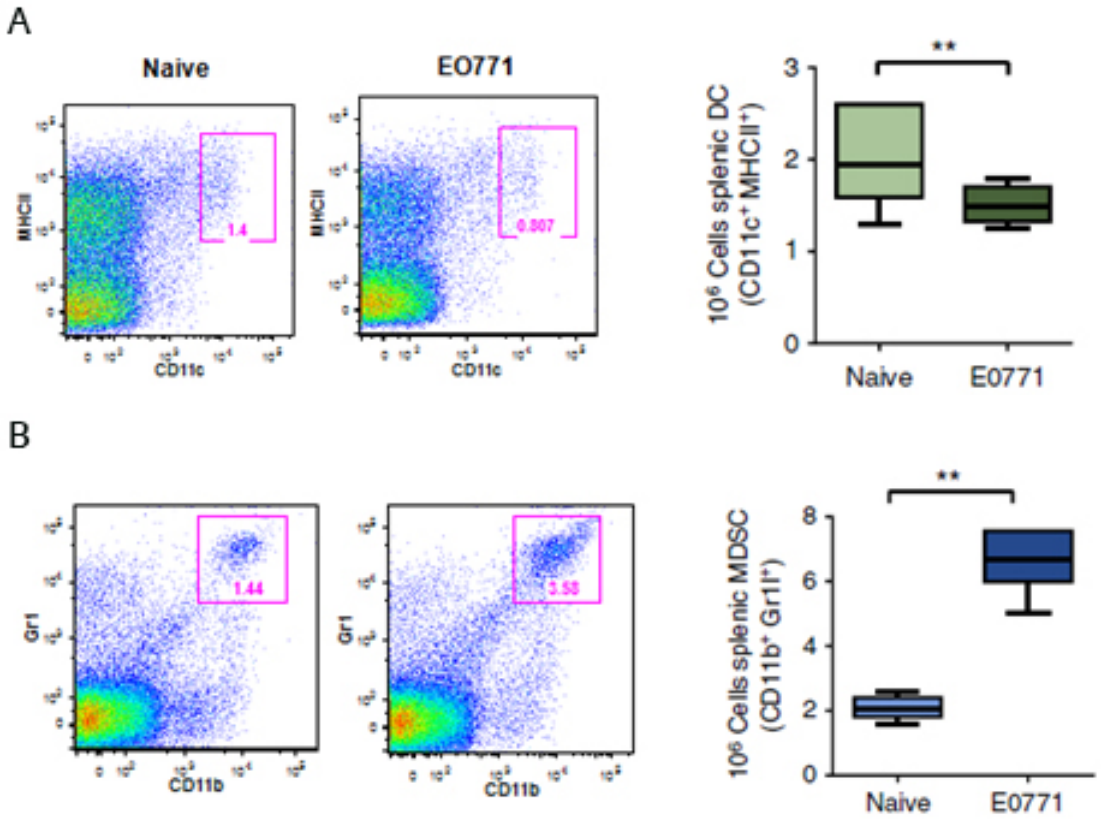


Figure 4.1. Flow cytometry analysis of spleens from naïve and EO771 mammary adenocarcinoma-implanted mice (Day 21 post implantation) for (A) frequency and absolute numbers of DC, and (B) frequency and absolute numbers of MDSC (Unpaired t-test, *p < 0.05, ***p < 0.001).

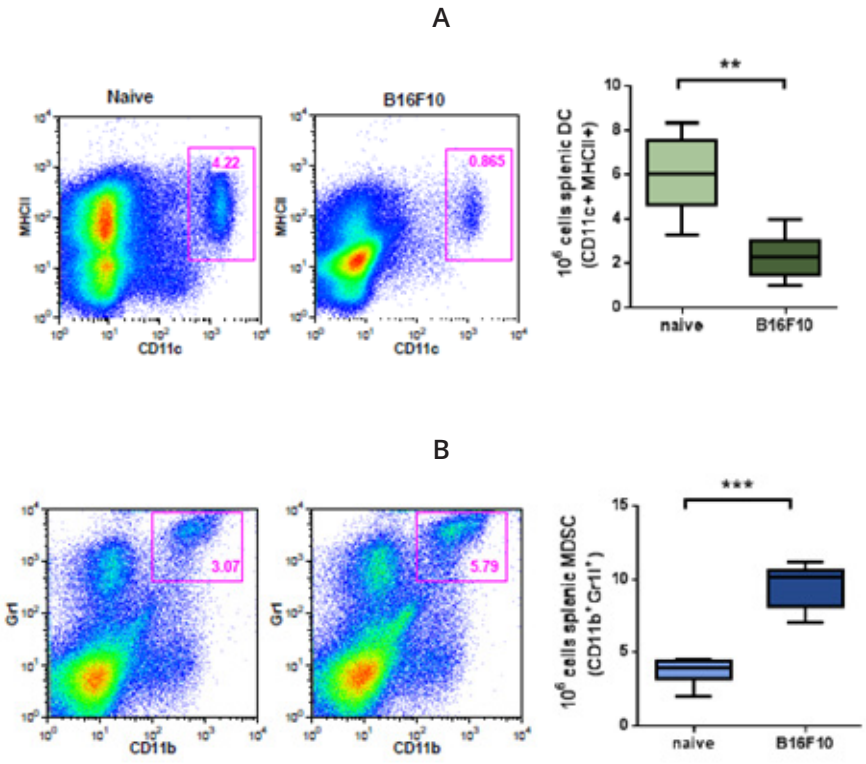


Figure 4.2. Flow cytometry analysis of splenic populations from B16F10 melanoma-implanted mice (Day 21 post implantation) (A) Frequency and absolute numbers of DC (Unpaired t-test $p < 0.01$). (B) Frequency and absolute numbers of MDSC (Unpaired t-test, ***$p < 0.001$).

As Id1 and Id3 upregulation in BM cells had been previously implicated in tumor and metastatic progression (Lyden et al., 2001; Gao et al., 2008; Kaplan et al., 2005), we sought to examine whether either of these transcriptional regulators were differentially expressed in DC and MDSC populations in tumor- versus non-tumor bearing mice. Splenic DC and MDSCs were isolated using fluorescence-activated cell sorting (FACS), and Id1 and Id3 expression was assessed by quantitative real time PCR (qPCR) analysis. We found that DCs isolated from non-tumor mice expressed very low to undetectable Id1, whereas Id1 expression was higher in MDSC from tumor-bearing mice compared with both control MDSC (Fig. 4.3) and DC from tumor-bearing mice (Fig. 4.3). Similarly to Id1, Id3 expression was higher in DC from tumor-bearing mice compared to DC from control mice, however, Id3 expression levels in MDSC from tumor-bearing mice were significantly lower compared to MDSC from non-tumor-bearing mice (Figure 4.3). We therefore focused our subsequent studies specifically on Id1.

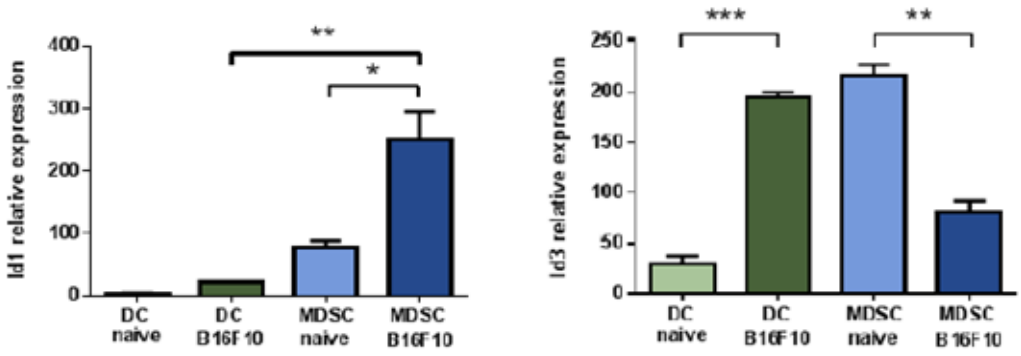


Figure 4.3. Id1 and Id3 mRNA levels in FACS-sorted splenic DC and MDSC populations, as determined by qPCR analysis, (n = 6, One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).

We then assessed Id1 protein levels in lysates from CD11b+ bead-sorted splenocytes isolated from naïve or B16F10-bearing mice. The western blot and densitometric analysis revealed a 6.1-fold Id1 upregulation at the protein level in B16F10-bearing CD11b+ splenocytes compared with controls (Fig.4.4 A, B). We also sought to examine if Id1 expression is associated with a particular MDSC subtype – monocytic or granylocytic. Assessment of Id1 mRNA expression levels in FACS-sorted monocytic and granulocytic MDSC populations from spleens from naïve and B16F10-bearing mice on days 7, 14 and 21 following implantation showed that increased Id1 expression is associated with both monocytic and granulocytic subsets, with increased levels in the monocytic subset in the earlier phase of tumor growth and the granulocytic subset in the advance metastatic stage (2.5-fold and 3.5-fold respectively, Fig. 4.4 C). Similar experiments were performed with DC and MDSC FACS- sorted splenic populations from the EO771 mammary adenocarcinoma model and the Id1 expression profile was comparable to the one observed in the B16F10 model (Fig. 4.4 D).

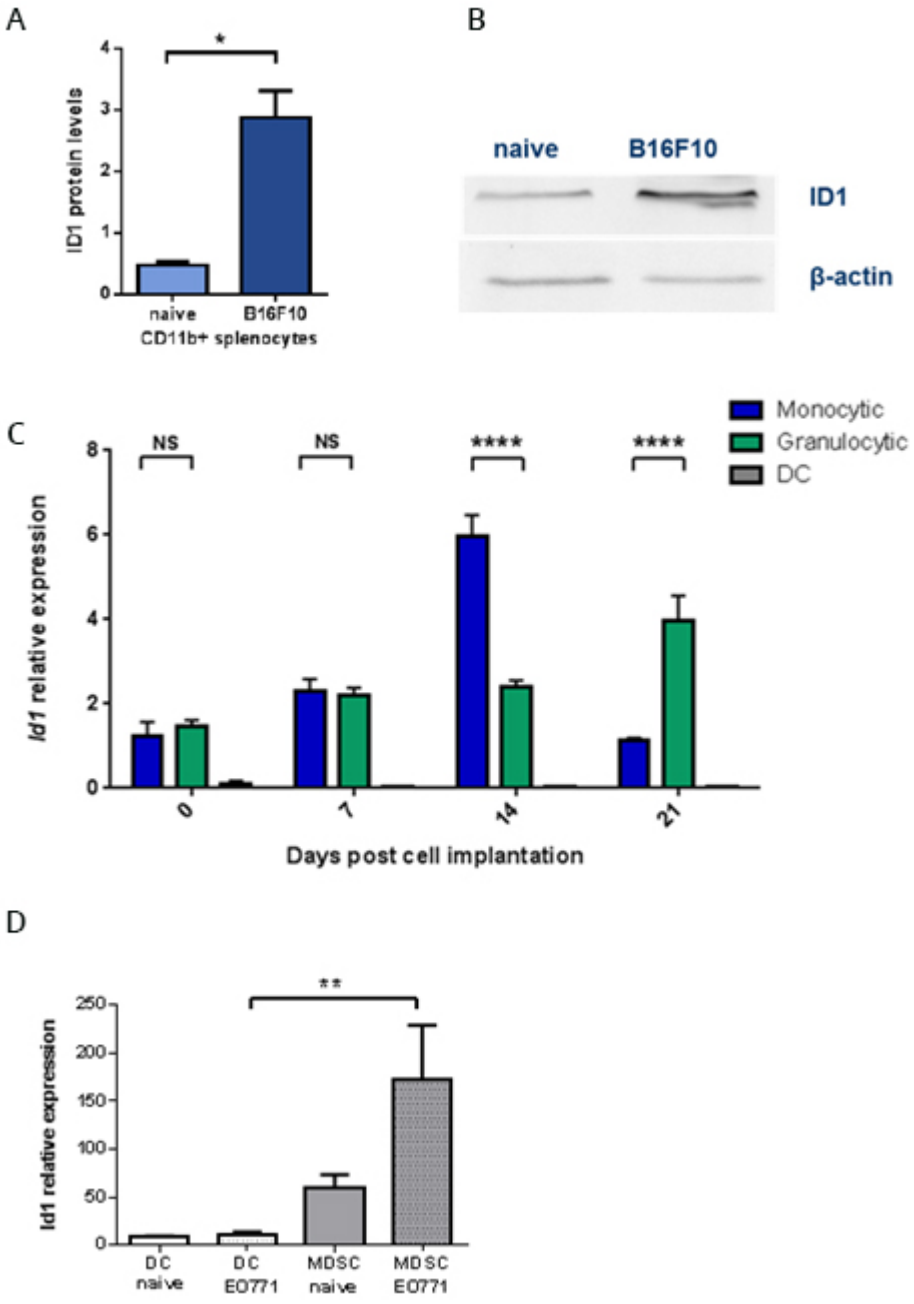
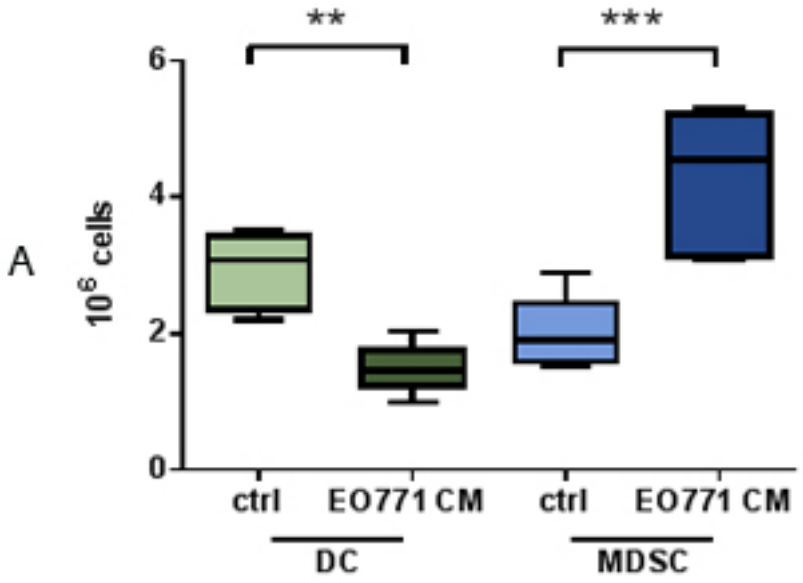


Figure 4.4. (A) Id1 protein levels in lysates from naïve and B16F10 bearing CD11b+ bead sorted splenocytes as determined by western blot and densitometric analysis (Unpaired t-test $p < 0.01$); (B) Id1 protein levels in lysates from naïve and B16F10 bearing CD11b+ bead sorted splenocytes as determined by western blot (representative results from three independent experiments); (C) Id1 mRNA expression levels of FACS-sorted splenic DC (CD11c+), monocytic (CD11b+Ly6C+) and granulocytic (CD11b+Ly6G+) MDSC populations from spleens from naïve and B16F10 melanoma-bearing mice, on days 7, 14 and 21 following implantation as determined by qPCR analysis (means ± SEM, n = 5, One-way ANOVA, ****$p<0.001$); (D) Id1 mRNA expression levels of FACS-sorted splenic DC and MDSC populations from spleens from EO771 mammary adenocarcinoma-implanted mice, as determined by qPCR analysis (means ± SEM, n = 6, One-way ANOVA, *$p < 0.05$).

As BM precursors give rise to all mature immune cells present in secondary lymphoid organs in vivo, we developed an in vitro model that mimics this differentiation process (BMDC assay). To determine whether differences in myeloid differentiation were due to circulating tumor-secreted factors, lineage negative (Lin-) hematopoietic progenitors were isolated from the bone marrow of C57BL/6 mice and cultured for 6 days in the presence of EO771 tumor-conditioned media (TCM) or control media. Using flow cytometry on day 6 of culture, we observed a decrease in the absolute DC numbers that differentiated in the presence in TCM, compared with control media (Fig. 4.5 A). In contrast, an increase in absolute MDSC numbers was observed on day 6 of culture with TCM compared to control media (1.8-fold; Fig. 4.5 A). Experiments performed with B16F10 TCM revealed a similar imbalance in DC versus MDSC frequencies concurrent with Id1 upregulation (Fig. 4.5 B). Gene expression analysis after 6 days of in vitro differentiation in B16F10 TCM revealed that Id1 mRNA expression levels were significantly higher in WT (Lin-) cells differentiated in the presence of B16F10 TCM compared to control media (4.9-fold; Fig. 4.5 C).



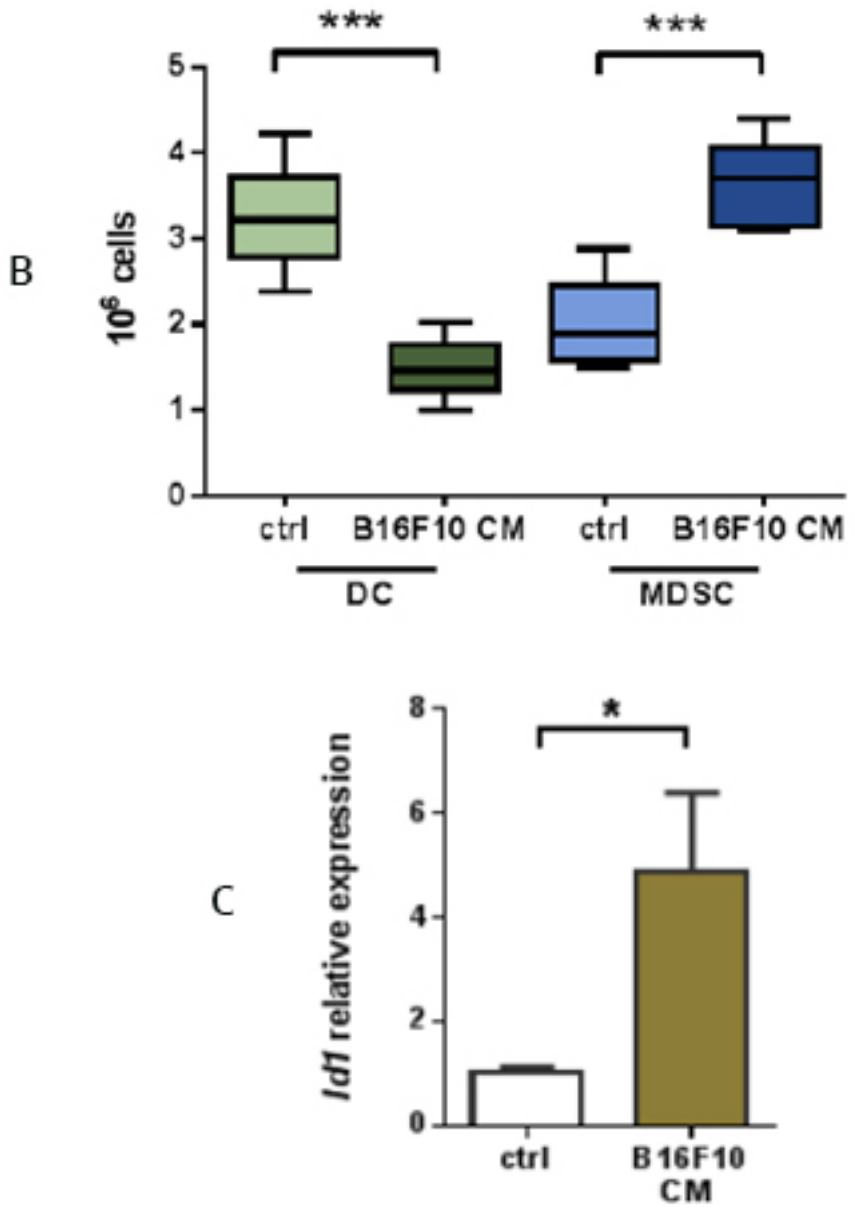
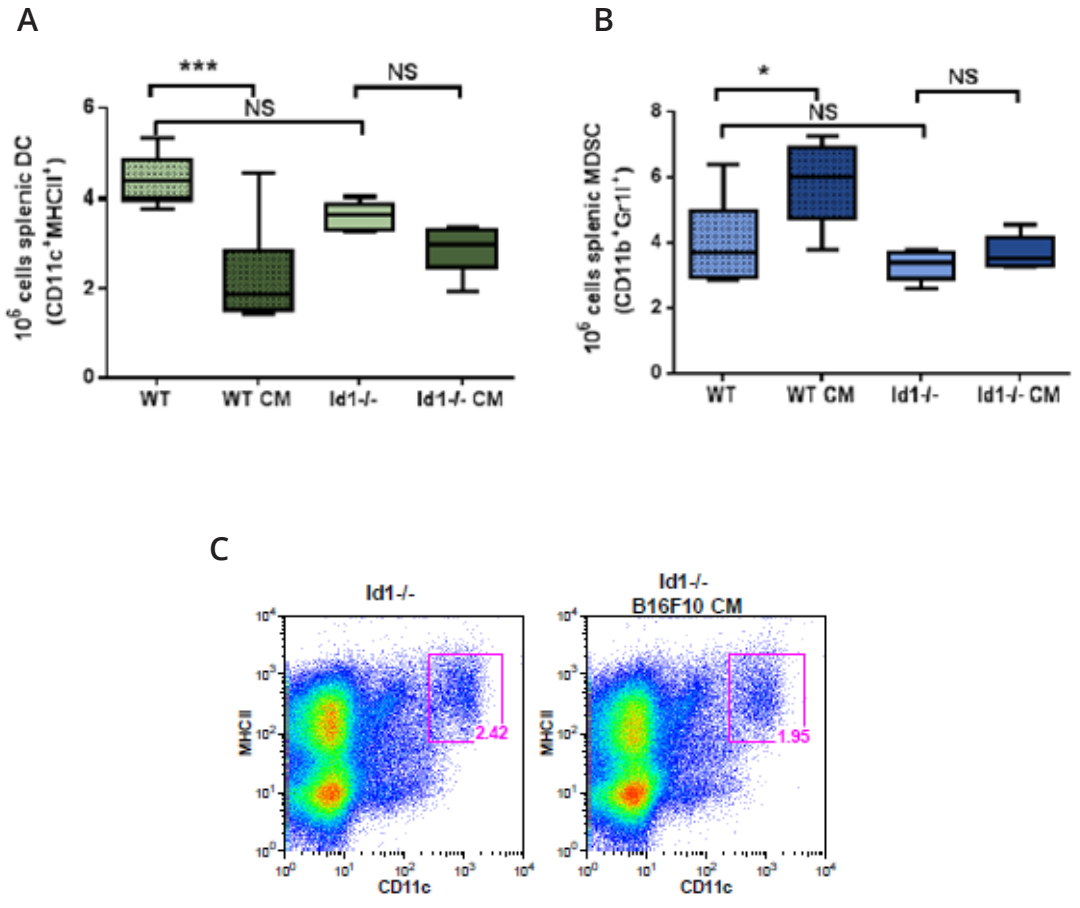


Figure 4.5. (A) In vitro differentiation of Lin- hematopoietic progenitors isolated from C57BL/6 mice, cultured for 6 days in the presence of EO771 mammary adenocarcinoma TCM (25% v/v) and analyzed for DC and MDSC content by flow cytometry (means ± SEM, n = 6, Unpaired t-tests, **p < 0.01, ***p < 0.001); (B) In vitro differentiation of lin- cells, cultured for 6 days in the presence of B16F10 melanoma TCM (25% v/v), and analyzed for DC and MDSC content by flow cytometry (n = 6, ANOVA, ***p < 0.001). (C) Id1 mRNA relative expression levels of Day 6 Lin- cells differentiated in the presence B16F10 conditioned media compared to control media, as determined by qPCR analysis (means ± SEM, n = 6, Unpaired t-test, *p < 0.05).

