# DUAL NEGATIVE ROLES OF C/EBPα IN THE EXPANSION AND PRO-ANGIOGENIC FUNCTION OF MYELOID DERIVED SUPPRESSOR CELLS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

December, 2012

Nashville, Tennessee

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To my Grandmother, Claire Louise Jaenke

9/27/29 - 11/11/11

ACKNOWLEDGEMENTS

I am grateful to my thesis committee for taking the time to invest in my graduate education, and for their truly helpful thoughtful input and constructive criticism. Thank you for being so generous with your time and for going above and beyond the call of duty. This would not have been possible without you. Thank you for your feedback on my presentations and writing. Dr. Hann, thank you for serving as my chair. Dr. Chen, thank you for agreeing to be my co-mentor and for welcoming me into your group. Dr. Sealy, your expertise was invaluable. Dr. Tansey, thank you for believing in me. Dr. Zutter, thank you for asking hard questions, they made me a better scientist.

I want to acknowledge Charles Lin for his contributions to this work. Beyond the financial and intellectual support, he taught me to be independent, to work hard and to continually strive to get better. I also want to acknowledge the members of the Lin laboratory. Laura DeBusk is a truly caring person, always willing to listen or talk. Hanako Kobayashi taught me qPCR (and many other things). I learned many of the techniques that made this work possible from Yongfen Min. Candice Shaifer's positive attitude was infectious and she was a pleasure to be around. Angela Boutte helped guide me through graduate school with advice and encouragement. I especially want to acknowledge Kimberly Boelte Pope, who has done as much to make this work possible as anyone at Vanderbilt. Beyond her direct and irreplaceable contributions to this work, she has been the older sister I never had; a matchmaker, mentor and devoted

colleague and friend who gave up more than one Friday night in order to help me. I miss our happy hours and conversations and I am grateful for her friendship. I also want to acknowledge the members of the Chen laboratory; you always made me feel welcome and served me well as my adopted lab.

I want to acknowledge the Cell and Developmental Biology Department at Vanderbilt University. In addition to cultivating an atmosphere of collaboration and collegiality, this department supported and encouraged me to finish this work. I want to express my sincere gratitude to Kathy Gould and Matthew Tiska, the former and current Director of Graduate Studies. Without their assistance, I would not have completed my dissertation. Thank you.

I would also like to acknowledge and thank my friend Dave George. Your counsel, support and love came at a time of true need. This was not possible without you.

Finally, I want to thank my wife, Amanda. You have been my strongest supporter and I could not have done this without you. You believed in me when I didn't believe in myself, encouraged me when I needed encouraging, comforted me when I needed comforting and challenged me when that was needed. You have been with me nearly from the beginning of this journey. I know I have put you through a lot, yet you never gave up on me. I love and appreciate you more each day and am so grateful to have been entrusted with you for this life. Not long after we started dating, Kim told me "She's good for you." I could not agree more and I look forward to facing life's challenges and celebrations with you!

This work was supported by the Biochemical and Chemical Training Grant for Cancer Research (5 T32-CA09582) and grants from NIH (CA108856, NS45888 and AR053718).

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AML: acute myeloid leukemia

BMDC: bone marrow-derived cell

CBC: complete blood count

C/EBPα: CCAAT/enhancer binding protein alpha

CFU: colony forming unit

CLP: common lymphoid progenitor

CMP: common myeloid progenitor

CN: C/EBPα conditional null (*Cebpa*$^{flox/flox}$*; LysMCre*$^{+/+}$ genotype*)*

DC: dendritic cell

EMT: epithelial to mesenchymal transition

FACS: fluorescence-activated cell sorting

FGF: fibroblast growth factor

GM-CSF(R): granulocyte- macrophage colony stimulating factor (receptor)

GMP: granulocyte-monocyte progenitor

Hbg: hemoglobin

HCT: hematocrit

HIF-1α: hypoxia-inducible factor-1 alpha

HRE: hypoxia-responsive element

HSC: hematopoietic stem cell

IFN: interferon

IL-(R): interleukin (receptor)

iNOS: inducible nitric oxide synthase

IRF-8: interferon regulatory factor-8

LIP: liver-enriched transcriptional inhibitory protein

LLC: Lewis Lung Carcinoma

M-CSF(R): macrophage colony stimulating factor (receptor)

MACS: magnetic activated cell sorting

MDSC: myeloid-derived suppressor cell

MEP: megakaryocyte/erythroid progenitor

MMP: matrix metalloproteinase

NF-κB: Nuclear Factor-KappaB

NO: nitric oxide

NK: Natural Killer cell

PCR: polymerase chain reaction

RBC: red blood cell

STAT: signal transducer and activator of transcription

TAM: tumor-associated macrophage

TCM: tumor-conditioned medium

TGF-β: transforming growth factor-beta

TNF-α: tumor necrosis factor-alpha

VEGF: vascular endothelial growth factor

WBC: white blood cell

WT: wild-type (*Cebpa*$^{flox/flox}$*; LysMCre*$^{-/-}$ genotype)

# CHAPTER I

# INTRODUCTION

## Cancer

Cancer is a term for a group of diseases characterized by unrestrained growth and spread of abnormal cells that, if unchecked, can result in death. It is the second most common cause of death in the United States, exceeded only by heart disease and accounting for nearly 1 of every 4 deaths [1]. Globally, one in eight deaths is due to cancer; cancer causes more deaths than AIDS, tuberculosis, and malaria combined. This year 1,596,670 Americans will be diagnosed with cancer and 571,950 are expected to die from it. In the US, nearly 1 in 2 men and 1 in 3 women will develop cancer in their lifetime. The 5-year relative survival rate for all cancers diagnosed between 1999 and 2006 is 68%, an improvement from 50% in 1975-1977 that reflects progress in diagnosing certain cancers at an earlier stage and advances in treatment [1]. However, we are still far from the goal of eliminating cancer as a major cause of sickness and death.