DELETION OF THE ID1 GENE RESTORES MYELOID DIFFERENTIATION DEFECTS IN VITRO AND IN VIVO

To assess whether Id1 is a direct regulator of MDSC and DC differentiation during tumor progression, we performed a series of experiments using Id1-/- mice. As Id1-/- mice have well-documented tumor angiogenic defects and abnormal tumor growth (Lyden et al., 2001), we performed daily injections of B16F10 melanoma TCM and control media over 21 days to systemically supply an equal amount of tumor-derived factors in both Id1-/- and WT control mice.

B16F10 TCM injections led to a significant reduction in splenic DC in WT versus TCM treated WT mice (1.9-fold; Fig. 4.6 A) that was comparable to the DC population reduction observed in tumor-bearing hosts. A non-statistically significant reduction in the DC population was detected in Id1-/- mice injected with TCM versus control media (1.16-fold; Fig. 4.6 A, C). Likewise, similar to the splenic MDSC expansion observed in tumor bearing hosts, WT mice injected with TCM exhibited an increase in MDSC compared to naïve mice (1.5-fold; Figure 4.6 B), with both monocytic and granulocytic populations equally affected across groups (Figure 4.5 E,F) whereas no expansion in MDSC was seen with TCM injection in Id1-/- mice (Figures 4.6 B, D).



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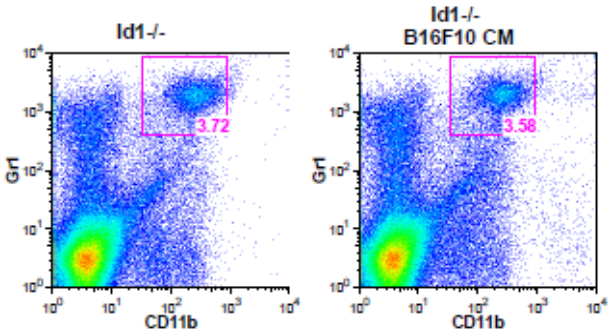
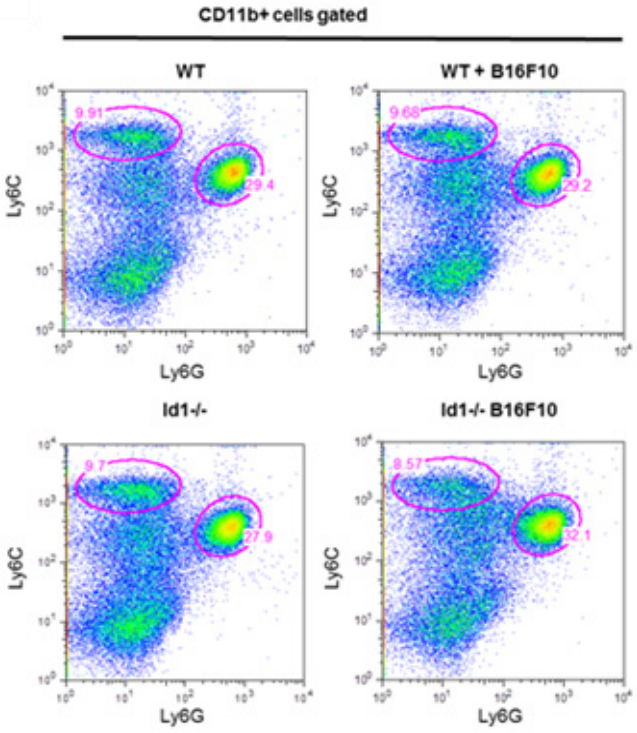

Id1-/-
Id1-/-
B16F10 CM
3.72
3.58
Gr1
CD11b

E


CD11b+ cells gated
WT
WT + B16F10
Id1-/-
Id1-/- B16F10
9.91
29.4
9.68
29.2
9.7
27.9
8.57
32.1
Ly6C
Ly6G

F

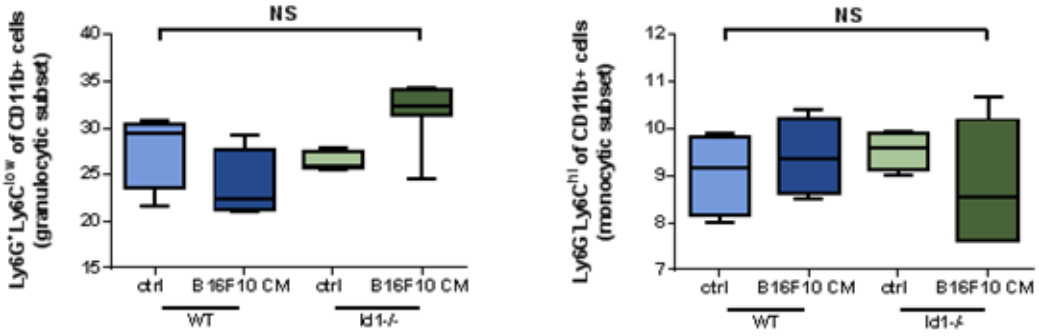


Figure 4.6. Flow cytometry analysis of spleens from WT and Id1-/- mice that received daily injections of B16F10 melanoma-derived TCM or control media for (A) absolute numbers of DC (One-way ANOVA, ***p < 0.001, NS: not significant) and (B) absolute numbers of MDSC levels (One-way ANOVA,, *p < 0.05, NS: not significant). (C) Example frequency plots of DC and (D) Splenic MDSC isolated from Id1-/- mice injected daily with B16F10 melanoma-derived TCM or control media; (E) Flow cytometry analysis of granulocytic and monocytic MDSC subsets from WT and Id1-/- splenocytes of mice injected daily with B16F10 TCM or control media over 21 days; (F) Summary graphs of granulocytic and monocytic subset levels across all animal groups (One-way ANOVA, not significant).

In summary, genetic ablation of Id1 largely restored terminal myeloid differentiation, as daily injections of B16F10 TCM prevented the DC reduction and MDSC expansion that was observed in WT controls that also received daily injections of B16F10 TCM. These data suggest that Id1 has a critical role in mediating the myeloid differentiation defects caused by tumor-derived factors in vivo and support our previous findings in steady state Id1-/- mice where we observed an increase in terminal myeloid differentiation in the peripheral lymphoid organs and lower frequencies of common myeloid progenitors (CMPs) in the BM of Id1-/- mice (Chan et al., 2009).

To further validate the role of Id1 in impairing myeloid differentiation, we employed the BMDC assay using Id1-/- cells. In contrast to the results obtained with WT cells, we detected a significant increase in Id1-/- DC numbers when BM progenitors were cultured in the presence of B16F10 melanoma TCM (1.4-fold; Fig. 4.7 A) and no significant difference in MDSC numbers compared to control-media cultures of Id1-/- cells, indicating that Id1 has a causal role in the myeloid differentiation impairment observed in the presence of tumor-derived factors both in vitro and in vivo. Gene expression analysis after 6 days of in vitro differentiation in B16F10 TCM revealed that the up-regulation of S100a8 and Vegfr1 - two established markers

of immature myeloid status (Kusmartsev et al., 2008; Kaplan et al., 2005; Sinha et al., 2008; Hiratsuka et al., 2008) - was abrogated in Id1-/- Lin- cells compared to WT cells (Figure 4.7 B). This suggested that in the absence of Id1, myeloid maturation is promoted. Id3 expression levels were not found to be significantly different between WT and Id1-/- cells cultured with TCM, (Fig. 4.7 B) excluding any potential compensatory mechanisms by Id3.

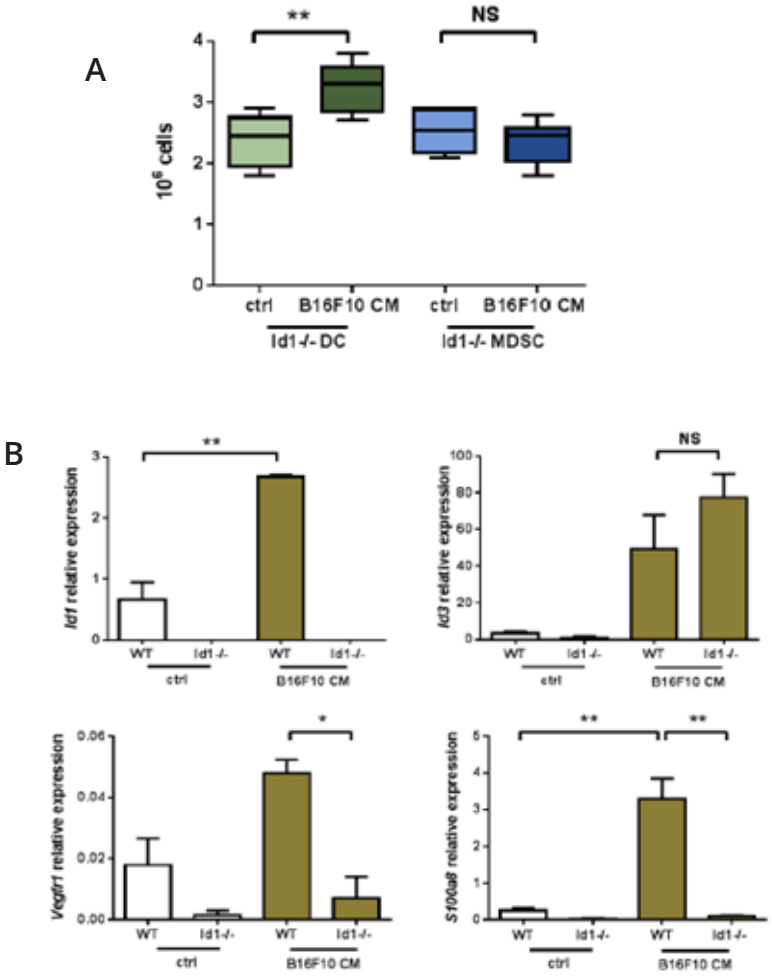


Figure 4.7. In vitro differentiation of Lin- hematopoietic progenitors isolated from Id1-/- mice, cultured for 6 days in the presence of B16F10 melanoma TCM (25% v/v) and analyzed for DC and MDSC content by flow cytometry (n = 6, ANOVA, **p < 0.01, NS: not significant). (F) Gene expression analysis of Id1-/- and WT cells after 6 days of in vitro differentiation in the presence of TCM, as determined by qPCR analysis (means ± SEM, n = 6, One-way ANOVA, **p < 0.01, *p < 0.05, NS: not significant).

To further investigate the role of Id1 in primary tumor and metastatic progression, we transplanted Lin- bone marrow cells from Id1-/- or WT bone marrow into lethally irradiated WT recipients. Eight weeks following bone marrow transplantation, Id1-/- and control BM chimeric mice were inoculated with mCherry labelled B16F10 melanoma cells. Tumors from WT control chimeric mice showed a significant increase in volume compared to Id1-/- BM chimeric mice at endpoint (day 19) (5.3-fold; Figure 4.8 A). As B16F10 melanoma cells are known to metastasize to the lungs (Kaplan et al., 2005; Hiratsuka et al., 2008), lungs from Id1-/- and control BM chimeric mice were analyzed for metastatic tumor burden by qPCR quantification of mCherry- labelled B16F10 melanoma cells. Lungs of WT control chimeric mice had a 6-fold increase in metastatic tumor cells compared to the lungs of Id1-/- BM chimeric mice mice (Fig. 4.8 B). These data further demonstrate a critical role for Id1-expressing bone marrow derived cells in tumor and metastatic progression.

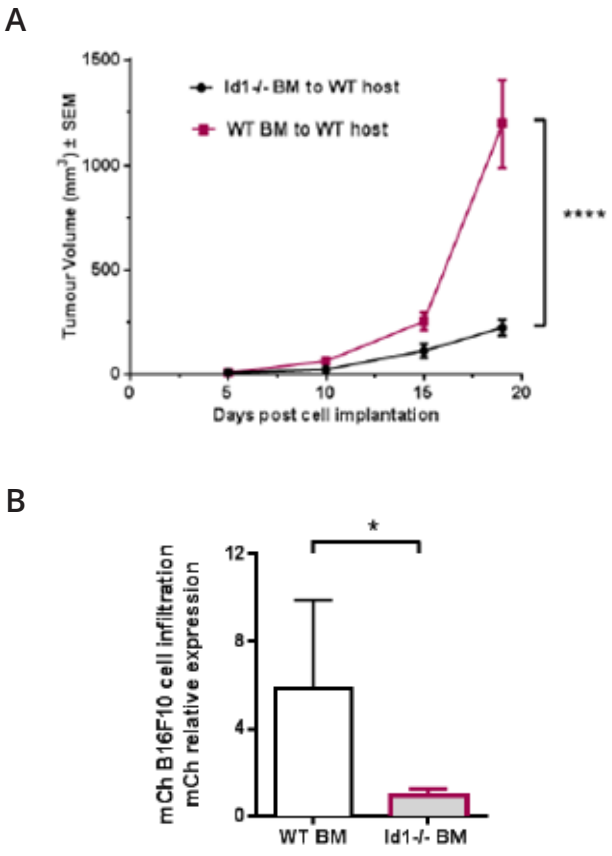


Figure 4.8. Analysis of primary tumour volume from Id1-/- and control BM chimeric mice following implantation of B16F10 melanoma cells (Two-way ANOVA, ****p < 0.0001). (H) Relative quantification of mCherry-labelled B16F10 melanoma cells in cryosections of lungs of Id1-/- and control BM chimeric mice measured by mCherry qPCR analysis (Unpaired t-test, *p < 0.05).

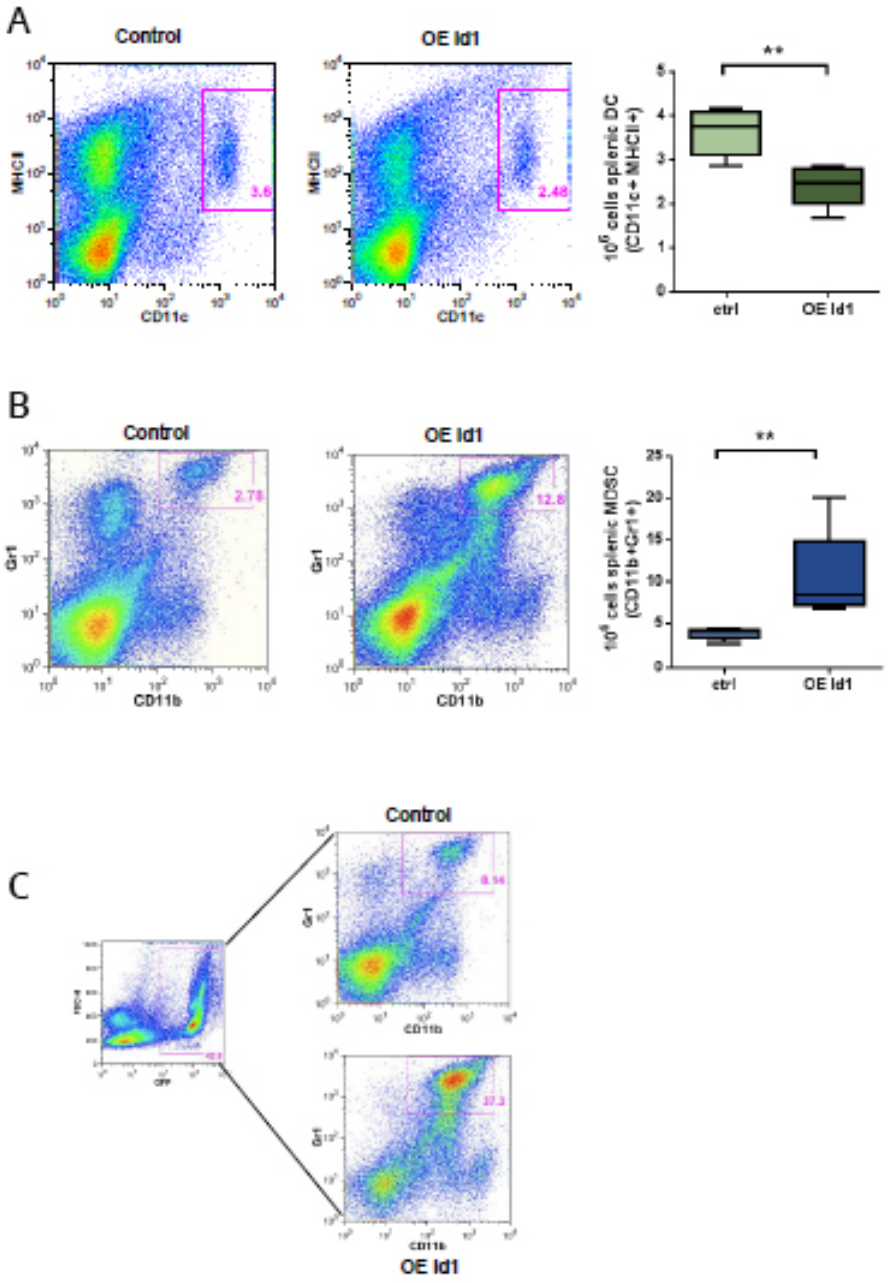
ID1 OVEREXPRESSION INDUCES MDSC ACCUMULATION

To determine whether Id1 is indeed responsible for the development and accumulation of MDSCs in response to tumor secreted factors, we transplanted lethally irradiated WT recipient mice with Lin- bone marrow cells from WT donor mice transduced with lentiviral vectors overexpressing Id1 (OE Id1) or control vectors (ctrl). Both vectors also encoded for green fluorescent protein (GFP) to track transduced cells. Six to eight weeks after transplantation, the bone marrow of recipient mice was reconstituted at a comparable reconstitution rate in both groups and over 90% of all cells in peripheral blood were positive for GFP.

Spleens from Id1-overexpressing and control vector mice were analyzed 8 weeks post-transplantation by flow cytometry for DC and MDSC levels. We found that, similarly to defects seen in tumor-bearing mice, Id1-overexpressing mice exhibited a decrease in splenic DC (1.5-fold; Figure 4.9A) and an increase in MDSC (2.7-fold; Figure 4.9B) compared to control vector mice with both granulocytic and monocytic populations equally affected. When assessing the percentage of MDSC in GFP-positive splenocytes, we observed that 37.3% (±8.43%) of GFP-positive Id1-overexpressing cells were CD11b+Gr1+ compared to 8.14% (± 2.43%) in GFP-positive control vector cells (Figure 4.9C), confirming our hypothesis that Id1 expression favours BMDC differentiation towards MDSC. These data also extend our previous observations in Id1 overexpressing mice where we observed an increase in the common myeloid progenitor frequency in the BM (Chan et al., 2009).

To further validate the role of Id1 in impairing terminal myeloid differentiation in vitro, we used the BMDC assay with WT cells transduced with lentiviral vectors overexpressing Id1 or control GFP only. After 6 days in culture, we observed a DC-MDSC imbalance similar to the one observed in cultures with TCM, with a significant decrease in DC numbers and a significant increase in MDSC numbers in Id1-overexpressing mice (4.0 and 2.1-fold respectively; Figure 4.9 D) thus confirming in an in vitro system our previous in vivo findings.

Gene expression analysis of Lin- cells transduced with Id1-overexpressing or control vectors after 6 days of in vitro differentiation showed a marked increase in Vegfr1 and S100a8 expression in Id1-overexpressing Lin- cells compared to control cells (Figure 4.9E) suggesting that Id1-overexpressing cells had a more immature phenotype than vector control cells.


A
Control
OE Id1
MHCII
CD11c
3.6
2.48
**
10^6 cells splenic DC (CD11c+ MHCII+)
ctrl
OE Id1
B
Control
OE Id1
Gr1
CD11b
2.78
12.8
**
10^6 cells splenic MDSC (CD11b+Gr1+)
ctrl
OE Id1
C
Control
OE Id1
Gr1
CD11b

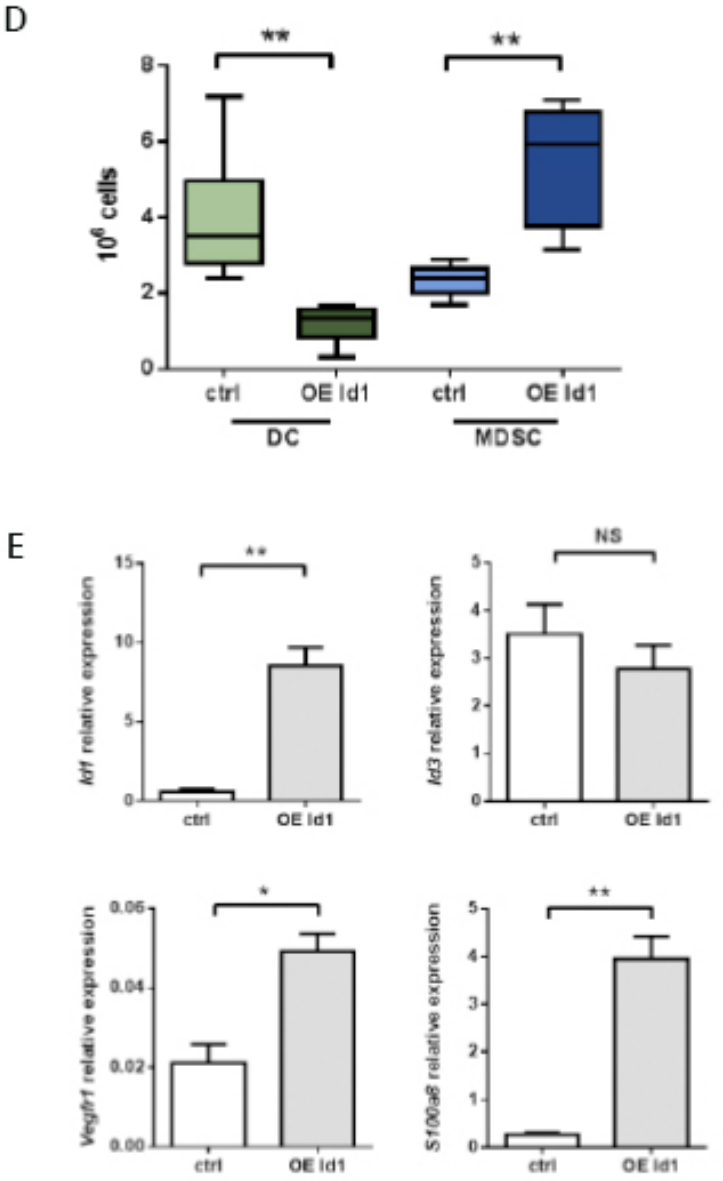


Figure 4.9. Flow cytometry analysis of spleens from mice transplanted with Id1-overexpressing and control-vector transduced Lin- bone-marrow cells for (A) frequency and absolute numbers of DC and (B) MDSC (Unpaired t-test, **p < 0.01). (C) Representative percentages of MDSC in GFP positive splenocytes from mice transplanted with Id1 overexpressing Lin- cells and control vector splenocytes. (D) In vitro differentiation of Lin- hematopoietic progenitors from C57BL/6 mice transduced with lentiviral or control and Id1 overexpressing vectors overnight, cultured for 6 days and analysed for DC and MDSC content by flow cytometry (n = 6, ANOVA, **p < 0.01). (E) Gene expression analysis of Lin- cells transduced with Id1 overexpressing and control vectors after 6 days of in vitro differentiation by qPCR analysis (means ± SEM, n = 6, Unpaired t-test, **p < 0.01, *p < 0.05, NS: not significant).