## Tumor Progression and Microenvironment

The process of malignant transformation, collectively known as tumor progression, can be broken up into steps. Each step is driven by mutations that activate or inactivate different genes. It is estimated that tumor cells posses $10^4$-

$10^5$ genetic changes [2]. The first step in tumor progression is initiation and growth. Here, mutations aberrantly activate genes involved in cell growth and division [3]. These genes are termed oncogenes. When functioning properly, tumor suppressors recognize improperly activated oncogenes and halt inappropriate cell division. However, additional mutations disable tumor suppressors, resulting in uncontrolled proliferation and division of normally quiescent cells.

The next step in tumor progression involves the recruitment and infiltration of non-malignant cells into the tumor. Rather than being a homogeneous collection of neoplastic cells, tumors also contain many non-malignant cells (Figure 1), [4]. Collectively, the cells, both malignant and non-malignant, and the conditions within a tumor, i.e. hypoxic, necrotic, etc., comprise the tumor microenvironment [5,6]. Fibroblasts and other stromal cells that support tumor growth are recruited to the tumor. In a process known as immune surveillance, the host immune system recognizes and attempts to halt tumor development [7]. Immune inflammatory cells including lymphocytes and monocytes infiltrate into the tumor [8]. However, tumors that progress evolve mechanisms to disable or re-program the immune cells in order to escape host immune surveillance [7].

Like other normal tissues, tumors need nutrients and oxygen in order to grow and divide. Tumors generally cannot grow larger than 1-2 mm without a blood supply, due to a lack of oxygen and other nutrients [9]. Thus, gaining access to the host vascular system and developing tumor blood vessels is an essential and rate-limiting step in tumor growth and progression [10]. The process of blood

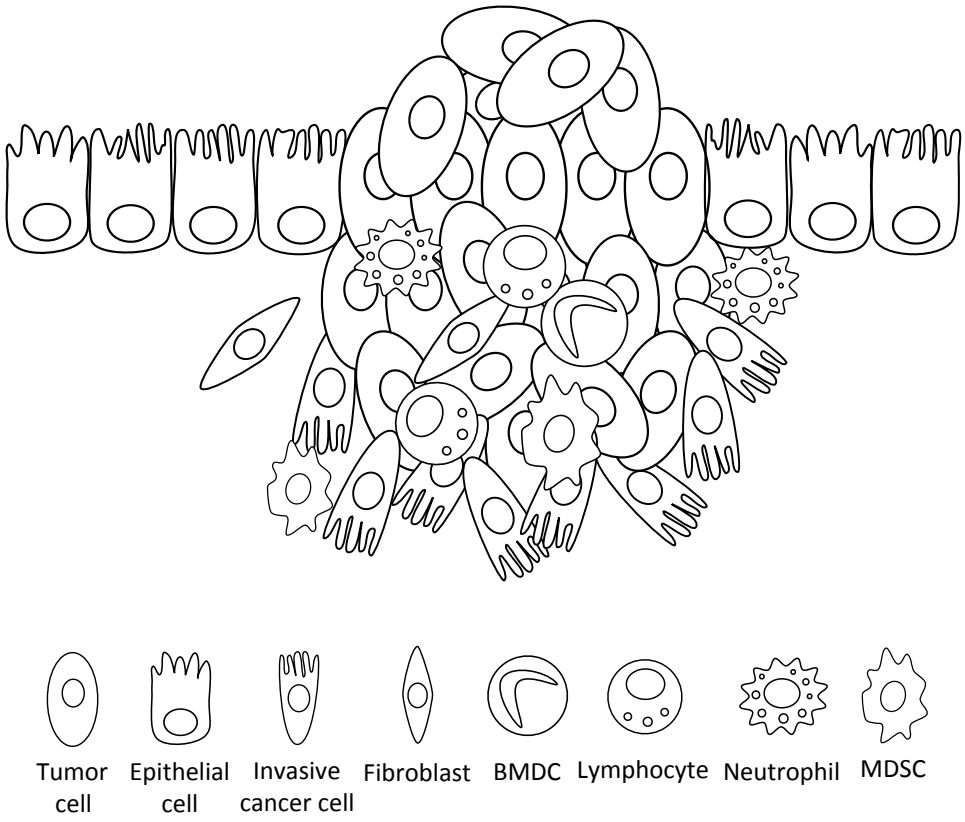


**Figure 1. The tumor microenvironment contains many non-malignant cells.** The tumor microenvironment refers to the different cell types present in the tumor as well as the tumor stroma, blood vessels, secreted molecules and the local conditions within the microenvironment (i.e. hypoxic, necrotic, etc.). In addition to malignant tumor cells, tumors contain fibroblasts, endothelial cells and pericytes that make up blood vessels, and numerous bone marrow-derived immune cells (BMDCs). Immune cells present in the tumor microenvironment include lymphocytes, macrophages known as tumor-associated macrophages (TAMs), neutrophils and myeloid-derived suppressor cells (MDSCs).

vessel spouting from pre-existing vessels is termed angiogenesis; the induction of tumor blood vessel growth is known as the angiogenic switch [10]. Hypoxia and other stresses induce tumors to secrete vascular endothelial growth factor (VEGF) and other angiogenic stimuli [11]. In response, endothelial cells sprout from existing blood vessels, proliferate and migrate towards the stimuli [12]. They adhere to one another as they migrate and mature into new vessel structures. This process is often disorganized in tumor angiogenesis, resulting in chaotic, irregular and leaky tumor blood vessels [13]. Tumor vessels also often lack pericytes and are therefore unstable [14].

The hypoxic and inflammatory microenvironment within a growing tumor applies selective pressure to on tumor cells escape [4,15,16]. Additional mutations allow tumor cells to produce matrix metalloproteinases (MMPs) [17] and/or to induce MMP production by tumor-associated myeloid-derived suprressor cells (MDSCs) [18]. MMPs degrade the basement membrane and extracellular matrix, resulting in VEGF and fibroblast growth factor (FGF) release, which further stimulates angiogenesis [19]. Additionally, this facilitates the escape and local invasion of tumor cells that have acquired motility in a process known as epithelial to mesenchymal transition (EMT) [20]. Tumor cells migrate along and towards blood vessels, often following a nutrient or oxygen gradient [21]. In a process known as intravasation, tumor cells pass between endothelial cells and enter the blood or lymphatic circulatory system [22]. Those cells that can survive in the blood stream and spread to a secondary site generally reach the capillary bed and adhere to the microvessel endothelium [23]. In a reversal of

EMT, tumor cells extravasate out of the vessel and colonize the secondary organ [24]. Tumor cells that encounter hospitable sites or that can adapt to the new microenvironment may to grow into metastases. However, to do so, these cells must overcome the same pressures of the primary tumor, i.e. immune evasion and angiogenesis [19].

## Myeloid-Derived Suppressor Cells

MDSCs which have also been referred to as myeloid immune-suppressor cells (MISCs) or myeloid suppressor cells (MSCs), are immature myeloid cells, comprised of monocytes/macrophages, granulocytes and dendritic cells (DCs) and other myeloid cells at earlier stages of differentiation [25,26]. In mice, MDSCs are defined by the simultaneous expression of CD11b (Mac-1), a myeloid macrophage/DC marker, and the granulocytic marker Gr-1. Furthermore, they lack or have reduced expression of markers of mature myeloid cells, low levels of major histocompatibility complex (MHC) class II and co-stimulatory molecules and suppress immune responses *in vitro* and *in vivo* [25]. Human MDSCs are more difficult to define phenotypically since humans lack the Gr-1 gene. As a result, studies have reported many different markers for human MDSCs. However, the consensus is that human MDSCs express the myeloid markers CD11b and CD33 with no or minimal expression of myeloid maturation markers [27]. MDSCs are increased in various pathological conditions including cancer [28]. MDSC accumulation has been documented in the peripheral blood of patients with melanoma, head and neck, breast, colon, renal, and non-small

cell lung cancers [29]. MDSCs are also significantly increased in the spleens, blood and bone marrow of mice bearing large tumors in an assortment of tumor models [18]. Furthermore, MDSC production and infiltration into tumors increased as tumor size increased and with the duration of tumor growth.

MDSCs suppress tumor immunity through multiple direct and indirect mechanisms, including inhibiting tumor-specific T-cell activation and proliferation through the production of arginase [30] and nitric oxide synthase (iNOS) [31]. Arginine is a substrate for both of these enzymes and it is also essential for T-cell proliferation and activation. Arginine metabolism by arginase and iNOS reduces the amount available to T-cells, resulting in T-cell anergy. MDSCs have also been demonstrated to suppress Natural Killer (NK) cell cytotoxicity via direct contact [32], impair dendritic cell maturation through the production of reactive oxygen species [33] and interact with macrophages to effect a tumor-promoting type 2 response [34]. MDSCs may also induce regulatory T cells and thereby promote immune tolerance of the tumor [35]. As such, the accumulation of MDSCs is often associated with poor prognosis. Increased circulating MDSC levels were shown to correlate with clinical cancer stage and metastatic tumor burden [36]. Gemcitabine treatment to reduce MDSCs enhanced antitumor immune activity in tumor bearing mice [37].

Recently, another tumor-promoting role has been demonstrated for MDSCs in tumor angiogenesis. MDSCs from tumor-bearing mice express higher levels of matrix metalloproteinase-9 (MMP-9), thereby increasing the bioavailability of VEGF [18] and promoting pericyte recruitment, which increases vascular stability

[38]. Moreover, MDSCs directly promote tumor angiogenesis by differentiating into endothelial cells and directly incorporating into the tumor endothelium *in vivo* [18]. MDSCs have also been implicated in mediating tumor refractoriness to anti-VEGF treatment [39]. MDSC-produced MMP-9 also enhances tumor cell invasion and metastasis [40].

## CCAAT/enhancer binding protein alpha (C/EBPα)

C/EBPα is the founding member of the CCAAT/enhancer binding protein family of transcription factors, all of which bind a similar DNA consensus motif. It was first identified as a heat-stable factor in rat liver nuclei that interacted with the CCAAT box motif found in some viral promoters. C/EBPα was also independently biochemically purified based on its specific interaction with a degenerate sequence common to many animal virus enhancers. Reflecting these interactions, it was eventually named CCAAT/enhancer binding protein (C/EBP) [41]. Upon cloning of the C/EBPα gene, detailed studies resulted in the discovery of the basic-leucine zipper (bZIP) class of DNA-binding and dimerization domain proteins [42]. Five other C/EBPs, all sharing the highly conserved C-terminal basic-leucine zipper domain DNA binding motif, have been identified. The six C/EBP family members are identified by Greek letters (α, β, γ, δ, ε, ζ), indicating the chronological order of their identification. Members of this family share N-terminal transactivation domain(s) and the C-terminal bZIP domain, which is made up of a basic-amino-acid rich DNA-binding region followed by the leucine rich “leucine zipper” dimerization motif. In fact, C/EBP family members share

greater than 90% sequence identity in the C-terminal bZIP domain [42]. C/EBPα forms homodimers and heterodimers with other C/EBPs as well as other leucine zipper transcription factors, including the Fos/Jun and ATF/CREB families [43], to precisely modulate the transcription of target genes [44]. Without dimerization DNA binding cannot occur and DNA binding specificity results from amino acid sequence of the basic region [44].