ID1-OVEREXPRESISING MDSCS INDUCE T-CELL SUPPRESSION

To determine the consequences of BMDC Id1 overexpression on other measures of systemic immune function, we examined levels of regulatory T-cells (Treg), a group of highly immunosuppressive cells that have been previously described to expand in response to MDSC (Serafini et al., 2008; MacDonald et al., 2005). Using flow cytometry analysis, we found that CD4+CD25+Foxp3+ Treg absolute numbers were significantly increased amongst splenocytes of Id1-overexpressing compared to control vector mice (1.6-fold; Figure 4.10A), supporting and extending our findings of an immunosuppressive role for Id1. As MDSCs can exert their immunosuppressive effects via both antigen specific and antigen-independent effects, we measured ROS production, thought to be one of the main non antigen-specific MDSC mediated immunosuppressive mechanisms (Lu et al., 2012), in Id1-overexpressing splenocytes by flow cytometry. Measurements of fluorescence levels of dichlorofluorescein, (DCF), a ROS-sensitive dye, indicated that splenocytes from Id1-overexpressing mice produce significantly higher levels of ROS than control vector splenocytes (1.7-fold; Figure 4.10 B), suggesting that non-antigen specific mechanisms are also involved in the immunosuppressive phenotype that is generated by Id1 overexpression. Next, we assessed antigen-specific immunosuppressive effects of Id1-overexpression on T-cell function using OVA antigen–specific T-cell co-culture models. Equal numbers of GFP+CD11b+Gr1+ cells isolated by FACS from Id1-overexpressing and control vector splenocytes were co-cultured in the presence of OVA257-264 peptide with OT-I splenocytes for 4 days. Quantification of proliferating (CFSElow), activated (IFNγ+) CD8+ antigen specific OT-I T-cells, showed a significant increase in T-cell proliferation in cultures with no CD11b+Gr1+ or control vector CD11b+Gr1+, but not with Id1 overexpressing CD11b+Gr1+ cells compared to control T-cell wells (no peptide) (Figure 4.10 C). We observed a significant increase in T-cell suppression by Id1 overexpressing CD11b+ Gr1+ cells compared to control vector (67.3% versus 5.3%, Figure 4.10 D). Furthermore, Th1/Th2 cytokine production analysis of conditioned media of splenocytes from Id1-overexpressing and control vector animals co-cultured in the presence of OVA323-339 peptide and CD4+ OT-II cells showed a marked decrease in Interferon IFN levels (5.3-fold; Figure 4.10 E) and a significant increase in interleukin-10 (IL-10) levels (1.9-fold; Figure 4.10 F) . Both assay systems indicated that Id1-overexpressing splenocytes and CD11b+Gr1+ cells, in particular, are able to directly suppress effector T-cell proliferation and activation and promote a tolerogenic T-cell phenotype.

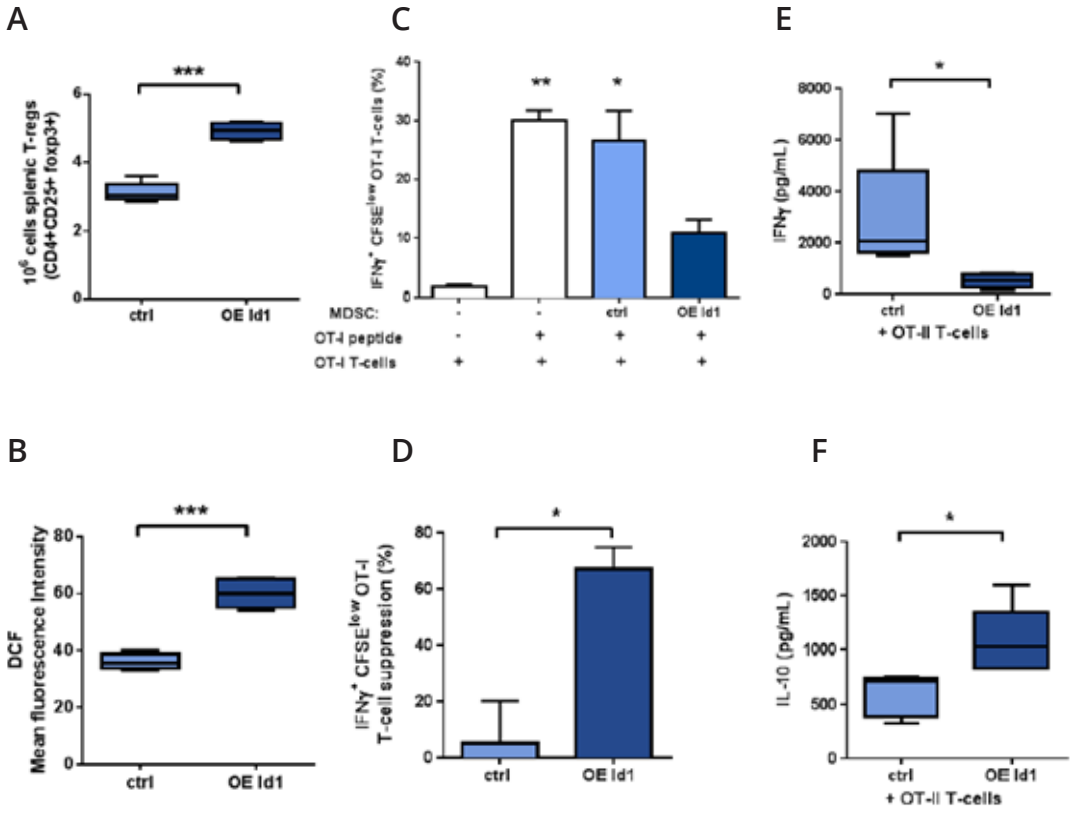


Figure 4.10. Id1 overexpression leads to an immunosuppressive phenotype and T-cell suppresision. Flow cytometry analysis of spleens from mice transplanted with Id1-overexpressing and control-vector transduced Lin- bone-marrow cells for (A) absolute numbers of regulatory T cells (CD4+CD25+Foxp3+; Unpaired t-test, ***p < 0.001), for (B) ROS production, as determined by mean fluorescence intensity levels of dichlorofluorescein (DCF), an ROS-sensitive dye (Unpaired t-test, ***p < 0.001). (C) CD8+ antigen specific T-cell proliferation functional assessment of GFP+ CD11b+ Gr1+ splenocytes from Id1-overexpressing and control vector animals co-cultured with OT-I splenocytes in the presence of OVA257-264 peptide. (ANOVA, **p < 0.01, *p < 0.05) (D) OT-I T-cell proliferation expressed as suppression induced by GFP+ CD11b+ Gr1+ splenocytes from Id1-overexpressing and control vector animals, relative to the no MDSC control wells. Data expressed as percentage T-cell suppression compared to no MDSC control. (Unpaired t-test, *p < 0.05) (E) Analysis of splenocytes from Id1-overexpressing mice and OT-II CD4+ T-cell co-cultures in the presence of OVA323-329 peptide for IFNγ levels (Unpaired t-test, *p < 0.05) and (F) IL-10 levels compared to splenocytes from control vector-treated mice and OT-II CD4+ T-cell co-cultures (Unpaired t-test, *p < 0.05).

D1-OVEREXPRESSING BMDCS PROMOTE PRIMARY TUMOR GROWTH AND METASTATIC PROGRESSION

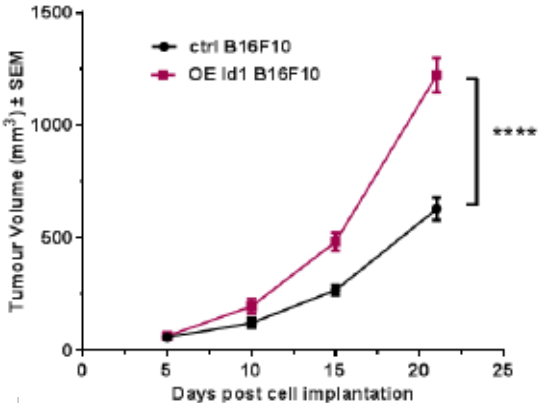
To determine if the functional effects exerted by Id1 overexpression can alter tumor progression, 8 weeks following bone marrow transplantation, Id1-overexpressing and control mice were inoculated with

mCherry labelled and non-labelled B16F10 melanoma cells. Tumor volume was measured during the model progression until day 21. Tumors from Id1-overexpressing mice had a significant increase in volume compared to control vector mice on day 21 (2.2-fold; Fig. 4.11 A). Quantification of vessels by platelet /endothelial cell adhesion molecule-1 (PECAM-1+) staining and BMDC infiltration by GFP+ cell quantification on B16F10 tumor sections, showed no statistically significant difference in vascularization or BMDC infiltration in the primary tumor of control mice compared with Id1-overexpressing mice implanted with B16F10 melanoma (Figures 4.11 B and 4.11C).

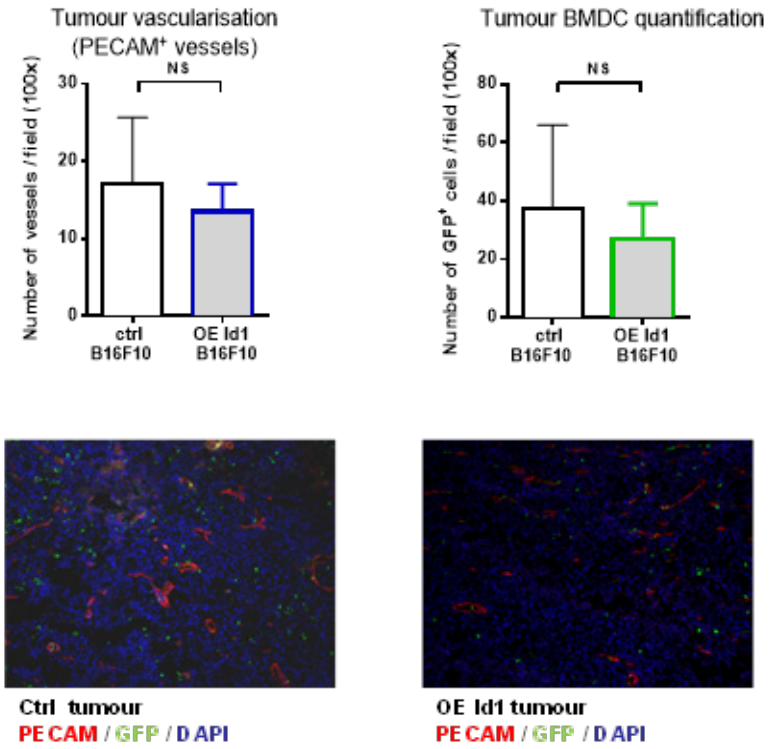
Lungs from Id1-overexpressing and control vector-transplanted mice were analyzed for metastatic tumor burden by quantification of mCherry-labelled B16F10 melanoma cells. Lungs of Id1-overexpressing mice had a 13-fold increase in metastatic tumor cells compared to the lungs of control vector-transplanted mice (Figure 4.11 D). Id1-overexpressing mice harboured significantly higher numbers of both micro- and macrometastatic lesions (Figures 4.11 E and 4.11 F).

When we assessed the immune function of Id1-overexpressing tumor-bearing mice, we found similar DC numbers but significantly elevated MDSC ($p<0.01$), Treg numbers ($p<0.001$) and ROS production (Figures 4.11 G, H, I, and J respectively) compared to control vector tumor-bearing mice. These findings demonstrate that Id1-overexpression in hematopoietic cells is associated with an immunosuppressive phenotype and significantly increased primary tumor growth and metastatic burden.

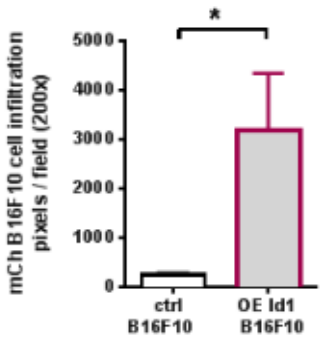
A



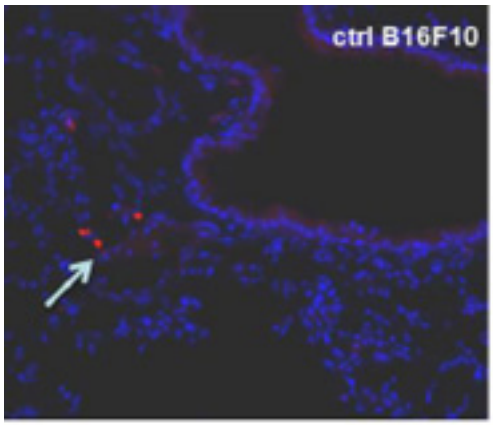
B

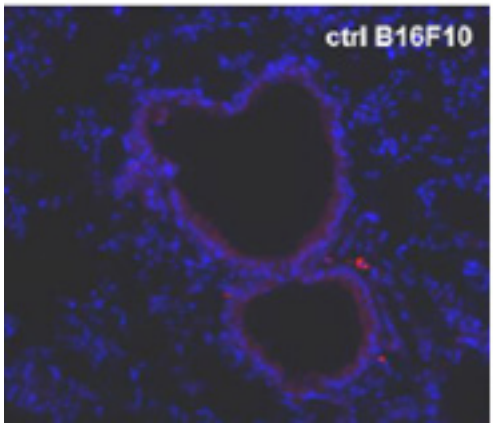

Tumour vascularisation
(PECAM+ vessels)
Number of vessels / field (100x)
NS
30
20
10
0
ctrl B16F10
OE Id1 B16F10
Tumour BMDC quantification
Number of GFP+ cells / field (100x)
NS
80
60
40
20
0
ctrl B16F10
OE Id1 B16F10
Ctrl tumour
PECAM / GFP / DAPI
OE Id1 tumour
PECAM / GFP / DAPI

C

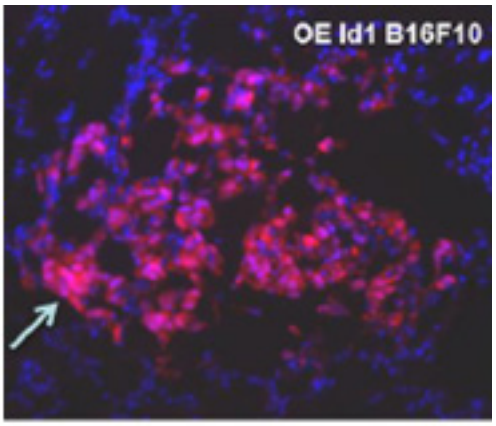

mCh B16F10 cell infiltration
pixels / field (200x)
*
5000
4000
3000
2000
1000
0
ctrl B16F10
OE Id1 B16F10

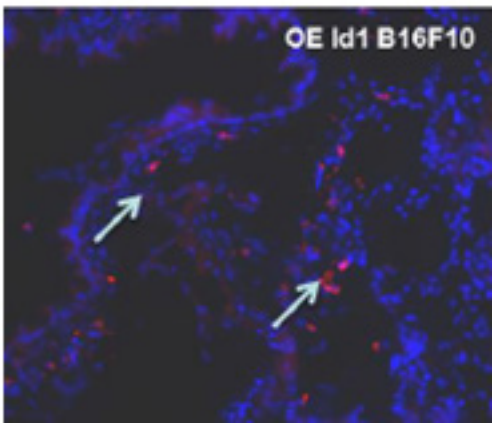
D


ctrl B16F10

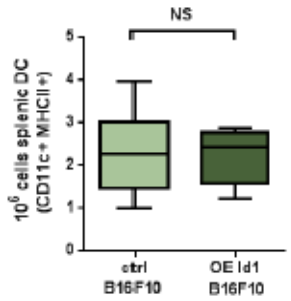

ctrl B16F10

E


OE Id1 B16F10


OE Id1 B16F10

F


NS
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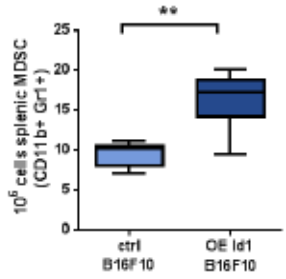
0
1
2
3
4
5
ctrl B16F10
OE Id1 B16F10

G


**
10^6 cells splenic MDSC (CD11b+ Gr1+)
0
5
10
15
20
25
ctrl B16F10
OE Id1 B16F10

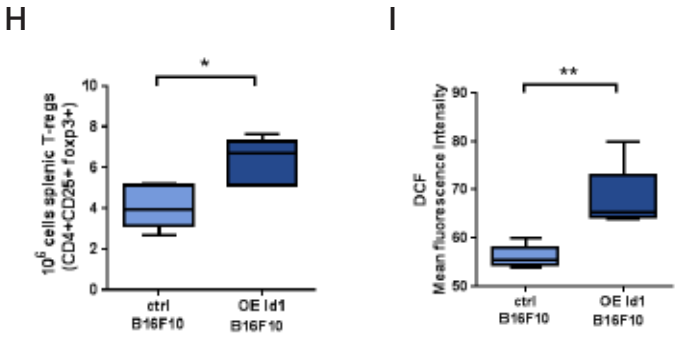


Figure 4.11. Id1-overexpressing BMDCs promote primary tumor growth and metastatic progression (A) Analysis of primary tumour volume from Id1-overexpressing mice and control vector mice following implantation of B16F10 melanoma cells (Two-way ANOVA, ****p < 0.0001). (B) Immunofluorescence analysis of vascularization of platelet/endothelial cell adhesion molecule-1 (PECAM-1) (Texas Red staining quantification) and bone marrow cell infiltration (GFP+ quantification) in primary B16F10 tumors from mice transplanted with control-vector and Id1-overexpressing-vector transduced lin- bone-marrow cells. Scale bar: 50 μm (Unpaired t-tests, NS: not significant). (C) Quantification of mCherry-labelled B16F10 melanoma cells in cryosections of lungs of bone marrow Id1-overexpressing mice and control vector mice measured as red pixels per field (Unpaired t-test, *p < 0.05). (D) Micro- and (E) Macrometastatic lesion formation of lungs in Id1-overexpressing mice and control vector mice. Flow cytometry analysis of splenocytes from bone marrow Id1-overexpressing and control-vector mice implanted with B16F10 melanoma cells for absolute numbers of (F) DCs, (G) MDSCs, (H) regulatory T-cells, and (I) ROS production (Unpaired t-tests: NS: non-significant, *p < 0.05, **p < 0.01).

ID1 IS UPREGULATED VIA A TGFB-DEPENDENT MECHANISM AND DOWN-REGULATES KEY GENES INVOLVED IN DC MATURATION

To identify upstream regulators of Id1 and downstream pathways affected by Id1 overexpression, we performed gene expression profiling of Id1-overexpressing and control BMDC using Affymetrix GeneChip arrays. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2280. Pathway analysis of the differentially expressed genes using Ingenuity Pathway Analysis (IPA) software identified TGFβ and IL-6 among the top predicted upstream regulators of Id1 overexpression-induced gene expression changes (P value: 6.38 x 10-29 and 2.98 x 10-21 respectively).

To confirm that TGFβ and IL-6 were able to up-regulate Id1 in a relevant cell system, we tested these molecules in addition to a series of candidate tumor-secreted factors previously implicated in MDSC expansion or Id1 up-regulation (Chambers et al., 2003; David et al., 2007; Liang et al., 2009; Strong et al., 2013) in the BMDC assay. We observed that culture with TGFβ, and to a lesser extent IL-6 and BMP-7, led

to Id1 up-regulation in BMDC (6.5-fold, 1.9, and 2.4 respectively, Fig. 4.12 A) confirming the two upstream pathway predictions of the microarray data analysis. Id1 mRNA expression levels were found to be significantly higher in Lin- cells differentiated in the presence of B16F10 TCM compared to control media (4.9-fold; Figure 4.12 B), whereas neutralization of TGFβ in B16F10 TCM largely prevented the up-regulation of Id1 by BMDC (Figure 4.12 B).

Pathway analysis of the differentially expressed genes using IPA software identified the DC maturation pathway as one of the canonical pathways most significantly affected by Id1 overexpression (P value: 1.69 x 10-3). Several key genes involved in DC maturation were found to be down-regulated following Id1 overexpression, including Cd83, Cd86, MHCII (HLA-DQA1 and HLA-DRB1), Fscn1, Stat4 and Irf8 (Icsbp) (Table 4.1).

Irf8 was of particular interest since it is a transcription factor that has been recently shown to restrict the generation of Gr-1+ granulocytic populations such as neutrophils and MDSCs (Waight et al., 2013) and to be responsible for initiating DC lineage commitment (Schonheit et al., 2013). To further investigate the relationship between If8 and Id1 and determine if Irf8 is a downstream mediator of Id1 function, we employed the BMDC assay to assess the expression of Irf8 in WT and Id1-/- BMDC in response to TGFβ and B16F10 TCM compared to control media. We observed a significant down-regulation of Irf8 expression in WT BMDC in response to TGFβ and B16F10 TCM (1.6 and 1.5-fold respectively, Figure 4.12 C), an effect that was abrogated in Id1-/- BMDC treated with TGFβ or B16F10 TCM (Figure 4.12 D). Furthermore neutralization of TGFβ in B16F10 TCM significantly reversed the down-regulation of Irf8 by BMDC (Figure 4.12 C). We therefore concluded that B16F10 TCM induces Irf8 down-regulation in BMDC via a TGFβ - Id1 dependent mechanism. To identify the specific cell population that is primarily responsible for the TGFβ-mediated Id1 upregulation and Irf8 downregulation we observed in the BMDC assay and to confirm whether these changes are occurring per cell or reflect overall cell population changes, we isolated DC and MDSC using FACS and assessed Id1 and Irf8 expression by qPCR analysis. We found that Lin- cells cultured in the presence of recombinant TGFβ generate MDSC expressing higher Id1 mRNA levels per cell (6.2-fold; Figure 4.12 E) and DC expressing lower Irf8 levels per cell compared to respective populations in control media cultures (5.9-fold; Figure 4.12 F) confirming the inverse relationship of Id1 and Irf8 in specific isolated populations.

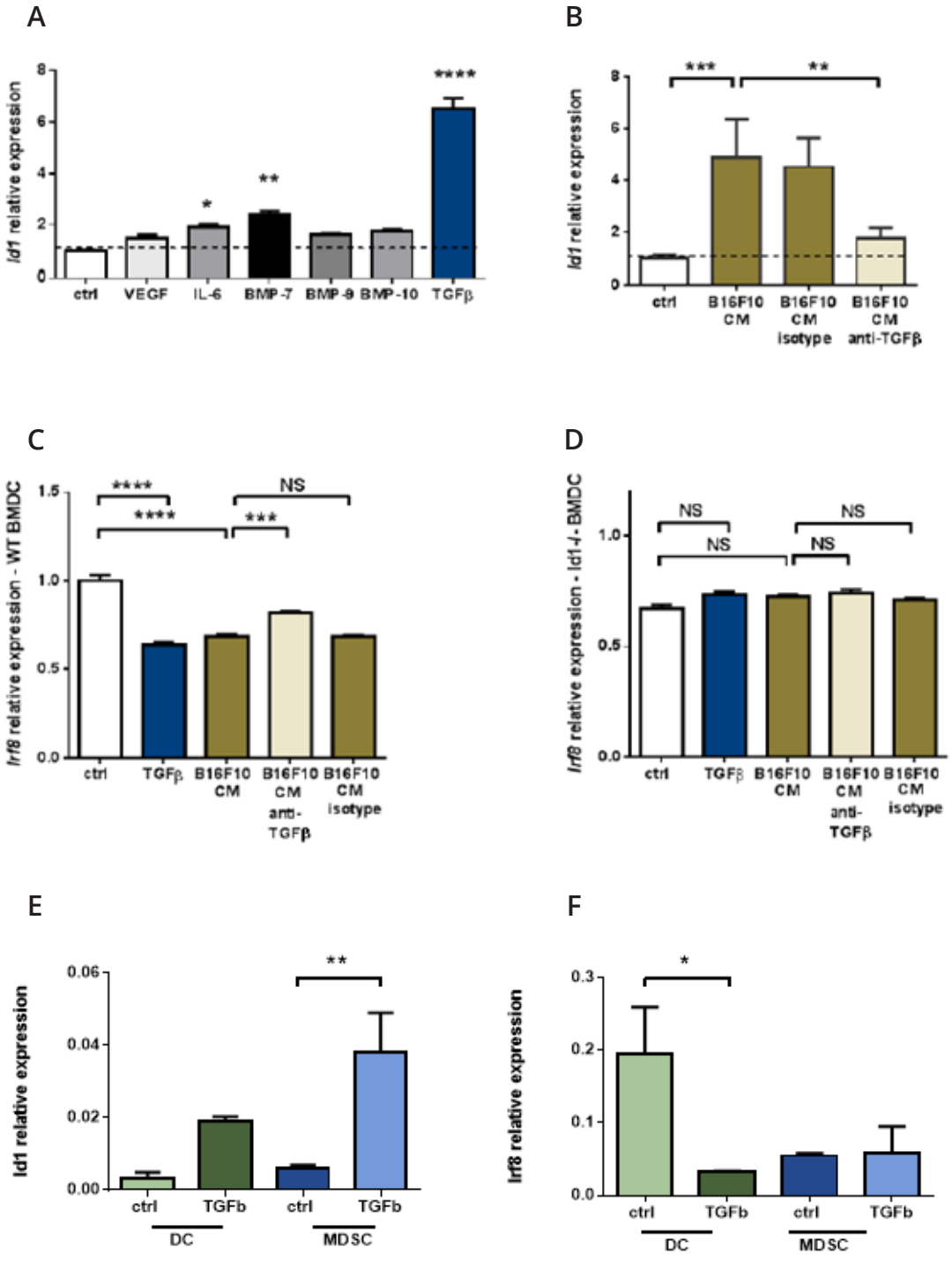

A
Id1 relative expression
ctrl
VEGF
IL-6
BMP-7
BMP-9
BMP-10
TGFβ
B
Id1 relative expression
ctrl
B16F10 CM
B16F10 CM isotype
B16F10 CM anti-TGFβ
C
Irf8 relative expression - WT BMDC
ctrl
TGFβ
B16F10 CM
B16F10 CM anti-TGFβ
B16F10 CM isotype
D
Irf8 relative expression - Id1-/- BMDC
NS
ctrl
TGFβ
B16F10 CM
B16F10 CM anti-TGFβ
B16F10 CM isotype
E
Id1 relative expression
ctrl
TGFb
DC
MDSC
F
Irf8 relative expression
ctrl
TGFb
DC
MDSC

Figure 4.12. Id1 is upregulated via a TGFβ-dependent mechanism and downregulates key genes involved in DC maturation (A) Id1 mRNA relative expression levels in Day 6 Lin- cells differentiated in the presence of 100 ng/μl of murine recombinant proteins (VEGF, IL-6, BMP-7, -9 and -10 and TGFβ compared to Lin- cells differentiated in control media, as determined by qPCR analysis (means ± SEM, n = 6, ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05). (B) Id1 mRNA expression levels in Day 6 Lin- cells differentiated in the presence of B16F10 conditioned media alone, with anti-TGFβ and anti-IgG compared to control media, as determined by qPCR analysis (means ± SEM, n = 6, ANOVA, ***p < 0.001, **p < 0.01). (C) Irf8 mRNA relative expression levels of Day 6 WT Lin- cells differentiated in the presence of 100 ng/μl of TGFβ, B16F10 conditioned media alone, with anti-TGFβ and anti-IgG compared to control media, as determined by qPCR analysis (means ± SEM, n = 6, ANOVA, ****p < 0.0001, ***p < 0.001). (D) Irf8 mRNA relative expression levels of Day 6 Id1-/- Lin- cells differentiated in the presence of 100 ng/μl of TGFβ, B16F10 conditioned media alone, with anti-TGFβ and anti-IgG compared to control media, as determined by qPCR analysis (means ± SEM, n = 6, ANOVA, not significant). (E) Id1 and (F) Irf8 mRNA expression levels of FACS-sorted DC and MDSC populations following in vitro differentiation of lin- cells, cultured for 6 days in the presence of recombinant TGFβ, as determined by qPCR analysis (means ± SEM, n = 5, One-way ANOVA, **p < 0.01, *p < 0.05).

4.5 DISCUSSION

Our work demonstrates a novel central role for Id1 in diverting normal myeloid cell differentiation from its intrinsic pathway of terminal differentiation to mature cells such as DC, towards a pathway that generates pathologically activated immature cells known as MDSCs (Gabrilovich et al., 2012; Talmadge et al., 2013; Cheng et al., 2008; Youn et al., 2013; Engblom et al., 2016) during tumor progression. We demonstrate that Id1 upregulation is responsible for generating an immunosuppressive macroenvironment and driving tumor progression. We also demonstrate that Id1 overexpression specifically by MDSC can directly suppress T-cell function, and identify TGFβ and IL-6 amongst the main factors responsible for Id1 up-regulation in BMDC. In light of our results, we propose the use of Id1 and its mediators as biomarkers of systemic immune dysfunction during tumor progression as well as candidates for targeted anti-tumor therapeutic strategies.

Cancer is often considered to be a reflection of 'embryonic memory'. Id genes are important in both embryonic neurogenesis and myocardial development (Ruzinova et al., 2003) and also regulate the self-renewal capacity of cancer-initiating cells (O'Brien et al., 2012). Id1 expression, in particular, correlates with both cancer progression and poor prognosis (Fong et al., 2004; Perk et al., 2005). Prior stu-

dies have demonstrated a role for Id1 in endothelial cell differentiation and tumor vasculogenesis (Lyden et al., 1999; Lyden et al., 2001), and progression from micro- to macrometastatic disease (Gao et al., 2008) via endothelial progenitor cell mobilization. This is the first study to implicate Id1 in the crosstalk between tumors and the host immune system via regulation of myeloid cell differentiation.

Tumor and host cells release multiple factors that perturb the myeloid compartment. These include VEGF, IL-4, IL-6, IL-10, IL-13, M-CSF and TGFβ which regulate likely redundant pathways mediating the maturation and expansion of MDSC at the expense of DC differentiation via transcription factors such as the signal transducer and activator of transcription 3 (STAT3) and CCAAT/enhancer-binding protein alpha (CEBPα) (Gabrilovich and Nagaraj, 2009; Sonda et al., 2011). Hence, here we examined several factors that have been implicated either in MDSC expansion or Id1 up-regulation in addition to factors that we identified as predicted upstream regulators of Id1-induced gene changes and we confirmed TGF-β and IL-6 as the main factors responsible for Id1 up-regulation. The link between TGF-β and Id1 appears to be context-dependent (Anido et al., 2010; Challen et al., 2010; Kang et al., 2003). Here, we show that in the case of BMDC, TGF-β is the primary tumor-derived factor responsible for Id1 up-regulation, as its neutralization largely abrogates Id1 expression in vitro.

Id1 has been shown to induce S100a8/9 and Vegfr1 expression, which have been previously associated with an immature myeloid phenotype. Specifically, the calcium-binding pro-inflammatory proteins S100A8 and 9 are thought to have key roles in myeloid differentiation and MDSC expansion (Cheng et al., 2008), whereas VEGFR1 is a marker of immature myeloid cells (Kaplan et al., 2005). These findings are also consistent with reports that VEGFR1+ cells may have impaired function in Id-mutant mice (Lyden et al., 2001) and that Id1-/- DCs are not responsive to VEGF treatment via VEGFR1 (Laxmanan, et al., 2005). These findings support our previous observation that increases in VEGFR1 and Id expression occur in BMDCs and are largely responsible for driving the metastatic process (Kaplan et al., 2005).

The transcriptional program driving MDSC differentiation is poorly understood, partly due to the heterogeneity of MDSC subsets (Youn et al., 2008; Youn et al., 2013). This study identifies Id1 as a new master transcriptional regulator of myeloid differentiation. Transcriptome analysis of Id1-overexpressing BMDC revealed the down-regulation of several genes thought to play a key role in DC maturation, such as those encoding the co-stimulatory molecules Cd83 and Cd86, and Irf8, a transcription factor that controls DC lineage commitment (Schonheit et al, 2013). Importantly, we demonstrate an inverse rela-

tionship and co-regulation between Id1 and Irf8. Humans with Irf8 mutations have a severe DC immunodeficiency syndrome (Hambleton et al., 2011) whereas in murine studies, Irf8 has been shown to impair the generation of Gr1+ granulocytic populations such as neutrophils and MDSCs, and to promote DC expansion and commitment (Waight et al., 2013; Becker et al., 2012). Moreover, Irf8 expression is decreased in MDSCs from tumor-bearing hosts and its overexpression leads to decreased MDSC level (Stewart et al., 2009), suggesting that Irf8 is an important regulator of MDSC differentiation during tumor progression. Our study provides novel insights into the molecular pathways that link the inhibition of DC maturation and MDSC expansion and identifies a previously unknown inverse relationship between Id1 and Irf8.

When examining the functional outcomes of systemic Id1-induced tumor immunosuppression, we identified both antigen non-specific and specific mechanisms by which Id1-expressing MDSC exert their immunosuppressive effects. Firstly, the increase in total ROS levels following Id1 overexpression comes in agreement with studies demonstrating that ROS are major factors in the inhibition of DC differentiation and MDSC expansion in tumor-bearing mice (Lu et al., 2012; Corzo et al., 2009). As VEGFR1 expression is also thought to be regulated by oxidative stress (Kusmartsev et al., 2004), these findings provide a mechanistic link between increased ROS and induced up-regulation of VEGFR1, and identify Id1 as the molecular link between the two phenomena. Second, another key mechanism of MDSC-induced immunosuppression is the activation and expansion of Treg (MacDonald et al., 2005; Huang et al., 2006). Although these mechanisms are not completely understood, they are thought to involve cell-to-cell contact and the production of cytokines, such as IFNγ, IL-10 and TGFβ (Huang et al., 2006). The significant decrease in IFNγ and increase in IL-10 detected in co-cultures of Id1-overexpressing splenocytes with naïve OT-II CD4 T-cells confirm the activation of antigen-specific immunosuppressive mechanisms. Finally, we demonstrate that Id1 overexpression in the CD11b+Gr1+ subset specifically induces antigen specific T-cell suppression, providing direct evidence of the functional consequences of Id1 overexpression in downstream effector immune responses.

Our study reveals for the first time a novel pivotal role for Id1 in tumor and metastatic progression and in controlling systemic tumor-induced immunosuppression, providing further insight into the therapeutic promise of Id1 targeting. Pharmacological inhibition of Id1 with technologies such as peptides or small interfering RNA would offer the advantage of selective targeting, therefore largely minimizing side effects. This new approach would offer the opportunity to re-examine immunotherapies in a new

improved setting. Targeting of Id1 or downstream pathways would provide a three-pronged therapeutic approach by reducing metastatic potential of the tumor itself, reducing tumor angiogenesis and finally restoring systemic immune function.

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5. CHAPTER FIVE
THE ROLE OF VEGFR1 IN REGULATING TUMOR ANGIOGENESIS AND METASTATIC PROGRESSION

EXPLORING THE ROLE OF **INTERLEUKIN-6** AND **OTHER HOST-ASSOCIATED FACTORS** IN **TUMOR PROGRESSION** AND **METASTASIS DEVELOPMENT**

5. CHAPTER FIVE
THE ROLE OF VEGFR1 IN REGULATING TUMOR ANGIOGENESIS AND METASTATIC PROGRESSION [5]

5.1 SUMMARY

The contribution of BMDCs to the tumor and metastatic microenvironments is well documented. We have shown that many recruited cells of the myeloid lineage express VEGFR1 and, as discussed in the previous chapter, increases in Id1 and VEGFR1 expression are largely responsible for driving the metastatic process. However, the functional role of VEGFR1 expression in these cells during tumor angiogenesis and metastasis remains unclear. In this chapter, we demonstrate that VEGFR1-deficient myeloid cells inhibit B16 melanoma growth and induce defects in blood vessel development. Gene expression analysis of myelomonocytic cells with inhibited VEGFR1 expression showed the upregulation of a potent anti-angiogenic factor, CXCL4 (platelet factor-4). For the first time, we show the upregulation of CXCL4 by BMDCs at the primary tumor and metastatic microenvironments and report CXCL4-/- mice exhibit enhanced primary tumor growth and drastic acceleration of experimental macrometastasis formation with increased blood vessel maturation. The anti-angiogenic effect of VEGFR1-deficient myeloid cells is CXCL4-dependent. Finally, lentiviral-mediated downregulation of VEGFR1 expression in the bone marrow drastically reduces the occurrence of advanced metastatic foci which is largely dependent on CXCL4 upregulation. Thus, our results clearly demonstrate an important function for VEGFR1-regulated CXCL4 expression by BMDCs in regulating angiogenesis at the primary tumor and metastatic microenvironments.

[5] Based on: Jared Wels, Maria Rosario Andre, Selena Granitto, et al. VEGFR1-regulated expression of CXCL4 in bone marrow-derived myeloid cells controls tumor angiogenesis and metastatic progression. Manuscript in preparation.

5.2 INTRODUCTION

As discussed previously, the cancer environment is comprised of tumor cells, as well as a wide network of stromal and vascular cells participating in cellular and molecular events necessary for invasion and metastasis. Of the stromal cell types comprising the tumor microenvironment, inflammatory cells derived from the bone marrow are particularly integral players in that they have been shown to contribute to such processes as blood vessel development, via local sprouting (angiogenesis) or de novo vessel formation (vasculogenesis), as well as local invasion and distant metastasis. The role of infiltrating of BMDCs and macrophages at the primary tumor is multi-faceted and in many cases provides a supportive environment for pre-existing malignant cells. The direct effects of myeloid BMDCs on tumor cells include promotion of tumor cell invasion, migration, and intravasation. However, one of the most documented roles of these cells is their ability to control tumor progression via regulating the switch of a tumor to an angiogenic stage.

In the PyMT model of mammary tumors, a significant infiltration of macrophages occurs at stages directly preceding those changes associated with angiogenesis (Lin et al., 2003). When given tumors, mice deficient in macrophages, through genetic deletion of the CSF-1 gene, manifest a delayed angiogenic switch (Lin et al., 2006). BMDCs of earlier maturation status have also been shown to play roles in tumor vessel development. For example, VEGFR1-positive myeloid progenitor cells lie in close association with forming tumor vasculature. Indeed, VEGFR1 inhibition reduced investment of vessels with perivascular cells, suggesting that VEGFR1+ cells confer vessel stability and promote tumor progression.

Activated myeloid progenitors and macrophages can release angiogenic factors such as VEGF-A, PDGF and angiopoietins, which serve to enhance vessel formation (Donovan et al., 2000; Okamoto et al., 2005; Otani et al., 2002). VEGF-A, considered the most potent angiogenic factor, is produced in significant quantities by myeloid BMDCs within the tumor (Leek et al., 2000; Lewis et al., 2000), which may be regulated by hypoxia as well as CSF-1 activation. Deletion of VEGF-A in myeloid cells inhibits high- density tumor vessel formation, resulting in vascular normalization (Stockmann et al., 2008). Notably, the growth of tumors in these mice is accelerated. Thus, myeloid cells are essential in the development and maturation status of the tumor vasculature. A mechanism by which myeloid cells may promote angiogenesis is by the paracrine release of angiogenic and/or anti-angiogenic factors, however the players and pathways involved are poorly understood.

It is now recognized that tumor-secreted signals not only induce dynamic alterations of the adjacent tumor microenvironment, but also stimulate distant changes, including those at sites of future metastasis (Chiang and Massague, 2008; Wels et al., 2008). Others and we have described an essential role for BMDCs in priming distant tissues for tumor cell implantation and proliferation (Erler et al., 2009; Hiratsuka et al., 2002; Hiratsuka et al., 2006; Kaplan et al., 2005; Mimori et al., 2008; Qian et al., 2009). Prior to the establishment of metastases, specific cells of hematopoietic origin mobilize from the bone marrow and engraft as cellular clusters into distant tissue sites by binding fibronectin in response to an array of chemokines derived from the primary tumor. Initial characterization of these BMDCs revealed the expression of surface markers including VEGFR1, c-Kit, and CD11b (Erler et al., 2009; Kaplan et al., 2005). Recruited myeloid cells with the cell surface receptor expression signature of CD11b+GR1-F4/80+VEGFR1high have also been shown to be direct mediators of tumor cell seeding and metastatic growth in mouse models of breast cancer (Qian et al., 2009). Despite the characterization of these cells and their role in tumor metastasis, there is a lack of understanding regarding the function of these cell surface markers and their potential metastasis-promoting activity.

Vascular endothelial growth factor receptor 1 (VEGFR1/flt-1), along with VEGFR2/flk-1 and VEGFR3/flt-4 comprise the three members of the VEGF receptor tyrosine kinase family activated by VEGF family ligands (PLGF, VEGF- A, VEGF-B, VEGF-C, and VEGF-D). Both VEGFR1 and VEGFR2 are expressed by vascular endothelial cells, however they play distinct roles during vasculogenesis. Although VEGFR1 binds VEGF with 10-fold higher affinity than VEGFR2, its tyrosine kinase activity is relatively weak compared with VEGFR2, and does not induce proliferative or migratory activity in these cells (Shibuya, 2001). Alternately, VEGFR1 appears to play a negative regulatory role in blood vessel formation, as VEGFR1 null mice die during development due to vascular overgrowth and disorganization (Fong et al., 1995). Notably, mice only lacking the intracellular tyrosine kinase domain (VEGFR1 TK-/-) develop normally with no vascular defects (Hiratsuka et al., 1998). VEGFR1 is also expressed by hematopoietic cell types including hematopoietic progenitor cells, monocytes and macrophages (Clauss et al., 1996; Hattori et al., 2002; Heil et al., 2000; Sawano et al., 2001). Within these hematopoietic cell types, VEGFR1 appears to play a more positive regulatory role, and is implicated in several pathologic processes including angiogenesis, metastasis, and inflammation (Kaplan et al., 2005; Kerber et al., 2008; Luttun et al., 2002). Activation of VEGFR1 on macrophages by VEGF-A promotes their migration and may provide a mechanism for recruitment of these pro-angiogenic cells to hypoxic tumor sites (Luttun et al., 2002). At the tumor microenvironment and metastatic sites, VEGFR1 is frequently used as a marker to denote recruited BMDCs that contribute towards tumor pro-

gression as well as the permissive microenvironment necessary for metastatic tumor seeding and outgrowth (Hiratsuka et al., 2002; Kaplan et al., 2005; Qian et al., 2009). The functional significance of VEGFR1 expression in these processes, however, remains controversial. Several studies have shown minimal effects of VEGFR1 pathway blockade on tumor angiogenesis and growth (Bais et al., 2010; Hiratsuka et al., 2002; Kaplan et al., 2005), whereas other recent works describe roles for VEGFR1 in BMDC recruitment and stimulation of solid tumor growth (Kerber et al., 2008; Muramatsu et al., 2010). At metastatic sites, others and we have reported that systemic blockade of VEGFR1, by inhibitory antibody or use of VEGFR1 TK-/- mice, significantly impairs metastatic progression (Bais et al., 2010; Hiratsuka et al., 2002; Kaplan et al., 2005). However, a report in a surgical model of tumor metastasis suggests VEGFR1 is not necessary for spontaneous metastasis formation (Dawson et al., 2009). Thus, further studies into VEGFR1 function within both the primary tumor and metastatic microenvironments are needed to understand the role of this predominant marker protein. To this date, no studies have specifically investigated VEGFR1 function on BMDCs within these microenvironments and/or VEGFR1-regulated crosstalk of BMDCs with other cells in the tumor stroma. In this chapter, we demonstrate that VEGFR1-deficient BMM cells inhibit tumor growth and macrometastatic progression due to anti-angiogenic activity and attribute this activity largely to the upregulation of the potent anti-angiogenic protein, CXCL4.

5.3 MATERIALS AND METHODS

CELL LINES AND MICE

The B16 melanoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained according to standard cell culture techniques.

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). mCXCL4-/- and hCXCL4+/- were generously provided by Dr. Mortimer Poncz and Dra. Anna Kowalska (University of Pennsylvania, Children's Hospital of Philadelphia). Generation of CXCL4 transgenic strains has been previously reported (Eslin et al., 2004; Zhang et al., 2001). CXCL4 mice were used for experiments after genotyping to confirm deletion of CXCL4 and/or presence of hCXCL4. All mice were maintained in the Research Animal Resource

Center of Weill Cornell Medical College. All animal procedures were approved and performed under the guidelines of the IACUC at Weill Cornell Medical College.