## The C/EBPα gene

C/EBPα is encoded as an intronless 2783 base pair gene [45] but two C/EBPα isoforms are generated due to utilization of alternate translation start codons [46]. C/EBPα is expressed in liver, adipose tissue, intestine, lung, adrenal gland, skeletal muscle, pancreas, placenta, prostate gland, mammary gland and peripheral blood mononuclear cells in similar levels in the mouse and human [44]. In addition to regulating transcription of target genes, C/EBPα has growth-arresting ability due to its protein-protein interactions and independent of its DNA-binding activity [47]. C/EBPα has been shown to interact with numerous cell cycle regulators, including p21 [48], Rb [49], E2F [50], and Cdk2 and Cdk4 [51]. Thus, C/EBPα is positioned at the crossroads of differentiation and proliferation control: it promotes differentiation by the up-regulation of lineage-specific genes and limits proliferation through its protein interactions [52,53].

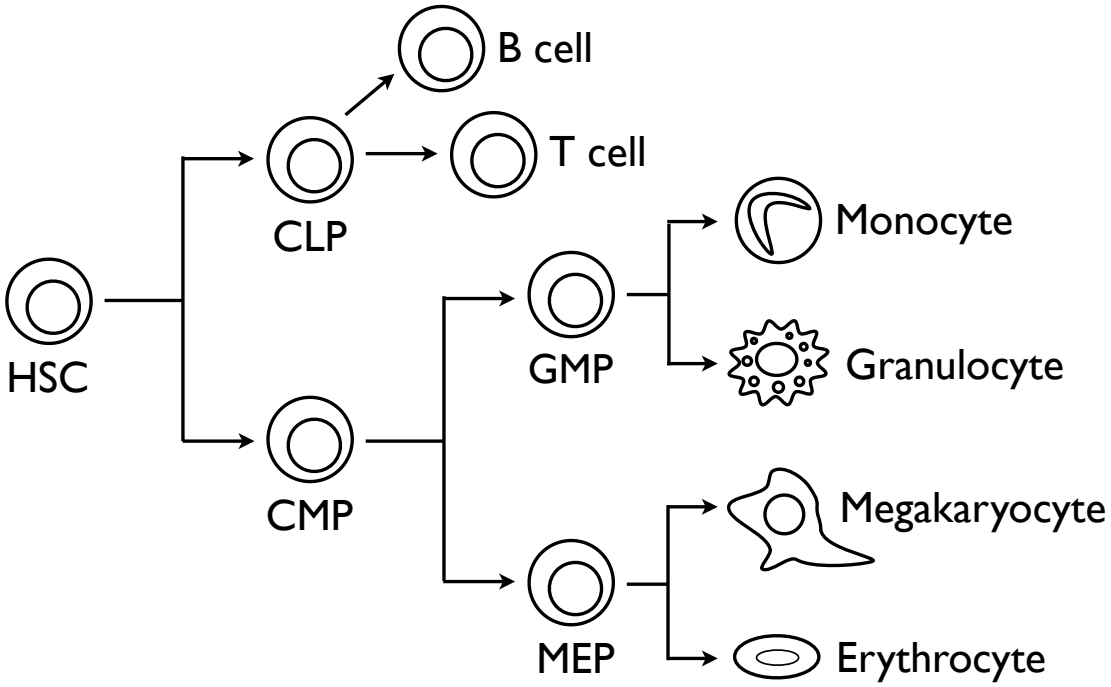


**Figure 2. Hematopiesis**. The formation of the different cellular components of blood, or hematopoiesis, initiates in the bone marrow. Hematopoitic stem cells (HSC) have the ability to self-renew and give rise to common lymphoid progenitors (CLP), which produce B and T cells, and the common myeloid progenitor (CMP). CMPs further differentiate into granulocytes-monocyte progenitors (GMP), which can produce granulocytes and monocytes, or megakaryocyte-erythrocyte progenitors (MEP) that can become megakaryocytes erythrocytes.

## Role of C/EBPα in hematopoiesis

Hematopoiesis, the formation and development of blood, involves the step-wise acquisition of a specific lineage identity, accompanied by a reduction in self-renewal capacity (Figure 2) [54]. Within hematopoiesis, C/EBPα is expressed predominantly in immature cells, including hematopoietic stem cells (HSC), common myeloid progenitors (CMP) and granulocyte-monocyte progenitors (GMP) [55]. C/EBPα plays a critical role in regulating the balance between proliferation and growth arrest in hematopoietic progenitors [56]. C/EBPα accomplishes this by inhibiting differentiation of erythroid cells while promoting myeloid differentiation in multipotent progenitor cells [57]. C/EBPα is required for the differentiation of bipotential GMPs from multipotential CMPs, as mice lacking C/EBPα have normal CMP numbers but lack GMPs and all subsequent stages of myeloid differentiation [56]. Once the GMP stage of hematopoiesis has been reached however, C/EBPα is no longer required for maturation to more differentiated granulocytes and monocytes [58]. C/EBPα has other roles in hematopoiesis including regulating hematopoietic stem cell self-renewal [58] and directing monocytic development from myeloid progenitors [59]. While C/EBPα expression in undetectable in megakaryocyte/erythroid progenitors (MEP) and common lymphoid progenitors (CLP), forced expression of C/EBPα in MEPs and CLPs redirected them to the myeloid lineage. This revealed that C/EBPα has the ability to activate latent myeloid differentiation programs and affect multilineage homeostasis [60].

## C/EBPα target genes

C/EBPα regulates the expression of the myeloid growth factor receptors, macrophage-colony stimulating factor receptor (M-CSFR) [61], interleukin-6 receptor (IL-6R), granulocyte colony-stimulating factor receptor (G-CSFR) [62] and granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) [63,64]. C/EBPα also induces the expression of lactoferin and collagenase, neutrophil-specific secondary granule proteins [65], and the microRNA miR-223, an important modulator of myeloid differentiation [66].