BONE MARROW TRANSPLANTATION

Bone marrow cells were harvested by flushing femurs and tibias. Lineage negative cells were isolated using a progenitor enrichment kit from StemCell Technologies. Lin- cells were plated in Stemspan serum-free medium (StemCell Technologies) supplemented with IL-3 (20 ng/ml), IL-6 (10 ng/ml), Flt-3L (10ng/ml), and SCF (100ng/ml) (Peprotech). 5x105 cells were transduced with concentrated lentivirus at MOI of 25-50 with 8ug/ml polybrene for 12 hours. 5x105 cells were injected via tail vein into four week-old recipient C57BL/6J mice lethally irradiated with a single dose of 950 rad of whole-body irradiation 24 hours before. After 6 weeks mice were bled to assess blood cell counts. Mice were then injected subcutaneously with 1x106 B16 melanoma tumor cells transduced with lentivirus overexpressing the red fluorescent protein, mCherry.

PREPARATION OF LUNGS FOR QUANTIFICATION OF METASTASIS

After the indicated time post-tumor implantation, mice were sacrificed and lungs were perfused with 3ml of PBS through the right ventricle. The right upper lobe of the lung was fixed in 1.6% paraformaldehyde/ 20% sucrose in PBS for 24 hours and then embedded in OCT. The rest of the lung was saved for mRNA extraction for qPCR analysis. To analyze metastatic burden, lungs frozen in OCT were sectioned at 0.8 uM and coversliped using Invitrogen Prolong Gold with Dapi. Micrometastatic lesions, defined by foci consisting of 20 or more mCherry-positive tumor cells, were counted in 6 lung sections/mouse. Individual seeding tumor cells were quantified by counting solitary mCherry-positive tumor cells in random 100x fields (6 fields/section, 6 sections/mouse).

ISOLATION OF BONE MARROW-DERIVED MYELOID CELLS

BM cells were harvested from 8-week old C57BL/6 mice by flushing tibias and femurs with 1% BSA in PBS with 2mM EDTA. Mononuclear cells were obtained by FIcoll (Invitrogen) gradient centrifugation at 240xg for 20 minutes. Cells were then plated in RPMI with 20% horse serum 10% FBS + 30 ng/ml M-CSF for 2 days. Non-adherent cells were removed, and adherent cells were scraped off the plates and seeded at 5x105 cells/well in a 24-well plate. 24 hours later, cells were transduced with appropriate lentiviral vector at MOI of 10-20 for 8 hours. Media was replaced with RPMI basal media + 2ug/ml puromycin. Puromycin selection was carried out for 2 days before cells were used for appropriate assay.

MYELOID/TUMOR CELL CO-CULTURES

7.5x104 RAW 267.4 or BMM cells were seeded in the bottom well of a 24-well 0.4uM transwell plate (Corning) in DMEM with 2%FBS or RPMI 20% Horse Serum 10% FBS, respectively. Twelve hours later, 1x105 B16 cells were plated in the upper chamber of the transwell in DMEM with 2%FBS. Co-cultures were allowed to go for 24-48 hours, after which conditioned media was collected and cell lysates were obtained by removing the upper chamber and resuspending cells in 200mL of RNAqueous lysis buffer (Ambion).

MATRIGEL ASSAY

300ul of phenol-red free Matrigel (BD Bioscience) was thawed and mixed with 200 uL conditioned media derived from appropriate BMM cells/B16 cells co-culture. Matrigel was injected subcutaneously into C57BL/6 mice and excised 7 days after implantation. Pictures were taken using Nikon SMZ800 dissecting microscope. Matrigel plugs were embedded into OCT and sectioned at 0.9 uM. Frozen sections were then stained for CD31 as described.

CXCL4 ELISA

Conditioned media derived from myeloid cells (BMM or RAW 267.4) cultures with or without B16 cells were passed through 0.2um and snap frozen in liquid nitrogen. ELISA kit for CXCL4 was purchased from R&D systems and used according to manufacturer's instructions.

RNA ISOLATION AND QUANTITATIVE PCR

mRNA from BMMs or RAW cells were obtained using RNAqueous Micro Kit (Ambion) according to manufacturer's protocol. Lung mRNA was isolated using Trizol reagent (Invitrogen). DNAse treatment using Turbo DNAse kit (Ambion) was performed for 40 minutes. cDNA was synthesized using Superscript cDNA kit (Invitrogen) according to manufacturer's instructions. VEGFR1, VEGF, CXCL4 and PEDF expression were quantified by qPCR performed on a 7500 Real-Time PCR system (Applied Biosystems), using Taqman primer/probes obtained from Applied Biosystems.

LENTIVIRAL CONSTRUCTS

Mouse pKLO.VEGFR1 shRNA, CXCL4 shRNA, VEGF shRNA and non-targeting control (Scramble) lentiviral vectors were purchased from Open Biosystems. The shRNA constructs were designed to include a hairpin of 21 base pair sense and antisense stem and a 6 base pair loop. Expression of the hairpin sequence is driven by the human U6 promoter. Hairpin sequences targeting VEGFR1 were, 5′ CGTGACCTTTAATCGTGCTTT 3′ (shVEGFR1#1) and 5′ GCCTCAGATCACTTGGTTCAA 3′ (shVEGFR1#2). Hairpin sequence targeting mCXCL4 was 5′ CCCGAAGAAAGCGATGGAGAT 3′ and non-targeting (scramble) hairpin sequence was 5′ CAACAAGATGAAGAGCACCAA 3′. Additionally, a puromycin selection marker is driven by the human phosphoglycerate kinase promoter (PGK) allowing for selection of cells with incorporation of the shRNA vector by incubation with 2ug/ml of puromycin. Virus production was performed as previously described (Dull et al., 1998).

IMMUNOFLUORESCENCE

Tissues were fixed in 1.6% paraformaldehyde/20% sucrose in PBS for 24 hours at 4ºC and then embedded in OCT compound and stored at -80ºC. Tissues were then sectioned at 0.8um using a cryostat and placed on Superfrost Plus glass slides (VWR). Slides were incubated in 10% normal donkey serum (Vector ImmunoResearch) in PBS/0.1% Triton X-100 for one hour. Primary antibodies diluted in PBS were then added for overnight incubation at 4ºC. Slides were washed three times with PBS and then incubated with anti-Rat secondary antibody conjugated to FITC or Cy3 for 2 hours at room temperature. After three washes in PBS, slides were coversliped using Prolong Gold anti-fade reagent with DAPI (Invitrogen). Visualization was performed using fluorescent microscope (Nikon Eclipse E800) and Retiga camera (QImaging) through IPLab version 3.65a imaging software (Scanalytics). Confocal images were acquired using Nikon D-Eclipse C1si confocal system and EZ-C1 3.6 software.

For immunofluorescence, frozen sections were post-fixed with acetone and stained with antibodies against CD11b (eBioscience), VEGFR1 (Santa Cruz), CXCL4 (R&D), or CD31 (BD Bioscience).

IN VIVO BM MYELOID CELLS/B16 CELL CO-INJECTION EXPERIMENT

BM myeloid cells transduced with appropriate shRNA vectors were isolated as described above. 5x105 BMM cells were mixed with 1x105 B16 cells in 300uL of Matrigel (BD Biosciences). Matrigel containing mixed BMM and B16 cells were injected subcutaneously into C57/B6 mice. Tumors were measured every other day with a caliper and were allowed to grow for 17 days after which they were excised and fixed in 1.6% PFA 20% sucrose in PBS overnight. Tumors were then embedded in OCT, sectioned at 0.8uM, and stained for CD31.

FLOW CYTOMETRY

Right lung was collected after perfusion with PBS by injection in the right ventricule. Tissue was minced

into small pieces, and incubated in 4mg/ml collagenase/dispase in PBS for 30-45 minutes in an agitating water bath at 37ºC. Tissue was then passed several times through a 21-gauge syringe needle after which it was filtered with 100- and 40-um filters (BD Biosciences), in order to form a single cell suspension. Cells were resuspended at a concentration of 1x107 cells/mL in 1%BSA in PBS and stained in total volume of 100uL for 20 min incubation with appropriate antibodies. The antibodies used were anti-CD11b, anti-GR-1 and anti-CD11c (BD biosciences) and anti-F4/80 (eBioscience). Cells were then washed and resuspended in 500uL PBS. Flow cytometry analysis was performed using a BD FACSCalibur and FloJo software was used for data analysis. BD FACS Aria was used for cell sorting experiment. Cells were sorted into RPMI 2%FBS, spun at 1300 rpm for 5 minutes, and then resuspended in RNAqueous lysis solution (Ambion).

IN VITRO ENDOTHELIAL CELL SURVIVAL ASSAY

1x104 2H-11 (ATCC) cells were plated in a 96-well plate in DMEM 10%FBS. Twenty-four hours later, media was changed to serum-free DMEM or to conditioned media-derived from B16 cells cultured in serum-free DMEM for 24 hours. 2x104 RAW cells transduced with scramble control or VEGFR1 shRNA were then either co-plated with 2H-11 cells, or plated in their own well. 48h later, cell viability was measured using CellTiter 96 Cell Proliferation Assay (Promega) as per manufacturer's protocol. Survival of 2H-11 cell was calculated by measuring using the equation= (abs 490nmRAW+2H-11 –abs 490nm RAW)/ (abs 490nm 2H-11), where RAW+2H-11 is co-culture of the two cell types, and RAW or 2H-11 are single cell cultures.

MICROARRAY

mRNA from RAW 267.4 cells co-cultured with B16 or LLC cells were processed using the RNAEasy mini kit (Qiagen) according to the manufacturer's protocol, including the optional DNAse treatment. RNA quantity was measured using a Nanodrop spectrophotometer and quality was assessed using a Bioanalyzer (Agilent). One microgram of high quality RNA was then submitted to the Microarray Core Facility of Weill Cornell Medical College, where the samples were labeled and hybridized to an Affymetrix GeneChip

Mouse Genome 430A 2.0 Array. Analysis of differential gene expression was performed using Genespring software (Agilent). Genes differentially up or downregulated by a factor of at least 2-fold were first identified. Of these, 23 genes were found to be statistically significant based on one-way ANOVA analysis on 3 replicate samples in each group.

STATISTICAL ANALYSIS

All statistical analysis were performed using GraphPad Prism 5 software. The statistical significance of comparisons between control and experimental groups was tested by Student's t test (unpaired, two-tailed), and results were considered statistically significant at p values < 0.05.

5.4 RESULTS

MYELOID CELLS LACKING VEGFR1 POSSESS ANTI-ANGIOGENIC ACTIVITY AND INHIBIT B16 MELANOMA GROWTH

In order to assess the functional role of VEGFR1 expression on BM-derived myeloid cells in the early tumor microenvironment, we implanted matrigel plugs consisting of normal or VEGFR1-deficient BMDCs with B16 tumor cells at a ratio of 5:1. This assay would create an artificial microenvironment that would accurately assess BMDCs impact on initial tumor outgrowth.

In order to generate BM-derived myeloid cells, whole BM was isolated and treated ex vivo with M-CSF to differentiate them to the myeloid lineage. Resultant cells expressed CD11b, F4/80, and VEGFR1 and were negative for CD11c and GR1. BM-derived myeloid cells were then transduced with lentiviral vectors containing a scramble shRNA or shRNA targeting VEGFR1 (Figure 5.1 A). Interestingly, we found that tumor cells in plugs containing myeloid BMDCs transduced with VEGFR1 shRNA grew significantly slower compared to controls, with most significant difference in tumor volume observed at day 17 after implantation (Figure 5.1 B). Notably, B16 cells

grew equally well in in vitro cocultures with control or VEGFR1-deficient myeloid BMDCs, suggesting this effect to be dependent on other in vivo stromal components. In addition, we observed defects in vessel formation within these tumors, as determined by quantification of CD31+ vessels (Figure 5.1 C,D). To determine if the observed defect in high density vessel formation was due to secreted factors derived from VEGFR1-deficient myeloid BMDCs, matrigel plugs mixed with BMDC conditioned media were implanted subcutaneously. After 10 days, we observed a clear reduction in the number of recruited CD31+ cells at the periphery of the matrigel plugs commixed with conditioned media derived from VEGFR1-deficient myeloid cells (Figure 5.1 E,F). These results demonstrate that VEGFR1-expression by myeloid BMDCs modulates B16 tumor outgrowth via the angiogenic-regulatory function of these cells, which can be attributed to the secretion of paracrine factors.

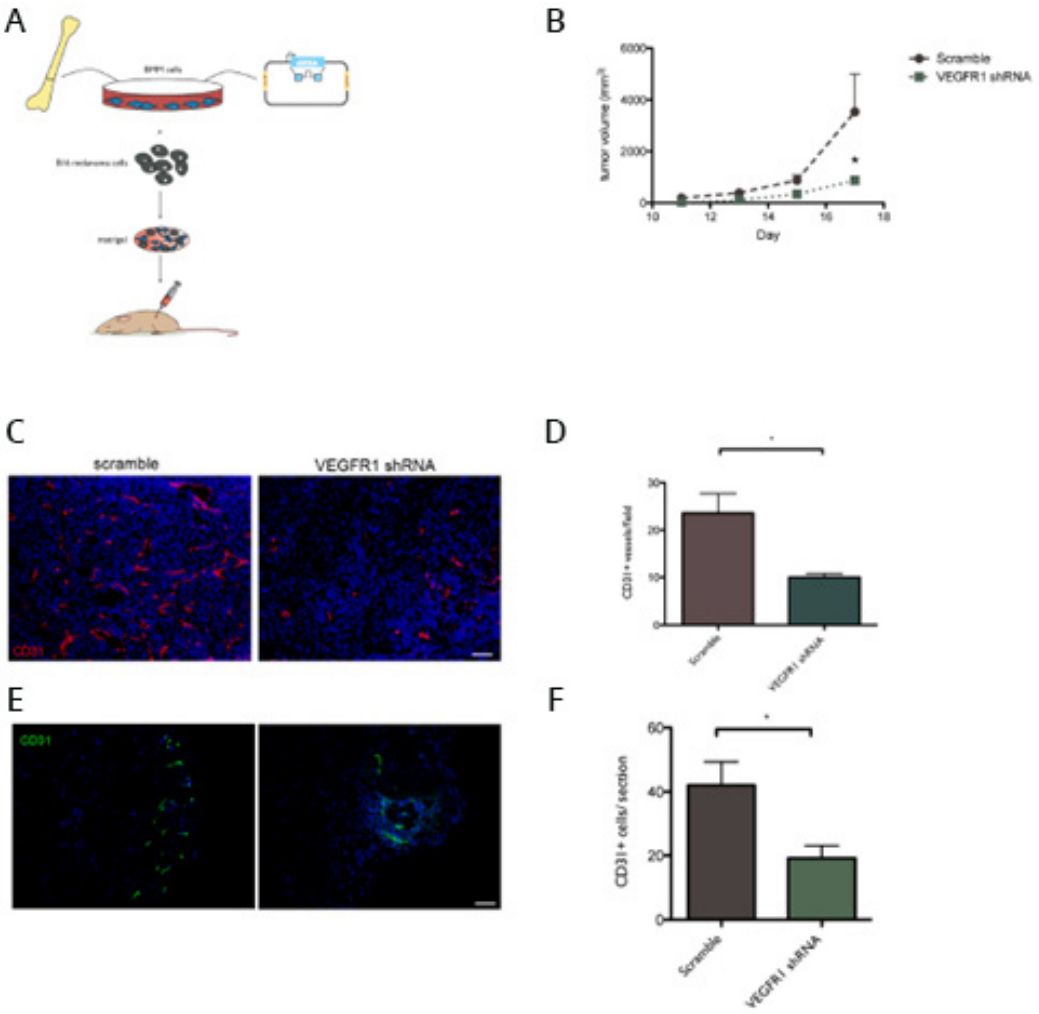


Figure 5.1. Myeloid cells lacking VEGFR1 possess anti-angiogenic activity and inhibit B16 melanoma growth. (A) Scheme illustrating method of BMM-B16 co-innoculation experiment. (B) Volume of B16 subcutaneous tumors mixed with scramble control or VEGFR1 shRNA transduced BMMs. (n=3, $p<0.05$). (C) CD31 immunostaining of vessels in BMM cell co-injected tumors (scale= 100um). (D) Quantification of microvessel density of tumors 17 days after (CD31+ vessels/field, $p<0.05$). (E) Immunofluorescence of CD31+ cells infiltrating into matrigel plug mixed with conditioned media derived from control or VEGFR1- deficient BMM cells ($p<0.05$).

ANTIANGIOGENIC FACTOR, CXCL4, IS HIGHLY EXPRESSED IN CD11B+ MYELOMONOCYTIC CELLS AND IS REGULATED BY VEGFR1 EXPRESSION

Given the apparent role of VEGFR1 expression by myeloid BMDCs in supporting a pro-angiogenic microenvironment, we sought to identify putative VEGFR1-regulated factors involved in this activity. To achieve this, we set up an in vitro co-culture system with VEGFR1+ myeloid cells and B16 tumor cells (Figure 5.2 A). Briefly, RAW 264.7 monocyte/macrophages expressing endogenous levels of VEGFR1 (Matsumoto et al., 2002) were transduced with the VEGFR1 shRNA lentivirus and co-cultured with tumor cells for 48 hours. RNA from RAW cells was then isolated and submitted for microarray analysis (Figure 5.2 A). Statistical methods identified 23 genes that were differentially upregulated or downregulated by a factor of at least 2 in VEGFR1-deficient RAW cells compared to controls (Figure 5.2 B). Of particular interest was CXCL4, or platelet factor 4, the most upregulated secreted chemokine and a well- characterized angiostatic factor (Maurer et al., 2006; Slungaard, 2005). CXCL4 was found to be secreted at high levels by BM myeloid cells as determined by an angiogenesis-specific proteonomic array. Though predominantly expressed by megakaryocytes and packaged into platelets (Bikfalvi, 2004; Maurer et al., 2006), recently CXCL4 was reported to be expressed by activated human monocytes and macrophages (Schaffner et al., 2005). CXCL4 has been shown to inhibit growth of various tumors (Bello et al., 2002; Maione et al., 1991; Sharpe et al., 1990; Tanaka et al., 1997), and metastasis (Kolber et al., 1995) in vivo, predominantly due to its antiangiogenic activity and not effects on tumor cell proliferation. Given the well-established function of CXCL4 as an angiogenesis-regulatory protein and its previously uncharacterized expression in tumor -associating myeloid BMDCs, we focused our attention on this factor to determine its potential VEGFR1-regulated activity in the tumor and metastatic microenvironments.

The upregulation of CXCL4 in VEGFR1-deficient RAW cells was verified utilizing two separate VEGFR1-targeting shRNAs (Figure 5.2 C). In addition, VEGFR1 knockdown induced CXCL4 upregulation in primary BM myeloid cells on both the mRNA and protein level (Figure 5.2 E,F). Notably, CXCL4 upregulation was increased 2-4 fold in VEGFR1-deficient myeloid cells co-cultured with B16 tumor cells, suggesting tumor-derived factors amplify this upregulation (Figure 5.2 D,F). Together these data indicate the negative transcriptional regulation of a highly anti-angiogenic factor by VEGFR1 expression in BM-derived myeloid cells. Such regulation implicates VEGFR1 in playing an important role in providing a permissive environment for tumor vessel development.

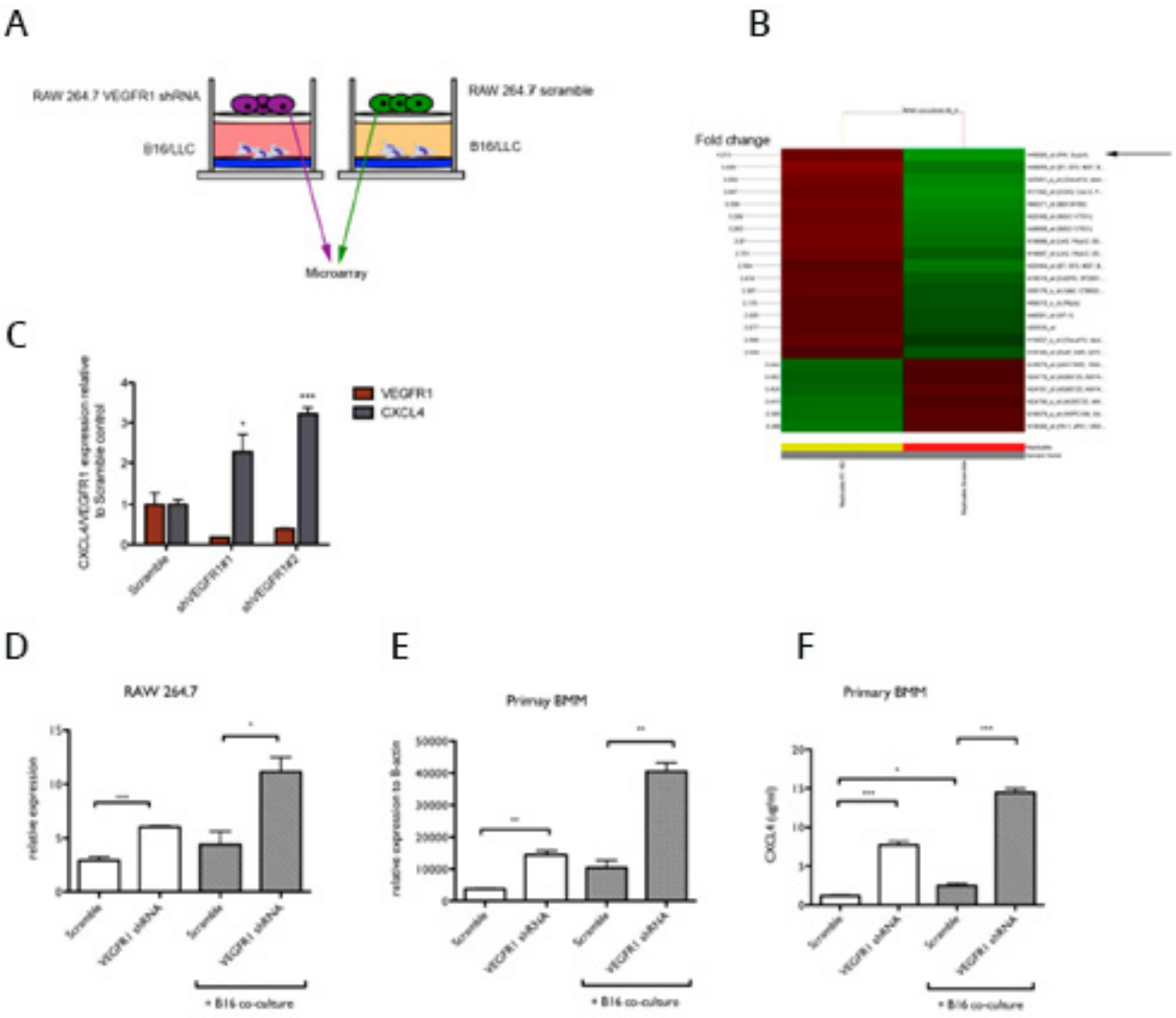
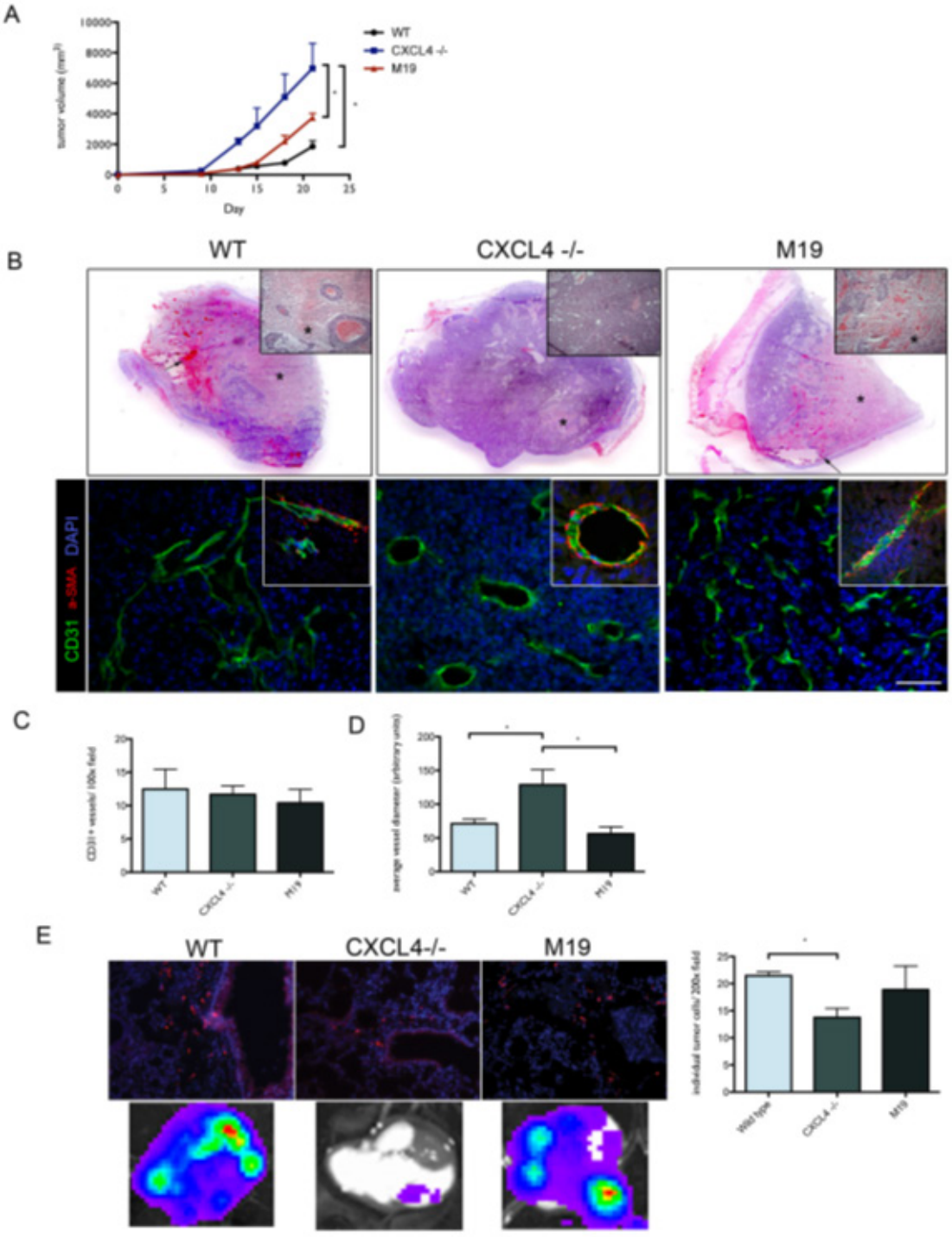


Figure 5.2. Antiangiogenic factor, CXCL4, is highly expressed in CD11b+ myelomonocytic cells and is regulated by VEGFR1 expression. (A) Scheme showing in vitro co-culture performed using the myeloid RAW 264.7 cell line. RAW cells transduced with VEGFR1 shRNA or scramble control virus were plated on the upper chamber of a 0.4uM transwell plate. These cells were cultured with B16 melanoma cells for 48h, after which RNA was isolated from RAW cells and submitted for microarray analysis. (B) Condition clustering analysis displaying 27 genes statistically significantly up or downregulated in control vs. VEGFR1 shRNA conditions (ANOVA p< 0.05, n=3). (C) QPCR for VEGFR1 and CXCL4 in RAW cells transduced with two different shRNAs targeting VEGFR1. Expression relative to scramble control. (D) and (E) Quantitative PCR analysis of CXCL4 expression from RAW cells (D) or BMM cells (E) alone or in co-culture with B16 cells for 48 h. (n= 3) (F) Elisa assay measuring levels of secreted CXCL4 in BMM cells transduced with either control or VEGFR1 shRNA vector alone and after co-culture with B16 melanoma cells for 48h. (*p<0.05, ** p<0.01, *** p<0.001).

Primary tumors and experimental metastases in CXCL4-/- hosts have accelerated growth and possess mature, normalized vasculature. The angiostatic activity of CXCL4 was first identified in 1990 by Sharpe et al., who demonstrated that purified recombinant human CXCL4 inhibited blood vessel proliferation in the chicken chorioallantoic membrane in a dose dependent manner (Maione et al., 1990). Since then, several reports have described recombinant forms and derivative peptides of CXCL4 as having clear anti-tumor and anti-metastatic effects in various tumor models due to its inhibitory activity on the development of tumor vasculature (Kolber et al., 1995; Li et al., 2003; Maione et al., 1991; Sharpe et al., 1990; Struyf et al., 2007; Tanaka et al., 1997; Yamaguchi et al., 2005; Yoshimitsu et al., 1995). In addition, various mechanisms have been reported to explain CXCL4 inhibitory action on endothelial cell proliferation, migration, and survival, including the binding of proangiogenic growth factors, integrins expressed on endothelial cell surface, and activation of distinct signaling pathways (Aidoudi et al., 2008; Hagedorn et al., 2001; Jouan et al., 1999; Perollet et al., 1998; Sulpice et al., 2004). Despite this in depth understanding of CXCL4 effect on tumor progression and its mechanism of action, all the previously mentioned studies have investigated the action of exogenously expressed CXCL4 on tumor progression and metastasis. Therefore, there is little understanding of endogenous sources of CXCL4 and their role in a non-therapeutic setting. In order to investigate the effect of host-derived CXCL4 on these processes, we challenged CXCL4 knockout (CXCL4-/-) mice with B16 melanoma tumors.

As anticipated, B16 primary tumors grew strikingly faster in CXCL4 -/- hosts compared to WT controls (Figure 5.3 A). Additionally, re-expressing human CXCL4 in a CXCL4 -/- background (M19) significantly rescued primary tumor growth, indicating a direct role for CXCL4 in the observed phenotype. Histology of primary tumors in CXCL4-/- host appears significantly more viable than WT controls, with fewer areas of necrosis and hemorrhaging vasculature (Figure 5.3 B, top). No significant difference in microvessel density was observed between groups (Figure 5.3 C), however, we noticed a drastic difference in the phenotype of tumor vasculature. CD31+ tumor vessels in CXCL4-/- mice possessed larger lumen area and appeared significantly more mature with complete pericyte coverage (Figure 5.3 B bottom, 5.3 D). Notably, accelerated primary tumor growth associated with a more mature vascular phenotype has been reported previously in mice lacking VEGF-A expression in myeloid cells (Stockmann et al., 2008).

Unexpectedly, when we assessed the effect of CXCL4 knockout on metastatic progression we observed fewer seeding tumor cells and associated macrometastatic development in CXCL4-/- mice compared to controls (Figure 5.3 E). In order to determine if the apparent disparity in primary tumor growth vs. metastatic progression was due to defects in the dissemination stage, we bypassed this dissemination step by injecting a small number (5x104) of tumor cells directly into circulation via tail vein. In this situation, we noticed a significant acceleration in metastatic lesion outgrowth in lungs of CXCL4-/- mice 15 days after tumor injection (Figure 5.3 F). Metastatic lesions in CXCL4-/- were increased in number and possessed enhanced vascularity with significant pericyte recruitment (Figure 5.3 G). Together, these experiments argue for a role of endogenously expressed CXCL4 in inhibiting primary and metastatic tumor growth by blocking tumor vessel development and maturity, yet promote tumor cell dissemination likely by contributing toward leaky vasculature.


A
WT
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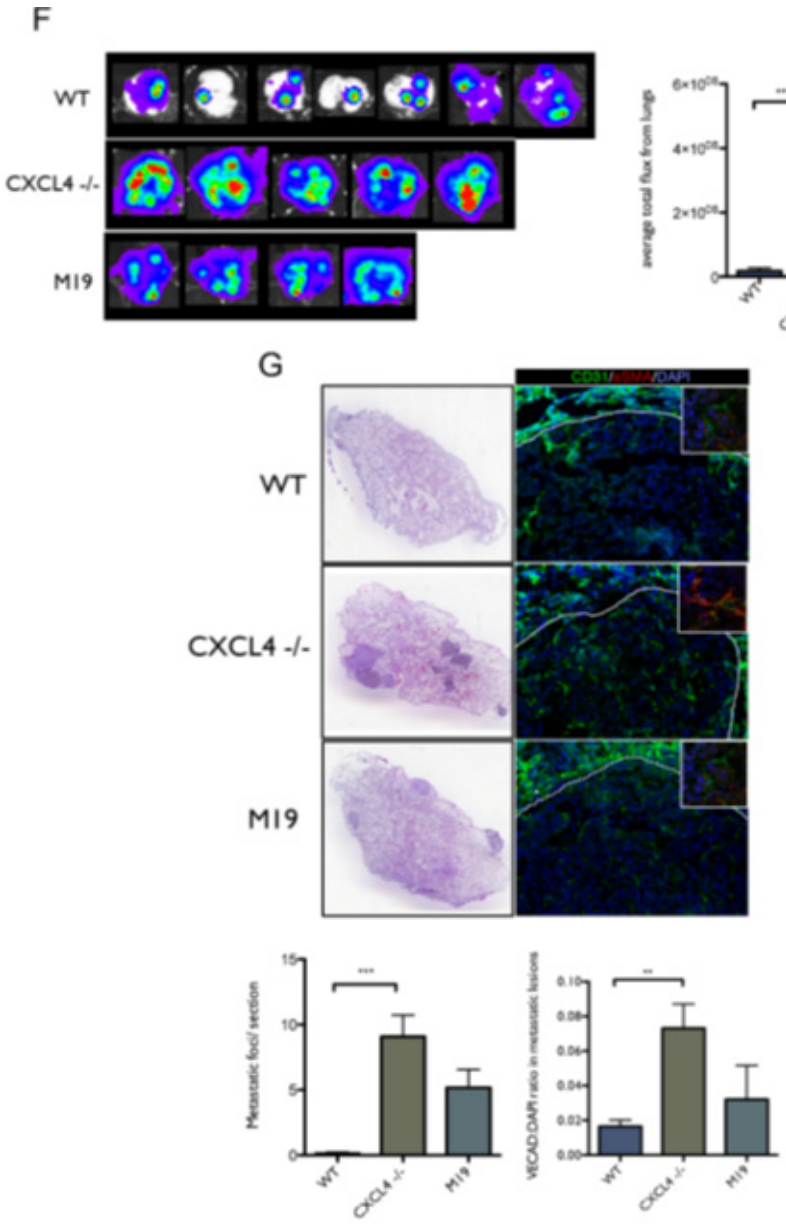


Figure 5.3. Primary tumors and experimental metastases in CXCL4-/- hosts have accelerated growth and possess mature, normalized vasculature. (A) B16 melanoma primary tumor volumes in WT, CXCL4-/-, and M19 mice (n=4-6, p<0.05) B) Tumor histology determined by H&E (top) and immunofluorescence for CD31 and a-SMA (bottom). Asterisks indicate regions of necrosis. Arrows indicate regions of hemorrhaging vessels. (C) Quantification of microvessel density in B16 tumors. (D) Quantification of vessel lumen diameter in WT, CXCL4-/-, and M19 mice (E) Seeding Mcherry+ B16 cells in the lung at day 21 after tumor implantation in WT, CXCL4-/-, and M19 mice (top) Bioluminescence of late stage metastatic lungs after tumor resection (bottom). (F) Bioluminesence imaging of macrometastatic disease 15 days after tail vein injection of 5x104 B16 cells. (n= 4-8, ****p<0.0001, *p<0.05). (G) H&E of lungs indicating levels of macrometastases (left). Immunofluorescence of VECAD+ vessels and aSMA pericyte coverage within macrometastatic lesions in WT, CXCL4-/-, and M19 mice (right).

MYELOID CELL-DERIVED CXCL4 REGULATES ANGIOGENIC PHENOTYPE AND GROWTH OF B16 MELANOMA TUMORS

Given the observed expression of CXCL4 in BM-derived myeloid cells in vitro, we were interested to determine the expression of CXCL4 by recruited BMDCs in the tumor microenvironment in vivo. By immunofluorescence, we observed an upregulation of CXCL4 by recruited GFP+ BMDCs at later stages of tumor progression (Supplemental Figure 5.4 A, top). At these stages, CXCL4 was expressed by GFP+ BMDCs at the tumor periphery as well as by BMDCs in close association with VECAD+ nascent tumor vasculature (Figure 5.4 A). To determine CXCL4 expression in myeloid cell types, GFP+ BMDCs were sorted for CD11b and GR1 and expression of CXCL4 was determined by QPCR. Confirming our imaging data, tumor-associating myeloid cells upregulated CXCL4 at later stages, with CD11b+GR1- cells expressing the highest levels of this chemokine (Figure 5.4 B).

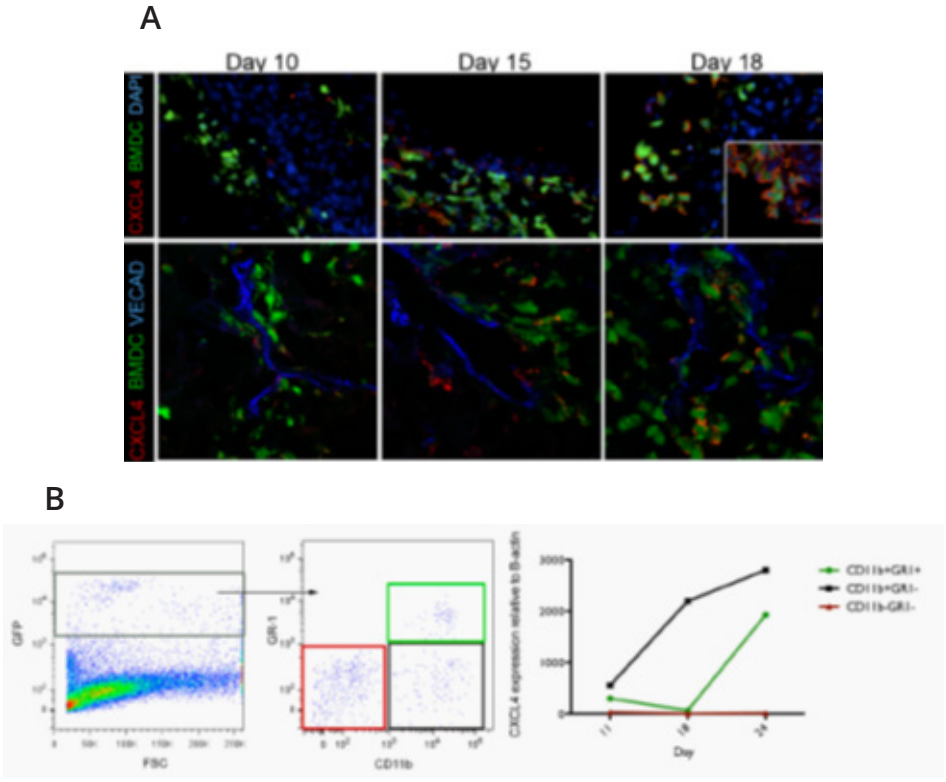


Figure 5.4. (A) Immunofluorescence for CXCL4 at the periphery (top) and center (bottom) of primary B16 melanoma tumors at Day 10, 15, and 18 after tumor injection. BMDCs are marked by GFP expression. (B) FACS analysis for GFP+,-CD11b and GR1 in cells derived from B16 melanoma tumors (left). RNA from sorted cell populations was collected and analyzed for CXCL4 expression (right). (C) Angiogenic cytokine array measuring levels of cytokine expression from BMM cell conditioned media. Graph highlights 4 of the most highly expressed angiogenic factors.

In order to test the effect of VEGFR1-regulated CXCL4 expression in BM-derived myeloid cells on tumor angiogenesis and progression, we repeated the myeloid BMDCs /B16 cell commixing experiment utilizing WT and CXCL4-/- BMDCs. Briefly, myeloid BMDCs isolated from WT or CXCL4-/- hosts were transduced with scramble or VEGFR1 shRNA-containing lentivirus. BMDCs were mixed at a ratio of 5:1 with B16 cells in matrigel and implanted subcutaneously into WT mice. At 14 days post-injection, tumors commixed with VEGFR1-deficient BM-derived myeloid cells were significantly smaller than WT controls as previously observed (Figure 5.5 A). However, VEGFR1 knockdown in CXCL4-/- cells failed to inhibit primary tumor growth (Figure 5.5 A). In fact, we observed a moderate increase in growth of tumors commixed with CXCL4-/- or CXCL4-/- VEGFR1 shRNA BM-derived myeloid cells (Figure 5.5A) .These results argue that CXCL4 expression is responsible for the inhibitory effect on tumor proliferation observed in VEGFR1-deficient BMM cell commixed tumors.

We next analyzed tumor histology and vascular phenotype of tumors commixed with myeloid BMDCs. Notably, the histology tumors commixed with CXCL4-/- or CXCL4-/- VEGFR1 shRNA BMM cells were mostly viable and contained smaller areas of necrosis compared to WT controls (Figure 5.5B, top). In addition, vessels in CXCL4-/- BM-derived myeloid cell-containing tumors appeared to have larger lumen area and were significantly less leaky compared to tumors mixed with WT or VEGFR1 shRNA BMM cells as determined by dextran permeability (Figure 5.5B-E). In general, the histology and vascular characteristics of tumors commixed with CXCL4 -/- BM-derived myeloid cells photocopied tumors in CXCL4-/- hosts. These results suggest that myeloid BM cell-derived CXCL4 expression has the capacity to inhibit tumor vascular maturation and ultimately regulate tumor growth and progression.

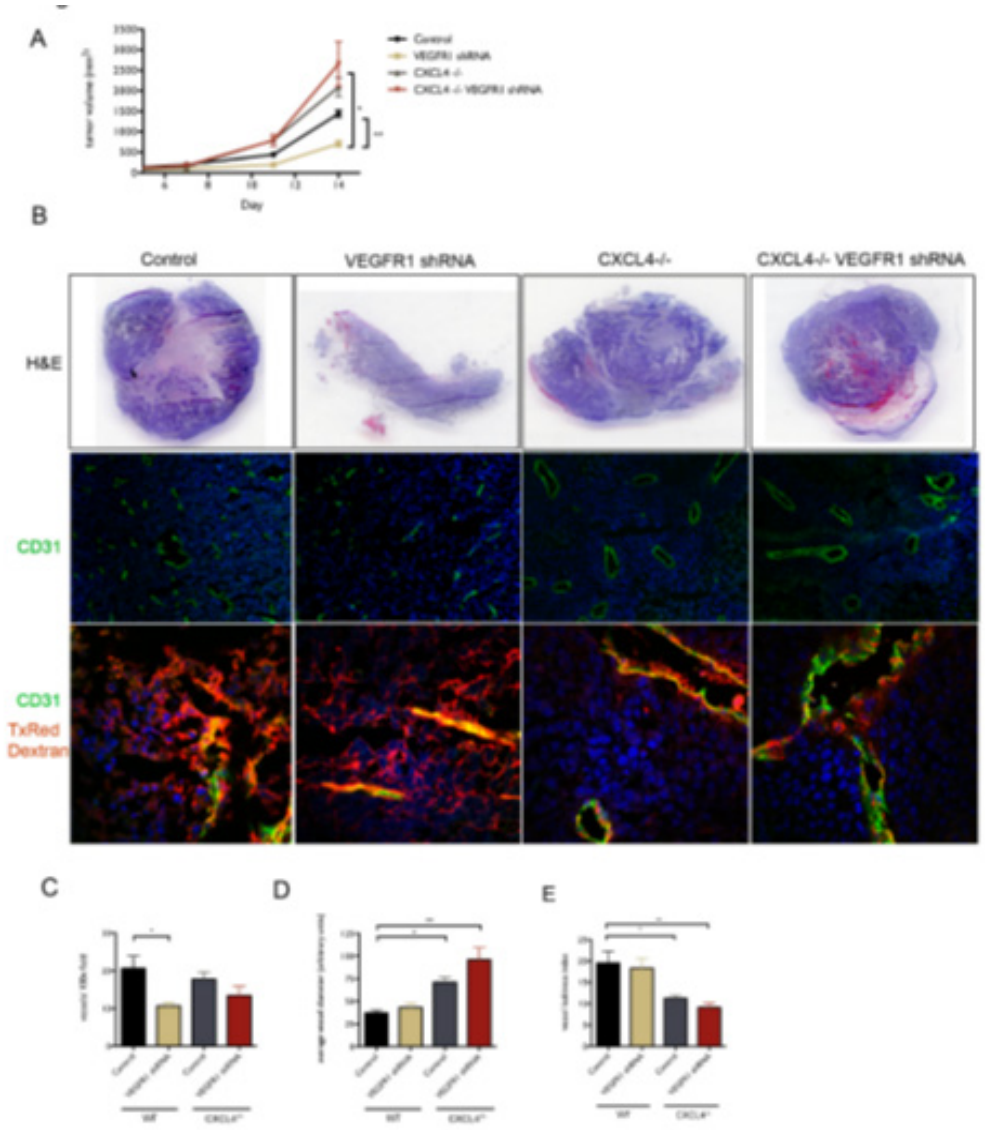


Figure 5.5. Myeloid cell-derived CXCL4 regulates angiogenic phenotype and growth of B16 melanoma tumors
(A) Growth of BMM + B16 commixed tumors. WT or CXCL4-/- BMMs transduced with control or VEGFR1 shRNA lentivirus were commixed with B16 melanoma cells and implanted subcutaneously in matrigel (n=3-4, *p<0.05, **p<0.01) Experiment was repeated 3 times with similar results. (B) Tumor histology (H&E, top), CD31 immunofluorescence (middle), and dextran vessel permeability (bottom) of B16+BMM commixed tumors. (C-E) Quantification of microvessel density, average vessel lumen diameter, and vessel leakiness in co-mixed tumors (*p<0.05, **p<0.01).