## C/EBPα and cancer

Given its important role in hematopoiesis, it is not surprising that mutations resulting in the loss of C/EBPα function have been found in acute myeloid leukemia (AML). The C/EBPα gene is mutated in 7-9% of all AML cases. Additionally, the AML1-ETO fusion protein, found in 12-15% of AML patients, appears to suppress C/EBPα expression, and the PML-RARα fusion, which blocks myeloid transcription factors including C/EBPα, occurs in 6-7% of AMLs [67]. Reduced C/EBPα expression has also been observed in many solid tumors, and it has been identified as a tumor suppressor in multiple tissues [68].

## Summary and Dissertation Goals

MDSCs play an important role in cancer progression and elucidating the mechanisms involved in the accumulation and function of these cells is important in the fight against cancer. To that end, a microarray comparing splenic Gr-1+CD11b+ cells from tumor-bearing mice with tumor-free mice found C/EBPα expression was reduced more than 4-fold in the tumor derived cells. Based on published reports, we hypothesized that tumors induce MDSC expansion through down-regulation of C/EBPα in myeloid cells. In Chapter II, the role of C/EBPα as a negative regulator of MDSC expansion was investigated. Deletion of myeloid C/EBPα in mice yielded an increase in myeloid progenitors and a reduction in mature myeloid cells. Upon inoculation with tumor cells, splenic MDSC production was enhanced nearly two-fold in mice lacking C/EBPα in myeloid lineage cells, while myeloid progenitor production was reduced, perhaps because more progenitors became MDSCs in the absence of C/EBPα.

In Chapter III, we sought to determine whether C/EBPα is a negative regulator of the immune suppressive and pro-angiogenic properties of MDSCs. MDSC infiltration and tumor vascularization was significantly greater in C/EBPα conditional null mice, resulting in markedly accelerated tumor growth. In an experiment where equal numbers of MDSCs were injected with tumor cells into mice, C/EBPα ablation resulted in an enhancement in the pro-tumor MDSC phenotype: tumor growth and tumor angiogenesis was significantly greater. In order to understand the mechanism behind our observations, we measured the

expression of genes involved in MDSC-mediated immune suppression and angiogenesis. We found that C/EBPα deletion resulted in upregulation of MMP-9, VEGF and iNOS expression as well as increased NO production. However, there was no difference in arginase expression or immune suppression. Given that NO can also regulate angiogenesis, we concluded that C/EBPα does not play a major role in MDSC-mediated immune suppression.

As a whole, our findings reveal dual negative roles for C/EBPα in the expansion and pro-angiogenic gene expression in MDSCs and suggest that overcoming these functions through C/EBPα inhibition may be a critical step in MDSC maturation. Our work indicates that therapy aimed at restoring C/EBPa expression in MDSCs may be a viable weapon in the fight against cancer.

# CHAPTER II

## C/EBPα REGULATES MYELOID DERIVED SUPRESSOR CELL EXPANSION


### Abstract

Myeloid-derived suppressor cells, a population of immature myeloid cells, are greatly expanded in cancer patients and tumor-bearing mice. They infiltrate into tumors and modulate the tumor microenvironment. In an effort to identify molecular mediators responsible for expansion and the tumor-promoting function of MDSCs, we discovered C/EBPα expression was significantly reduced in MDSCs from tumor-bearing mice compared to non-tumor-bearing hosts. Tumor-conditioned medium down-regulated C/EBPα expression, suggesting tumor-secreted factors inhibit the gene expression. Consistent with the function of C/EBPα in regulating the balance between proliferation and growth arrest in hematopoietic progenitors, myeloid lineage specific deletion of C/EBPα resulted in significantly enhanced MDSC proliferation and expansion, as well as an increase of myeloid progenitors and a decrease of mature cells. Taken together, our results suggest that C/EBPα functions to negatively regulate MDSC production and that tumor-induced C/EBPα down-regulation in myeloid lineage cells results in MDSC expansion.

# Introduction

MDSCs are a heterogeneous population of immature myeloid cells that are greatly overproduced in numerous human cancer patients MDSCs are also systemically expanded in the mice bearing implanted or spontaneous tumors. MDSCs are recruited to tumors where they promote tumor growth through mechanisms of immune suppression and angiogenesis in the tumor microenvironment. As hallmarks of cancer, angiogenesis and immune suppression are essential for tumor growth and progression. MDSCs possess both tumor-promoting properties and are an intriguing target for cancer therapy.

While researchers have long observed the dramatic expansion of MDSCs in tumor-bearing hosts, the molecular mechanisms regulating MDSC expansion under tumor conditions remain less clear. We discovered C/EBP$\alpha$ expression is greatly reduced in MDSCs. Given the central role of C/EBP$\alpha$ in regulating myeloid differentiation, we hypothesized that C/EBP$\alpha$ is a negative regulator of MDSC production and/or function.

## Materials and Methods

### *MDSC Isolation*

3LL lung cancer cells or B16 melanoma cells were subcutaneously (s.c.) implanted in C57Bl/6 mice for 3 weeks (3LL) or 30 days (B16). After sacrificing the mice, tumor tissues were removed and digested with collagenase A and hyaluronidase (both from Sigma Aldrich, St. Louis, MO) at 37°C overnight. Single cell suspensions were prepared from tumor and spleen tissues. MDSCs were isolated by sequential antibody labeling and separation by magnetic column, using anti-Gr1-PE (Cat. # 130-091-932) and anti-PE (Cat. # 130-48-801) multisort beads, followed by CD11b (Cat. # 130-093-634) MicroBeads (all from Miltenyi Biotech, Auburn, CA).