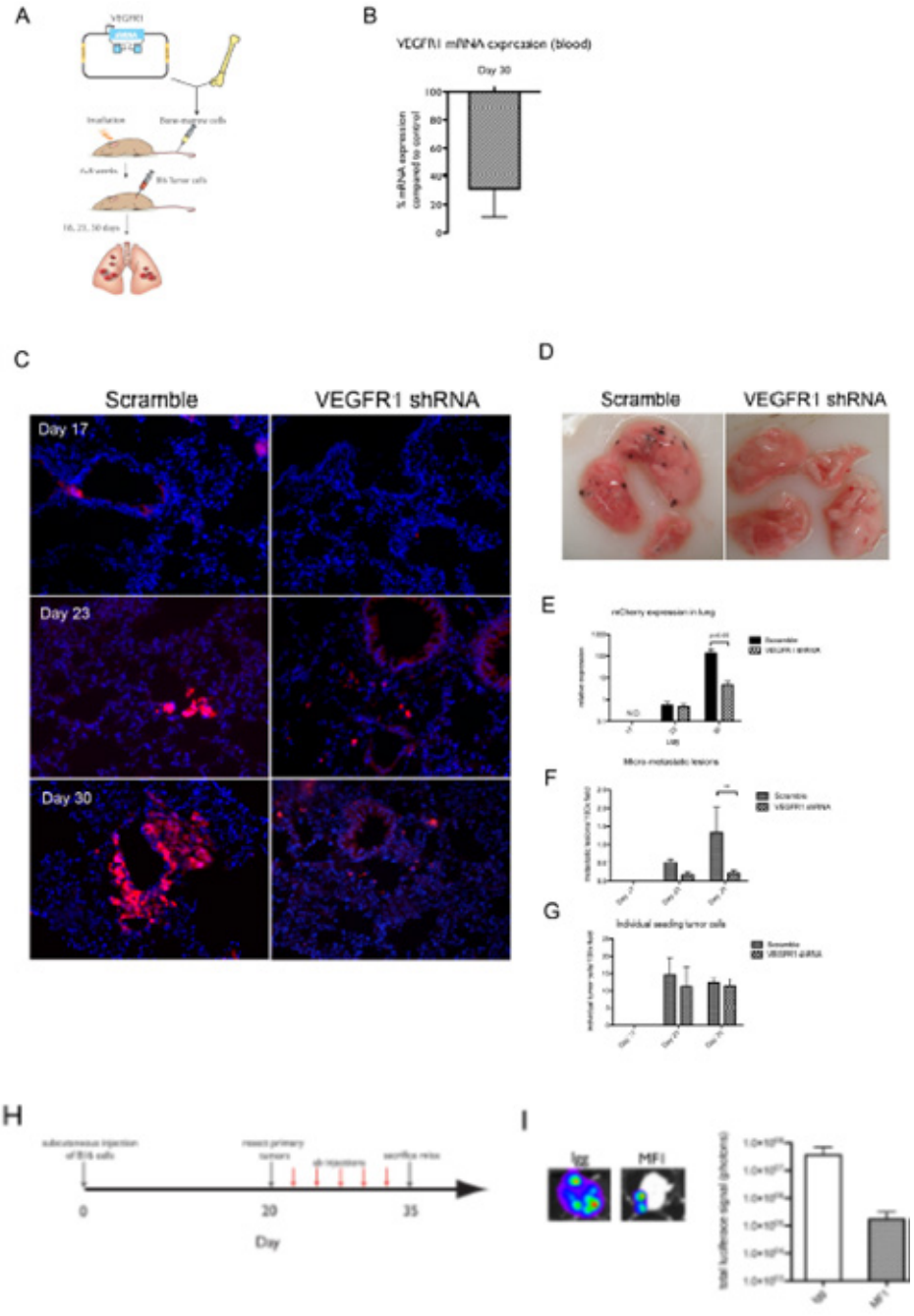
INHIBITION OF VEGFR1 IN THE BM BLOCKS THE PROGRESSION OF MACROMETASTASIS AND IS CXCL4 DEPENDENT

To assess the role of VEGFR1 expression by BM-derived myeloid cells in the metastatic microenvironment, we tested the effect of BM-specific VEGFR1 inhibition on spontaneous metastasis in the B16 tumor model. Briefly, lineage negative cells derived from C57/B6 donors were transduced ex vivo with either a lentiviral vector containing either VEGFR1 shRNA or a non-targeting control hairpin (scramble) and transplanted into lethally irradiated recipient mice (Figure 5.6 A). VEGFR1 expression in the blood of transplanted animals was reduced 70%, on average, compared to controls (Figure 5.6 B). Mice had normal hematopoietic reconstitution after 6-8 weeks, and were challenged with subcutaneous injection of mCherry-B16 melanoma cells. Tumors were allowed to grow for 17, 23, or 30 days. Inhibition of VEGFR1 by shRNA did not significantly alter primary tumor growth or weight, though a slight yet significant reduction in microvessel density was observed. Levels of CD11b+GR1+ and CD11b+Gr1- cells mobilized into the blood were not significantly different in VEGFR1 shRNA mice compared to controls. Additionally, infiltration of CD11b+ cells into the pre- metastatic regions of the lung was still present in VEGFR1 knockdown mice, and at a frequency similar to control mice. Consistent with recent reports (Dawson et al., 2009), these results suggest that recruitment and homing of these BMDCs to pre-metastatic sites may be reliant on VEGFR1- independent mechanisms (Erler et al., 2009; Kaplan et al., 2005).

To quantify metastatic burden, we analyzed lungs of tumor-bearing mice at day 17, 23 and 30-post tumor injection. Fluorescent microscopy and visual inspection of the lungs revealed a pronounced reduction in the progression of visible metastatic lesions in VEGFR1 shRNA-transduced mice as compared to controls (Figure 5.6 C and 5.6 D). As late as day 30-post tumor inoculation, VEGFR1 shRNA mice possessed single, scattered tumor cells, however lacked the appearance of developed metastatic foci (Figure 5.6 C). Total tumor burden as well as number of micro and macro metastatic lesions in the lung was dramatically reduced by day 30 in VEGFR1 shRNA-transduced animals (Figure 5.6 E,F). Notably, the number of individual seeding tumor cells were comparable in VEGFR1 shRNA-transduced and control mice at all time points (Figure 5.6 G). These data demonstrate that VEGFR1 expression by BMDCs present at the metastatic microenvironment is essential for the maintenance and progression of colonizing tumor cells into micrometastatic and metastatic foci.

To verify the role of VEGFR1 in promoting macrometastatic progression within the metastatic microenvironment, we determined the effect of inhibiting VEGFR1 after initial tumor cell seeding. Briefly, B16 tumors were allowed to grow for 20 days after which tumors were surgically resected. Blocking antibodies targeting VEGFR1 (MF1) or IGG control were then administered every two days until 15 days after resection when mice were sacrificed and metastatic burden was assessed (Figure 5.6H). By bioluminescence imaging, MF1 treated mice possess overall less metastatic burden in the lung compared to IGG controls (Figure 5.6I). Metastatic lesions were less abundant and were smaller in size in MF1 treated mice (Figure 5.6J). These experiments confirm the role for VEGFR1 in providing a permissive microenvironment for metastatic lesion outgrowth.

In order to determine the functional importance of CXCL4 in VEGFR1-regulated metastasis progression, we tested the metastatic potential of mice transplanted with bone marrow transduced with VEGFR1 shRNA alone or VEGFR1+CXCL4 shRNAs. CXCL4 shRNA lentiviral transduction showed efficacy in normalizing mRNA levels of CXCL4 in bone-marrow cells co- transduced with VEGFR1 shRNA. After bone marrow transplant and hematopoietic reconstitution, mice were challenged with B16 subcutaneous tumor injection and tumors were allowed to grow for 30 days. We did not observe significant differences in primary tumor growth or weight in any of the groups. Interestingly, mice with VEGFR1+CXCL4 shRNA bone marrow were able to develop significantly more metastatic lesions in the lungs compared to VEGFR1 shRNA alone transduced mice (Figures 5.6 K). VEGFR1+CXCL4 shRNA mice developed similar numbers of micrometastatic foci in the lungs compared to Scramble controls (Figure 5.6 K) resulting in a significant rescue of overall metastatic burden determined by mCherry expression (Figure 5.6 K), however not to levels of Scramble control mice. Together, these results indicate that VEGFR1-dependent regulation of CXCL4 expression in CD11b+ cells at the metastatic niche is a key pathway in providing a permissive environment for vascularization and subsequent progression to macrometastatic disease.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B
VEGFR1 mRNA expression (blood)
Day 30
% mRNA expression compared to control
C
Scramble
VEGFR1 shRNA
Day 17
Day 23
Day 30
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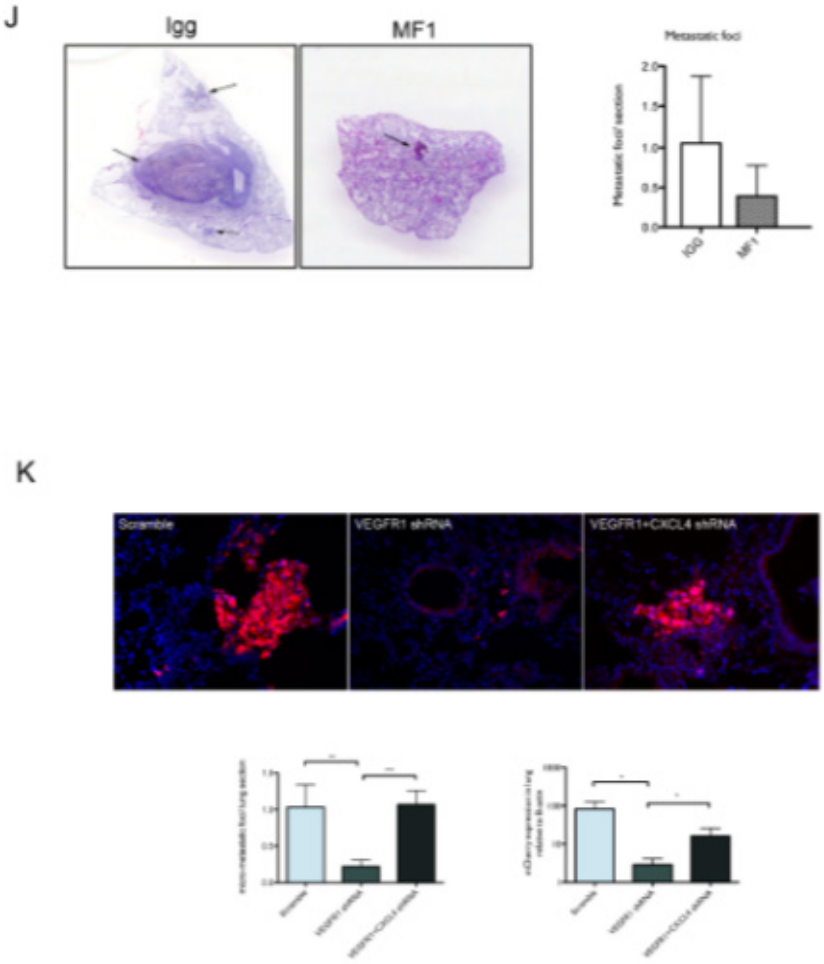

Figure 5.6. Inhibition of VEGFR1 in the BM blocks the progression of macrometastasis and is CXCL4 dependent. (A) Schematic showing experimental design to determine effect of VEGFR1 knockdown in BM on tumor progression and metastasis. Lineage-depleted bone marrow cells were transduced with a lentiviral vector driving ubiquitous expression of VEGFR1 shRNA. Transduced cells were used for bone marrow transplants. Six to eight weeks after transplantation, mice were given subcutaneous injections of mCherry-B16 tumor cells and lungs were analyzed at day 17, 23, and 30 of tumor burden. (B) mRNA expression of VEGFR1 in isolated blood cells of mice transplanted with VEGFR1 shRNA treated BM (C) Microscopy of mCherry+ B16 cells in the lung of Scramble control and VEGFR1 shRNA mice at Day 17, 23, and 30-post tumor injection. 200x (D) Images illustrating visible metastatic foci in lungs after 30 days of B16 melanoma tumor burden. (E) Quantitative PCR analysis of tumor-derived mCherry expression in lungs at day 17, 23, and 30 of tumor burden (n= 5-9, **p<0.01). (F) Quantification of micrometastatic lesions (defined by > 20 tumor cells) (top) and (G) Individual tumor cells visible/ per 100x field at day 17, 23, and 30 after tumor injection. Data are means +/- SEM. (H) Experimental scheme indicating time points of tumor resection and antibody administration. Primary B16 tumors were resected at day 20 and then MF1 or Igg injections were given every three days for 21 days. (I) Bioluminescence imaging of representative lungs from MF1 of Igg treated mice at day 15 post-resection (top). Quantification of total flux signal from lungs of mice (n=3-4) (bottom). (J) Histology of lungs showing development of metastatic lesions in either Igg or MF1 treated mice (left). Quantification of metastatic foci/ section in Igg and MF1 treated mice (right) (K) Fluorescence microscopy of mCherry+ B16 metastases in transplanted mice 30 days post tumor implantation (top). Quantification of micro-metastatic foci (cluster of > 20 mCherry+ cells) in lungs of mice transplanted scramble, VEGFR1 shRNA, or VEGFR1+CXCL4 shRNAs (bottom left). Foci/ lung section, 6 sections/ lung. QPCR for mCherry expression in lungs of transplanted mice after 30 days of tumor burden (bottom right, * p<0.05).

5.5 **DISCUSSION**

The role of VEGFR1 in regulating tumor progression and the metastatic process has been under question. Several studies have described VEGFR1 expression by myeloid cells that promote pathologic angiogenesis in conditions including tumorigenesis and inflammation (Du et al., 2008; Hiratsuka et al., 2001; Kerber et al., 2008; Kusmartsev et al., 2008; Lin et al., 2007; Murakami et al., 2006). In addition, VEGFR1 expressing populations of bone marrow-derived myeloid cells are key mediators of tumor cell extravasation, survival, and progression (Kaplan et al., 2005; Qian et al., 2009). However, the precise function of VEGFR1 expression by BMDCs and the mechanism through which this regulation occurs have not been characterized. Here, we report novel findings that indicate that VEGFR1 expression controls the angiogenic activity of tumor and metastasis-associating myeloid cells by suppressing the expression of a potent angiostatic chemokine. Such angiogenic regulation appears to play a profound role in controlling the primary tumor growth as well as the progression of colonizing metastatic cells into macrometastases.

The role of myeloid cells in regulating tumor angiogenesis is well recognized. BMDCs such as macrophages, neutrophils, eosinophils, and dendritic cells become mobilized, often in large numbers, to primary tumor sites where they've been shown to play an important role in driving angiogenic processes (Murdoch et al., 2008). Mechanisms by which they achieve this function includes the production of pro-angiogenic growth factors including VEGF, basic fibroblast growth factor, TNFα, interleukin 1β, CXCL4, cyclooxygenase 2, and matrix- degrading enzymes such as matrix metalloproteinase 7 (MMP7), MMP9, and MMP12 (Murdoch et al., 2008). VEGFR1+ cells, including those in the monocytic/macrophage lineage and their associated precursors, have been implicated in pro-angiogenic pathways as well. For example, VEGFR1+ macrophages are an important source of VEGF-A within the tumor as mice deficient in macrophages, through genetic deletion of the CSF-1 gene, manifest a delayed angiogenic switch (Lin et al., 2006). Other VEGFR1+ hematopoietic progenitor cells lie in close associated with forming vasculature and confer vascular stability (Lyden et al., 1999).

Several studies have reported differing findings with regard to the effect of VEGFR1 inhibition on tumor growth and angiogenesis. Works utilizing VEGFR1 blocking antibody or mice lacking the tyrosine kinase domain of VEGFR1 (VEGFR1 TK-/-) in various subcutaneous syngeneic tumor models have shown minimal effects on primary tumor growth (Bais et al., 2010; Hiratsuka et al., 2002; Kaplan et al., 2005; Lyden et al., 2001). Additionally, blockade of PLGF, a VEGFR1-specific ligand was shown to have no effect on angioge-

nesis during primary tumor growth in these models (Bais et al., 2010). Alternately, studies utilizing the same VEGFR1 TK-/- model have shown attenuation of angiogenesis and solid tumor growth in B16, HSML, and Gl261 tumor models (Kerber et al., 2008; Muramatsu et al., 2010). One likely explanation for this discrepancy is variation in tumor type dependency on BMDC VEGFR1 expression. For example, Muramatsu et al. found VEGFR1 inhibition to block B16 and HSML tumor growth whereas LLC proliferation was unaffected. These results implicate the role of other overlapping pro-angiogenic pathways that possess the capacity to compensate for VEGFR1 deficiency.

In our models of VEGFR1 inhibition through knockdown in BMDCs or use of blocking antibody, we observed a slight attenuation of vessel development in B16 primary tumors, however this effect was not significant enough to alter overall tumor growth or tumor cell dissemination to the lung. However, these methods drastically suppressed macrometastatic progression. Alternately, when tumor cells were co-implanted with myeloid BMDCs lacking VEGFR1 expression, we observed a significant reduction in tumor growth and vessel density. These results indicate that VEGFR1 influence on tumor proliferation is highly dependent on the microenvironmental context, specifically, when the stromal BM myeloid cells presence is high enough. This concept agrees with the drastic inhibition of macrometastatic progression after VEGFR1 inhibition since the environment in which metastatic tumor cells reside, the pre-metastatic niche, constitutes a high density of myeloid and progenitor cells (Kaplan et al., 2005).

Recent studies have challenged the importance of VEGFR1 in the metastatic process (Dawson et al., 2009), yet in contrast to this study, we observed a drastic reduction in macrometastatic disease in animals after VEGFR1 inhibition. Several factors may account for differing results. Though Dawson et al. use the same syngeneic tumors (B16), they employ model whereby tumors are implanted in the hind limb and resected by amputation of the leg. Such a dramatic surgery and removal of the primary tumor likely alters tumor-derived paracrine factors involved in setting up a permissive metastatic niche. Differing methods of VEGFR1 inhibition may also explain these apparent disparities. Both methods of VEGFR1 blockade (antibody and tyrosine kinase inhibition) utilized by Dawson et al. are strategies to systemically block VEGFR1 activity. Thus, this study does not directly assess the function of VEGFR1 in BMDCs. In addition, our antibody blockade experiments were aimed at targeting the events that allow for metastatic outgrowth after initial tumor cell colonization. Thus, the treatment regimen used to block this process, i.e. at later stages, may be more effective than starting antibody treatment at the onset of the experiment. Together, these studies suggest VEGFR1-independent pathways may be involved in earlier step including

myeloid cells recruitment to metastatic sites, yet emphasize the importance of VEGFR1 function at the metastatic microenvironment.

BM myeloid cell-derived CXCL4 regulation of angiogenesis

The anti-tumoral effect of CXCL4 is well described, as it has been shown to suppress the growth of several tumor types through its antiangiogenic activity (Bello et al., 2002; Kolber et al., 1995; Maione et al., 1991; Sharpe et al., 1990; Tanaka et al., 1997). The mechanism of this action has been attributed to interactions with angiogenic growth factors, such as VEGF and bFGF, integrins, and cell surface receptors to directly inhibit endothelial cell proliferation and migration. Until recently, CXCL4 has been recognized as a megakaryocyte- specific protein that is packaged into α-granules of platelets and released into the blood upon activation. However, CXCL4 is upregulated significantly in human monocytes upon inflammatory activation (Schaffner et al., 2005). Our data support the notion that cells of myeloid origin possess the capacity to express high enough levels of CXCL4 to affect pathological angiogenesis. Importantly, unlike platelets, the specific localization of VEGFR1+ cells to both the tumor microenvironment the metastatic niche within the lung parenchyma where newly seeded tumor cells reside provides an advantage to modulate metastatic progression through anti-angiogenic signals.

Our studies indicate that the pathological expression of CXCL4 expression in myeloid BMDCs is a key regulatory event for angiogenesis and subsequent metastatic progression. However, we believe this activity inhibits certain stages of metastatic progression, yet promotes others. In this model, VEGFR1 serves to promote an early pro-angiogenic burst to nascent tumors or colonizing metastatic tumor cells. As the tumor microenvironment matures, the combinatorial upregulation of both VEGF and CXCL4 in BM myeloid cells serves to disorganize the vasculature. Though this effectively reduces overall tumor volume, these tumors are highly hypoxic and possess a more aggressive phenotype. The combination of increasingly invasive and migratory tumor cells and highly permeable vasculature will lead to increased tumor intravasation and dissemination. After initially tumor cells seeding, analogous processes occurs with early VEGFR1+ cells promoting metastatic angiogenic switching and macrometastatic outgrowth. Therefore, CXCL4 effectively inhibits early angiogenic stages as well as vascular maturation which results in reduced overall tumor volume, however, promotes metastatic tumor cell dissemination steps. As with other emerging anti- angiogenic therapies, combinatorial targeting of this pathway will likely be necessary to effectively block all steps of tumor and metastatic progressiSSS

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6. CHAPTER SIX
THE ROLE OF EXOSOMES AND THE MICROENVIRONMENT IN DRUG RESISTANCE

EXPLORING THE ROLE OF INTERLEUKIN-6 AND OTHER HOST-ASSOCIATED FACTORS IN TUMOR PROGRESSION AND METASTASIS DEVELOPMENT

6. CHAPTER SIX
THE ROLE OF EXOSOMES AND THE MICROENVIRONMENT IN DRUG RESISTANCE [6]

6.1 SUMMARY

In the last decades, several studies demonstrated that the tumor microenvironment is a critical determinant not only of tumor progression and metastasis, but also of resistance to therapy. Exosomes are small membrane vesicles of endocytic origin, which contain mRNAs, DNA fragments and proteins, and are released by many different cell types, including cancer cells. Mounting evidence has shown that cancer-derived exosomes contribute to the recruitment and reprogramming of constituents associated with the tumor microenvironment. Understanding how exosomes and the tumor microenvironment impact drug resistance will allow novel and better strategies to overcome drug resistance and treat cancer.

In this chapter, we describe a technique for exosome purification from cell culture, fresh and frozen plasma and further analysis by electron microscopy, Nanosight microscope and Western-blot.