### *Quantitative RT-PCR*

RNA was isolated from cells using the RNeasy Kit from Qiagen (Cat. no. 74104, Valencia, CA), according to their protocol. cDNA was synthesized from 1 μg RNA with the iScript cDNA Synthesis Kit (Cat. # 170-8891, BioRad, Hercules, CA) according to the manufacturer's protocol. For quantitative real time PCR, SsoFast EvaGreen Supermix (Cat. # 172-5201, BioRad, Hercules, CA) and a CFX96 or MyiQ machine (BioRad, Hercules, CA) were used. The primer sequences used for C/EBP-α are ACGGCGGGAACGCAACAACA and GAAGATGCCCCGCAGCGTGT, for β-actin: GACAACGGCTCCGGCATGTGC and TGGCTGGGGTGTTGAAGGTC. After an initial denaturing (95°C for 2:00),

40 cycles of 5 s at 95°C and 9 s at 58.7°C were performed. Target and control reactions were performed in triplicate and the average Ct value was calculated. Relative changes in transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal control. Experiments were repeated three times.

*Cell lines*

32D myeloid cell line (# CRL-11346), Lewis lung cancer (3LL) cell line (# CRL-1642) and B16 melanoma (# CRL-6475) cell line (all murine, C57BL/6J mice) were purchased from ATCC (Manassas, VA). 32D cells were cultured in RPMI 1640 + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin () + 10% IL-3 Culture Supplement (Cat. # 354040, BD Biosciences, San Jose, CA) at 37°C, 5% $CO_2$. 3LL cells were grown in RPMI 1640 + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% $CO_2$. B16 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% $CO_2$.

*Mice*

C57BL/6J and LysMcre [69] mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice with a floxed *Cebpa* gene, called *Cebpa*$^{flox/flox}$ [70], were a gift from Peter Johnson (NCI, Frederick, MD). The animals were housed in pathogen-free units at the Vanderbilt University Medical Center, in compliance with IACUC regulations. We generated mice with myeloid-specific deletion of *Cebpa* by breeding *Cebpa*$^{flox/flox}$ mice to *LysMCre* mice, which express Cre

recombinase under the control of murine lysozyme M promoter. F1 LysMCre$^{+/-}$;*Cebpa*$^{fl/+}$ were then crossed to produce the genotypes used in the experiments. Mice used were between 6 and 10 weeks of age. The studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center and complied with all relevant institutional and federal guidelines and policies.

*Tumor allograft model*

B16 or 3LL cells (1 x $10^5$ cells) were injected subcutaneously into the left flank of C57Bl/6 mice. The size of tumors was determined by measurement of tumor dimensions at 2–3 day intervals using calipers. The equation volume = length x $(width)^2$ x 0.5 was used to calculate tumor volume. Tumor samples taken at days 21 (3LL) or 30 (B16) post-injection were flash frozen in Tissue-Tek OCT (Sakura, Torrance, CA) and prepared for histology. Experiments were repeated at least three times.

*Flow cytometry*

3LL lung cancer cells were implanted subcutaneously in mice for 3 weeks. Single cell suspensions from spleens of tumor-bearing mice were labeled with florescence-conjugated Gr-1 (Cat. # 553126, monoclonal, rat) and CD11b (Cat. # 553311, monoclonal, rat) antibodies (BD Biosciences, San Jose, CA) and analyzed by flow cytometry using a BD LSRFortessa or BD FACScan.

*Proliferation assay*

Cell proliferation was measured by BrdU incorporation using the BD Pharmingen FITC BrdU Flow Kit (Cat. # 559619, BD Biosciences, San Jose, CA). 3 weeks after subcutaneous implantation of 1 x $10^5$ cells 3LL tumor cells, mice were injected intraperitoneally with 2 mg of BrdU 2 hours prior to being sacrificed. Single cell suspensions were prepared from the spleens and labeled with florescence-conjugated Gr-1 (Cat. # 553126, monoclonal, rat), CD11b (Cat. # 553311, monoclonal, rat) and BrdU (Cat. # 559619e, monoclonal, rat) antibodies (BD Biosciences, San Jose, CA). BrdU incorporation in the Gr1+CD11b+ double-positive MDSC population was measured on a BD LSRFortessa cell analyzer. Assay was repeated twice.

*Immunohistochemistry*

Cryo-preserved tumor sections were blocked with M.O.M. solution (Vector Laboratories, Burlingame, CA), incubated with antibodies to CD31 or Gr-1 (BD Biosciences, San Jose, CA; 1:500 dilution or 1:200 dilution, respectively) overnight (4° C), and visualized by using Texas Red-conjugated goat anti-rat 2nd antibody (Jackson Immuno Research Laboratories, West Grove, PA; 1:10000 dilution). Images were captured with a fluorescent microscope. Vascular density and MDSC infiltration was calculated in 10 randomly selected fields and counted in a blinded manner. These experiments were repeated 3 times.

*Methylcellulose colony assays*

Bone marrow cells were isolated by flushing the tibias and femurs of mice with cold IMDM + 2% FBS and pooled from 2-3 mice per group. A single cell suspension was prepared and plated in Methocult 3234 methylcellulose semi-solid medium (Stemcell Technologies, Vancouver, BC) supplemented with 10 ng/ml IL-3, 10 ng/ml IL-6, and 50 ng/ml stem cell factor (SCF, all from R&D Systems, Minneapolis, MN) with and without erythropoietin (StemCell Technologies, Vancouver, BC; 3 U/ml). After 7-8 days incubation at 37°C in a $CO_2$ incubator, colonies were scored based on cell morphology and colony composition under an inverted microscope. Assays were performed in duplicate and repeated three times.

*Statistical Analysis*

All data were averaged, analyzed using the Student's t test (one-sided; paired, equal variance) and were expressed as mean ± standard error across experiments.