[6] Based on: Maria do Rosário André, Ana Pedro, David Lyden. Cancer exosomes as mediators of drug resistance. Methods Mol Biol. 2016. 1395:229-39

6.2 INTRODUCTION

THE TUMOR MICROENVIRONMENT AND THE ROLE OF EXOSOMES IN CANCER PROGRESSION

As discussed previously, solid tumors are complex, organ-like structures, consisting of cancer cells along with a supportive stroma composed of multiple non-malignant cell types, such as fibroblasts, endothelial cells, mesenchymal stem cells and immune cells, sustained by an extracellular matrix and a vascular network (Joyce & Pollard, 2009). Besides these different cell types, and the tumor-derived chemokines and growth factors mentioned before, exosomes are also important mediators of metastasis, being involved in a permanent crosstalk between the primary tumor and local/ distant host cells. Exosomes are small membranous extracellular vesicles, ranging in size between 40 and 100 nm in diameter, that contain microRNAs, messenger RNAs (mRNA), DNA fragments and proteins (Simpson et al., 2009). These small vesicles consist of a lipid bilayer membrane surrounding a small cytosol, are devoid of cellular organelles and are secreted by many kinds of cells, including tumor cells, reticulocytes and hematopoietic cells (Keller et al., 2006). Exosomes are formed by the inward budding of cells known as multivesicular endosomes. Fusion of these endosomes with the plasma membrane leads to the release of internal vesicles known as exosomes (Akers et al., 2013). The major role of exosomes seems to be the transport of bioactive molecules between cells, with consequences in targeted cell phenotypes. Exosomes are involved in the normal physiology of the body, including immune regulation, tissue repair and communication within the nervous system (Corrado et al., 2013). In cancer patients, the abundance of secreted exosomes suggests an important role of these mediators in cancer development. In fact, a positive correlation between increased exosome secretion and cancer stage and progression has been shown (Taylor & Gercel-Taylor, 2005). Exosomes travel to surrounding cells or distant tissues to execute important functions in tumor biology such as angiogenesis, immune suppression, induction of proliferation and transfer of genetic material (El Andaloussi et al., 2013). The transport of oncogenic proteins and miRNAs by exosomes released by tumor cells and the uptake of these oncogenic elements by non-malignant cells in the tumor microenvirnment can result in the transfer of oncogenic activity (Al-Nedawi et al., 2008). Work by Peinado et al. demonstrated that tumor-derived exosomes promote metastatic niche formation by educating bone marrow derived cells towards a more pro-vasculogenic and pro-metastatic phenotype, through the exosome-mediated transfer of the oncoprotein MET (Peinado et al., 2012). Further studies supported these results, and confirmed the importance of exosomes in

tumor growth, angiogenesis and metastatic development (Hood et al., 2011).

THE TUMOR MICROENVIRONMENT AND CHEMOTHERAPY RESPONSE

The acquisition of resistance to chemotherapeutic drugs continues to be a major obstacle in cancer treatment. Although it was believed for several years that drug resistance resulted primarily from selection of mutant tumor cells that were resistant to the cytotoxic effects of certain therapies, mounting evidence suggests that there is more to this story than once believed. Functional gene mutations that alter the expression of proteins involved in the uptake, metabolism and export of drugs are main causes of drug resistance, as are nonmutational (epigenetic) changes that can be associated to transient drug resistance. However, as discussed above, the tumor cell is only part of a complex group of constituents, and this tumor microenvironment is a critical determinant not only of tumor progression and metastasis, but also of resistance to therapy.

In 1998 Brown and Giaccia proposed that the microenvironment could be a major mechanism of drug resistance through the reduction of drug distribution throughout the tumor, therefore protecting high proportions of cells from damage induced by the drug (Brown & Giaccia, 1998). In fact, the tumor stromal components contribute to an increase in interstitial fluid pressure, and several studies have shown an association between high interstitial fluid pressure and poor drug penetration, with a suggested association to response to chemotherapy (Heldin et al., 2004). On the other hand, the increase in interstitial fluid pressure in association with an oncotic pressure gradient of almost zero, can lead to the extravasation of macromolecules, which can decrease the effectiveness of the treatment if the administered drug is lost at the tumor periphery (Netti et al., 1995).

Response to chemotherapy is also influenced by the vasculature, not only because the delivery of cytotoxic drugs can be impaired as a consequence of vascular disorganization (Durand, 2001), but also because this disorganized blood flow results in an abnormal and limited delivery of nutrients to the tumor, and the appearance of hypoxia (Galmarini et al., 2000; Tannock & Rotin, 1989). The first link between glucose deprivation and drug resistance was reported by Shen et al in 1987. They showed that in Chinese hamster ovary cells stress conditions that induced the endoplasmic reticulum

-resident stress proteins, such as hypoxia or glucose deprivation, were associated with significant resistance to doxorubicin. Moreover, it was shown that the removal of these conditions resulted in the disappearance of drug-resistance (Shen et al., 1987). Hypoxic conditions can lead to the activation of genes associated with angiogenesis and cell survival (Pouyssegur et al., 2006). The expression of these genes may result in an expansion of biochemically altered cells, with a drug-resistant phenotype. As an example, transient hypoxia has been shown to be associated with an increased expression of genes encoding P-glycoprotein and dihydrofolate reductase, which induces drug resistance, and with selection for cells that are deficient in DNA mismatch repair which increases their resistance to platinum-based chemotherapy (Rice et al., 1986). Furthermore, the limited supply of nutrients induces cell cycle arrest, with a consequent reduction of tumor cell proliferation rate (Hirst et al., 1979). As most chemotherapeutic drugs are more effective against proliferating cells, the slow growing cells localized most distant to the tumor vasculature have a high likelihood of becoming resistant to therapy (Valeriote & van Putten, 1975). Another known mechanism by which tumor stroma can influence drug resistance is through the interactions between tumor cells and the extracellular matrix. Work performed by Garrido et al., 1997, demonstrated that confluent cells in culture are more resistant to anticancer drugs than non-confluent cells (Garrido et al., 1997). Moreover, tumor cell adhesion to extracellular matrix mediated by integrins has been shown to protect small cell lung cancer cells from drug-induced apoptosis (Sethi et al., 1999).

In recent years, mounting evidence has suggested that certain growth factors and immune suppressor cells within the tumor microenvironment can induce tumor growth and mediate resistance to therapy. Straussman et al. demonstrated that in BRAF-mutant melanoma, hepatocyte growth factor (HGF) secretion by stromal cells was associated with poor response to BRAF inhibition. Furthermore, it was demonstrated that HGF plasmatic levels were inversely related to the response to BRAF inhibition in BRAF-mutant melanoma (Straussman et al., 2012). Recent work by Sun et al., suggests that microenvironment-mediated therapy resistance in the clinical management of prostate cancer may also arise from an adaptive, reciprocal signaling dialogue between the microenvironment and tumor cells. Specifically, it was shown that WNT16B was increased within fibroblasts exposed to cytotoxic drugs both in vitro and in vivo, and that in human tumors, WNT16B expression was associated with higher rates of disease recurrence after chemotherapy. Furthermore, when high expressing fibroblasts were co-culture with epithelial cells or xenograft tumors and then exposed to cytotoxic agents, there was a survival advantage as compared to cultures with low or absent WNT16B expres-

sing fibroblasts. This work demonstrated that WNT16B signals through a paracrine manner to tumor cells, increasing their proliferation and resistance to apoptosis (Sun et al., 2012).

More recently, the role of exosomes in drug resistance has begun to be explored. In a study published recently, MCF-7 (breast cancer) cells sensitive to docetaxel were exposed to exosomes extracted from the supernatant of a docetaxel-resistant MCF-7 variant. It was demonstrated that exosomes effectively transferred drug resistance characteristics from drug-resistant breast cancer cells to sensitive ones (Lv et al., 2014). Another study using breast cancer-derived exosomes reinforced these results, showing that adriamycin and docetaxel-resistant breast cancer cells may spread resistance capacity to sensitive cells by releasing exosomes and that these effects are attributed to the intercellular transfer of specific miRNAs (Chen et al., 2014). Moreover, it was demonstrated that docetaxel resistance in hormone refractory prostate cancer cells can be acquired by non-invasive cell lines also via exosomes (Corcoran et al., 2012). The addition of cisplatin (DDP) to A549 tumor cells (lung cancer cell line) has been shown to increase exosomes secretion and the interaction of these secreted exosomes with other cancer cells increased the resistance of these A549 cells to DDP (Xiao et al., 2014). This study also demonstrated that when A549 cells were exposed to DDP, the expression levels of several miRNAs and mRNAs reportedly associated with DDP sensitivity change significantly in exosomes, and that these changes probably mediate the DDP resistance of these tumor cells.

Exosomes may also contribute to chemotherapy resistance throw drug expulsion. Exosomes released from tumor cells have been shown to contain cisplatin, potentially redirecting the drug away from the nucleus where it would normally act, causing DNA damage, cell cycle arrest and apoptosis (Safaei et al., 2005). A recent study identified another method by which exosomes may contribute to chemotherapeutic resistance. It was observed that exosomes released from cancer cells might impede antibody and drug therapies by expressing cancer derived cell surface proteins that sequester the compound away from the target cell (Ciravolo et al., 2012)

Taken together, the current data suggests that accurate predictions of response to cancer treatment will be incomplete unless an integrative approach is undertaken. It seems proper to consider that more attention should be given to the role of the microenvironment in drug resistance, namely the role of exosomes in therapy resistance. Understanding how exosomes impact drug resistance

will allow novel and better strategies to treat cancer and prevent the emergence of drug resistance. In this chapter, we describe a technique for exosome purification from cell culture, fresh and frozen plasma and further analysis by electron microscopy, Nanosight microscope and Western-blot.

6.3 MATERIAL AND METHODS

Biological samples and filters:

- Cell lines
- Fresh plasma
- Frozen plasma
- 1.2µm nylon filters
- 0.22-µm filter

Reagents:

- Culture media
- 40% Tris/sucrose/D2O solution (40% sucrose cushion); 40 g protease-free sucrose, 2.4 g Tris base,50 ml D2O. Adjust pH to 7.4 with 10 N HCL drops. Adjust volume to 100 ml with D2O. Sterilize by passing through a 0.22-µm filter. Store up to 2 months at 4□C
- FBS, Hyclone
- PBS
- RIPA buffer; 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS,140 mM NaCl
- Protease inhibitor tablet (Roche)
- Antibodies against CD3, CD9 and MHC-I
- 2% PFA
- 200 nm phosphate buffer (pH 7.4)
- FormVar-carbon-coated grid
- 1% glutaraldehyde
- Aqueous uranyl oxalate

- 0.4% w/v uranyl acetate
- 1.8% w/v methyl celullose

Equipment:

- Sorvall Surespin 630 rotor
- Sorvall S100AT5 rotor
- Centrifuge
- Nanosight microscope
- Electron microscope
- SDS-PAGE equipment

Exosome purification from cell culture

The exosomes are purified by ultracentrifugation: the first steps are designed to eliminate large dead cells and large cell debris by successive centrifugations at increasing speeds. At each of these steps, the pellet is thrown away, and the supernatant is used for the following step (Figure 6.1). The final supernatant is then ultracentrifuged at 100,000 × g to pellet the small vesicles that correspond to exosomes. The pellet is washed in a large volume of PBS, to eliminate contaminating proteins, and centrifuged one last time at the same high speed.

1. FBS (Fetal Bovine Serum, Hyclone) is depleted of bovine exosomes by ultracentrifugation at 100,000 × g for 70 min (Sorvall Surespin 630 rotor)

2. Cells are cultured in media supplemented with 10% exosome-depleted FBS

3. Supernatant fractions are collected from 48—72 h cell cultures and pelleted by centrifugation at 500 × g for 10 min (see note 1).

4. The supernatant is centrifuged at 20,000 × g for 20 min.

5. Exosomes are then harvested by centrifugation at 100,000 × g for 70 min.

6. The exosome pellet is resuspended in 20 ml of PBS and collected by ultracentrifugation at 100,000 × g for 70 min.

7. The exosome pellet is resuspended in PBS and then stored at 4°C short term (1–7 days) or −20°C long term.

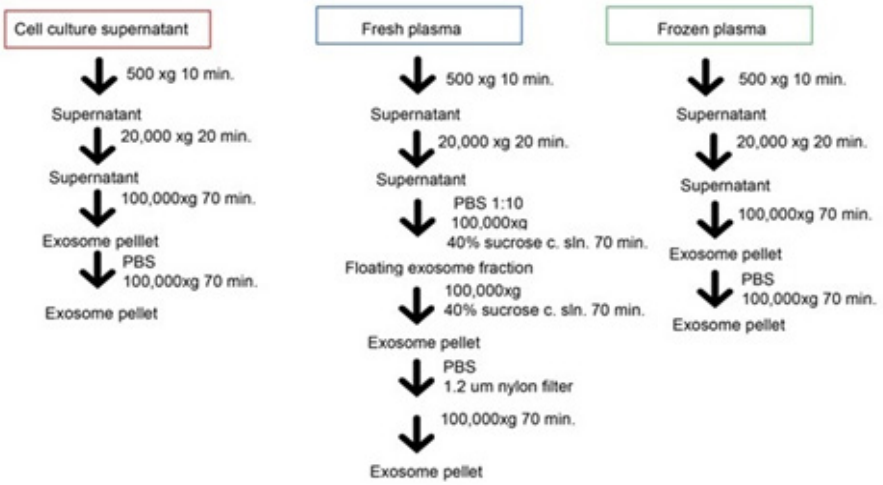

Figure 6.1. Exosome purification procedures for cultured cells, fresh plasma and frozen plasma

Exosome isolation from fresh mouse and human plasma

Circulating exosomes are isolated from mouse and human plasma in the same way as from cell culture with an extra purification step with a sucrose cushion and an additional filtration through 1.2 μm nylon filters before the last step of ultracentrifugation. The extra purification step with a sucrose cushion eliminates more contaminants, such as proteins nonspecifically associated with exosomes, or large protein aggregates, which are sedimented by centrifugation but do not float on a sucrose gradient. The filtration through 1.2 μm nylon filters will eliminate dead cells and large debris while keeping small membranes for further purification by ultracentrifugation.

1. Plasma is pelleted by centrifugation at 500 × g for 10 min.

2. The supernatant is centrifuged at 20,000 × g for 20 min.

3. The supernatant is diluted 1:10 in PBS.

4. Exosomes are then harvested by ultracentrifugation at 100,000 × g for 70 min on a 40% sucrose cushion solution.

5. The floating exosome fraction is collected again by ultracentrifugation as above.

6. The exosome pellet is resuspended in 20 ml of PBS and filtered through 1.2 μm nylon filters (GE)

7. The exosome pellet is collected by ultracentrifugation at 100,000 × g for 70 min.

8. The exosome pellet is resuspended in PBS and then stored at 4°C short term (1–7 days) or −20°C long term.

Exosome isolation for retrospective studies using frozen human plasma (see note 2)

1. 2 ml of cell-free frozen plasma is centrifuged at 500 × g for 10 minutes

2. Then the supernatant is centrifuged at 20,000 × g for 20 min

3. Exosomes are then harvested by centrifugation at 100,000 × g for 70 min.

4. The exosome pellet is resuspended in PBS and collected by ultracentrifugation at 100,000 × g for 70 min (Sorvall S100AT5 rotor).

5. The exosome pellet is resuspended in PBS and then stored at 4°C short term (1–7 days) or −20°C long term (see note 3).

Electron microscope analysis of exosomes

Exosomes purified as described above are fixed in 2% PFA (w/v) in 200 mM phosphate buffer (pH 7.4). Fixed exosomes are dropped onto a formvar-carbon–coated grid and left to dry at room temperature for 20 min. After washing in PBS, the exosomes are fixed in 1% glutaraldehyde for 5 min, washed in water, and stained with saturated aqueous uranyl oxalate for 5 min. Samples are then embedded in 0.4% w/v uranyl acetate, 1.8% w/v methylcellulose and incubated on ice for 10 min. The excess liquid is removed. The grid is dried at room temperature for 10 min and viewed at 20,000 and 50,000 magnification using an electron microscope (model 910, Carl Zeiss.).

Identification of exosome specific markers by Western-Blot analysis (see note 4)

Exosomes are lysed with RIPA buffer containing a complete protease inhibitor tablet (Roche). Lysates are cleared by centrifugation at 14,000 × g for 20 min. Supernatant fractions are used for Western blot. Protein extracts are resolved by SDS-PAGE and probed with the indicated antibodies. For Western Blot analysis the following antibodies are used to identify specific exosome markers: anti-CD3, anti-CD9 and anti-MHC-I.

Quantification of exosome size, distribution and number by LM10 nanoparticle characterization system (NanoSight)

The LM10 nanoparticle characterization system (NanoSight) equipped with a blue laser (405 nm) is used for real-time characterization of the vesicles.

Notes

Note **1.** Cell culture conditioned media can be centrifuged at 500 × g for 10 min, then 3000x g for 20 min and stored at 4oC or -80oC till use.

Note **2.** Plasma for retrospective studies is previously centrifuged at 3000xg for 20 minutes before storing at –80°C.

Note **3.** Exosomes isolated from conditioned media, fresh and frozen plasma can also be stored at –80°C. The pellets are ressuspended in 100 ul of PBS in the case of exosomes isolated from conditioned media, in the case of exosomes derived from plasma samples they are ressuspended in 50 µl of PBS.

Note **4.** Western-blot analysis should be supported by mass spectrometry analysis of the exosomes.

6.4 DISCUSSION

It is widely accepted nowadays that a variety of stromal cells are recruited to tumors, and that these cells not only play a crucial role in enhancing growth of the primary tumor, but also are determinant for metastatic dissemination to distant organs (Joyce & Pollard, 2009). To add more complexity to this process, exosomes are emerging as a relevant contributor to cancer progression (Zoller, 2016) and have been implicated in cancer chemoresistance, as discussed before.

Cancer drug resistance may stem from host factors (innate resistance) or be acquired by tumor cells that were initially responsive to cancer drugs. Either way, cancer drug resistance poses a significant challenge to physicians in their clinical practice, being responsible for treatment failure in over 90% of patients with metastatic cancer (Longley & Johnston, 2005; Holohan et al., 2013; Rueff & Rodrigues, 2016).

Understanding the precise role of exosomes in drug resistance will be critical to develop novel and better strategies to treat cancer and prevent the emergence of drug resistance. The methodology proposed above will enable a deeper knowledge about the biology of exosomes and their mechanism in drug resistance. Future studies will likely focus on the characterization of cancer exosomes heterogeneity and how this can benefit the understanding of changes in clonal expansion of cancer cells in response to therapies. Also, using exosomes as delivery vehicles for therapeutic agents is a very attractive concept once

this would allow a more directed and ideally less toxic cancer treatment option.

The last thee-decades have seen an exponential growth in exosomes directed research, and we believe this interest will be soon translated into direct benefit for our cancer patients.

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7. CHAPTER SEVEN
FINAL CONSIDERATIONS

EXPLORING THE ROLE OF **INTERLEUKIN-6** AND **OTHER HOST-ASSOCIATED FACTORS** IN **TUMOR PROGRESSION** AND **METASTASIS DEVELOPMENT**

7. CHAPTER SEVEN
FINAL CONSIDERATIONS

Metastatic disease poses a significant clinical challenge for any physician given the complexity of its treatment and also its incurable nature. Although in recent years the world was witness to substantial advances in the diagnosis and treatment of cancer, the overall prognosis of a patient with metastases remains very low, with 5-year survival rates below 20% for most stage IV cancers. Improvements in cancer survival are needed and will only be possible based on a deeper knowledge and a better management of metastatic disease.

Traditionally, cancer research has had its centre of attention in the tumor cells and alterations in their genes, but recently there has been a shift in focus beyond the tumor cells themselves to the surrounding stroma and matrix components in the tumor microenvironment and sites of future metastasis. It is now recognized that tumor cell-autonomous changes alone are not sufficient for the metastatic cascade to be efficient and that the tumor microenvironment plays a crucial role in this process. Tumor-stromal cells, more than innocent bystanders, provide advantage to the tumor through different mechanisms which not only enhance growth of the primary tumor, but also tumor cell dissemination and metastatic disease. In fact, it is increasingly apparent that BMDCs play a critical role in metastatic progression, being involved in several pathways that facilitate/ drive tumor progression. BMDCs are mobilized to pre-metastatic niches and, together with resident cells, create a suitable microenvironment for the engraftment of tumor cells and the formation of metastatic lesions. The pre-metastatic niche model advocates that these changes occurring in the pre-metastatic organs are crucial for tumor cells to engraft and constitute metastatic lesions at secondary sites.

Our work demonstrates that upregulation of the pro-inflammatory cytokine IL-6 in BMDCs during the early stages of tumor progression, is associated with increased levels of hematopoietic progenitor cells in the bone marrow microenvironment, with a subsequent mobilization and recruitment of immature myeloid cells to pre-metastatic niches. This increase in BMDCs in pre-metastatic organs renders this foreign microenvironment suitable for the proliferation of metastatic tumor cells. By transplanting IL-6-expressing BMDCs in IL-6 non-expressing mice and reverting the phenotype, we demonstrate that IL-6 expression in BMDCs is one of the driving events of metastatic development, reinforcing the requirement of the bone marrow microenvironment in the metastatic progression of solid tumors.

Furthermore, our work also demonstrates that IL-6 together with TGF-β, are amongst the main responsible factors for Id1 up-regulation in BMDCs, and that Id1 upregulation is responsible for generating an immunosuppressive macroenvironment, which is a main driver of tumor progression. In fact, our work demonstrates that upregulation of Id1 redirects BMDC differentiation towards Id1-high expressing MDSC with a reciprocal decrease in DC numbers. Moreover, Id1 overexpression down-regulates key DC differentiation pathway molecules such as Irf8 and leads to Treg expansion, increased ROS production and suppression of CD8 T-cell proliferation which in turn promote primary tumor growth and metastatic progression. Our work also shows that Id1 induces VEGFR1 expression, supporting our previous observation that increases in VEGFR1 and Id1 expression occur in BMDCs and are largely responsible for driving the metastatic process. However, the functional role of VEGFR1 expression in BMDCs during metastatic development had not been clarified before. Our work demonstrates that VEGFR1 expression controls the angiogenic activity of tumor and metastasis-associating myeloid cells by suppressing the expression of a potent angiostatic chemokine, CXCL4. Such angiogenic regulation appears to play a profound role in controlling the primary tumor growth as well as the progression of colonizing metastatic cells into macrometastases.

Our work supports the concept of a determinant role of the bone marrow in solid-tumor progression and metastasis. We show that, with STAT3 phosphorylation, IL-6 expression is increased in the bone marrow microenvironment and that this in turn is associated with an increase not only in the number of MDSCs but also in the number of hematopoietic progenitor cells, and with an increased recruitment of these immature myeloid cells to pre-metastatic niches. Furthermore, our work reinforces the relevance of BMDCs and the immune response during tumor progression, revealing a

critical role for Id1 in BMDC differentiation. We also demonstrate that VEGFR1+ myeloid BMDCs play an important role in regulating angiogenesis at the primary tumor and metastatic microenvironment.

These results support the evaluation of pharmacological strategies for targeting the tumor microenvironment and in particular the pathways explored in our work. Reducing myeloid-cell mobilization and recruitment to pre-metastatic niches using IL-6 therapies, restoring systemic immune function by targeting Id1, or inhibiting angiogenesis through CXCL4 pathway are all possible therapeutic paths that might be worth exploring further. Above all, our results reinforce the importance of the microenvironment in metastasis development and contribute to an increased knowledge of the biology of metastatic disease, crucial for a better management of our advanced cancer patients.

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