# Results

## *C/EBPα is significantly reduced in tumor-derived MDSCs*

MDSCs are vastly overproduced in cancer patients and tumor-bearing animals. They infiltrate into tumors and promote tumor growth by promoting tumor angiogenesis and immune suppression. To better understand what regulates MDSC expansion under tumor conditions, we isolated MDSCs from the spleens of C57/BL6 mice with or without Lewis Lung Carcinoma (3LL) tumors by magnetic activated cell sorting (MACS). MDSCs were also isolated from the tumors of the tumor-bearing mice. We compared C/EBPα expression quantitative PCR and found that C/EBPα was significantly reduced in MDSCs isolated from tumor-bearing mice compared to controls (Figure 3A). Furthermore, we found that C/EBPα expression was nearly undetectable in MDSCs isolated from the tumors.

## *C/EBPα is significantly reduced in myeloid cells cultured under tumor conditions*

Hypoxia is a hallmark of solid tumors. It promotes tumor growth and progression. To test whether hypoxia regulates C/EBPα expression in myeloid cells, we cultured 32D murine myeloid cells under hypoxic conditions (1% $O_2$). Interestingly, we found that C/EBPα expression was significantly reduced after 4 hours and even further reduced after 12 hours incubation (Figure 3B). In addition, we cultured 32D cells in a mixture of fresh media and medium conditioned by 3LL tumor cells (TCM). C/EBPα expression was significantly reduced after 4

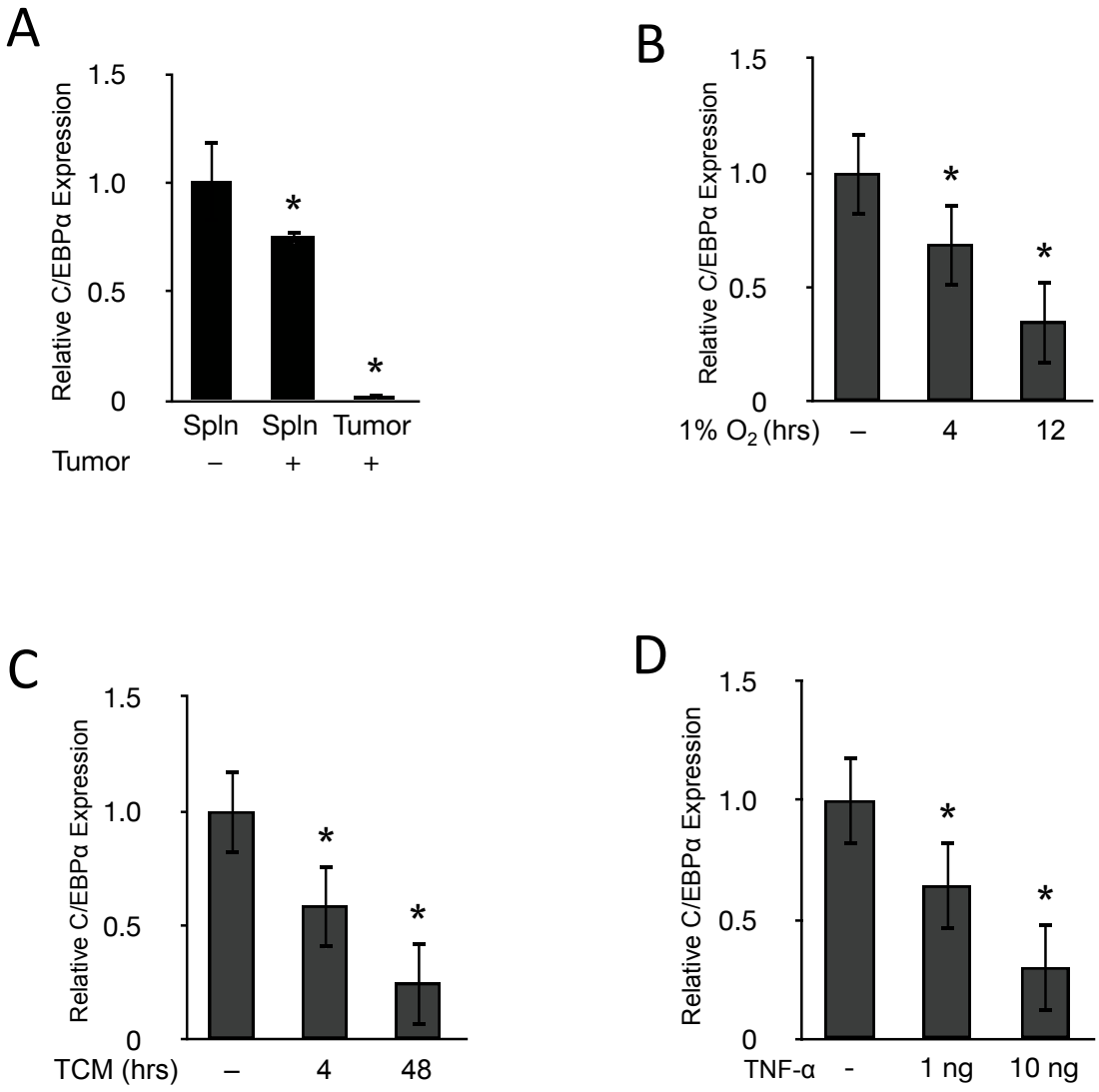


**Figure 3. C/EBPα is down-regulated in MDSCs from tumor bearing mice and in myeloid cells cultured under tumor conditions.** Gr-1+CD11b+ cells were isolated from spleens (spln) of C57BL/6 mice with (+) or without 3LL (-) tumors as well as tumor tissues (tumor). Gr-1+CD11b+ cells with greater than 95% purity were pooled from 5-7 mice, RNA was isolated and C/EBPα expression was measured by real-time PCR (A). 32D cells were cultured under normoxic or hypoxic (1% $O_2$) conditions. RNA was isolated and C/EBPα expression was measured by real-time PCR (B). Media was collected from 3LL tumor cells after 2-3 days in culture to make tumor-conditioned medium (TCM). 32D cells were cultured in a 50:50 mixture of TCM and fresh media for 4 or 48 hours. RNA was isolated and C/EBPα expression was measured by real-time PCR (C). 32D cells were treated with the TNF-α and cultured for 24 hours. RNA was isolated and C/EBPα expression was measured by real-time PCR (D). * $p < 0.05$. The experiment was done in triplicate and repeated twice.

hours in TCM and longer exposure to TCM further reduced C/EBPα mRNA levels (Figure 3C). Furthermore, treatment of 32D cells with the inflammatory cytokine TNF-α reduced C/EBPα expression by nearly half (Figure 3D).

*Conditional deletion of myeloid C/EBPα increases MDSC expansion under tumor conditions*

Based on the critical role of C/EBPα in regulating the balance between proliferation and growth arrest in hematopoietic progenitors [56], as well as the down regulation of C/EBPα in tumor derived MDSCs (Figure 3A), we suspected an inhibitory function for C/EBPα in MDSC expansion and tumor promotion. To test the hypothesis *in vivo*, we utilized a system of conditional myeloid-lineage gene deletion. C/EBPα was deleted in cells of the myeloid lineage by breeding *Cebpa*$^{flox/flox}$ mice with *LysMCre* mice, which express Cre recombinase under the control of a murine lysozyme M promoter. Murine lysozyme M is exclusively expressed in cells of the monocyte/macrophage and granulocyte lineages of hematopoietic differentiation [71] and its expression correlates with myeloid maturation, progressively increasing during myeloid differentiation [72].

The conditional deleted mice are healthy, fertile and grossly normal. We isolated Gr1+CD11b+ MDSCs from the spleens of wild-type littermates and *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ conditional null mice and confirmed C/EBPα deletion by real-time RT-PCR. In this system, C/EBPα was ablated in approximately 75% of the Gr1+CD11b+ MDSCs (Figure 4A). This reduction in C/EBPα mRNA levels was similar to what was observed in MDSCs isolated from tumor tissues (Figure

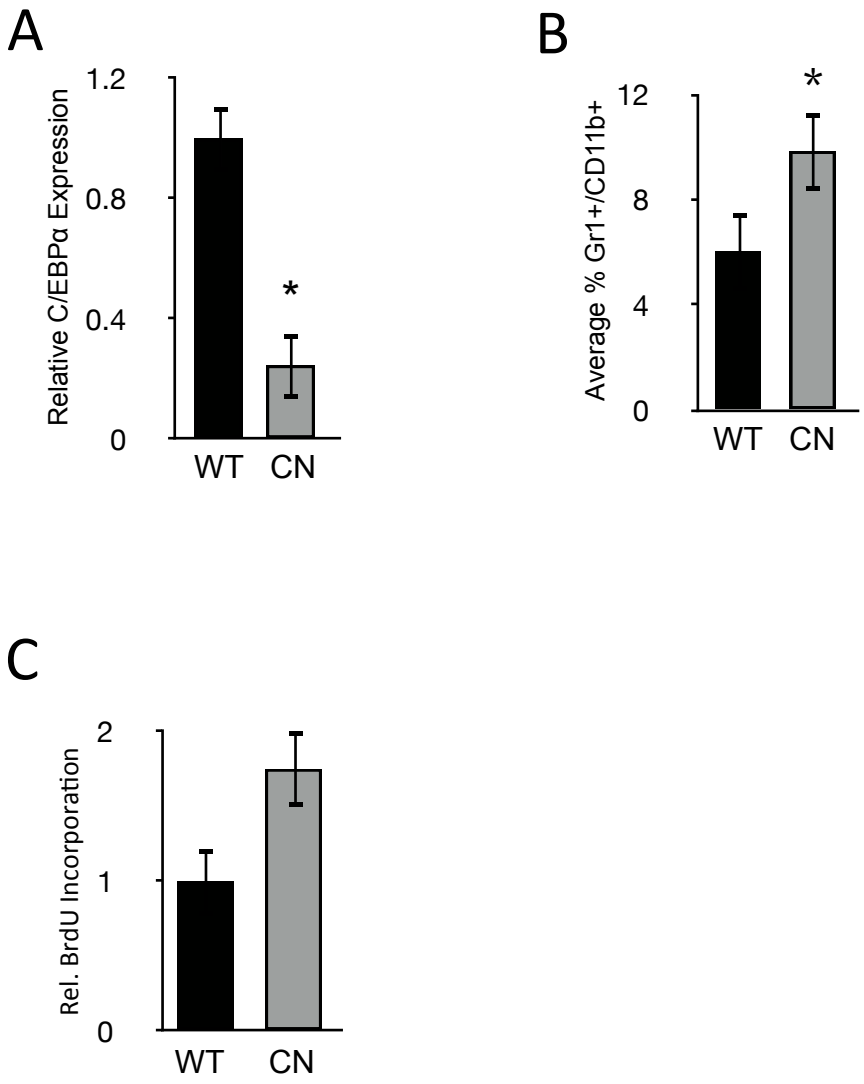


**Figure 4. MDSC expansion and proliferation is greater in C/EBPα conditional null tumor-bearing mice.** (A) Gr-1+CD11b+ cells were magnetically purified from spleens of C/EBPα$^{flox/flox}$ (WT) and C/EBPα$^{flox/flox}$;LysMCre (CN) mice, RNA was isolated and C/EBPα expression was analyzed by semi-quantitative PCR. Wild-type (C/EBPα$^{flox/flox}$) and myeloid conditional null (C/EBPα$^{flox/flox}$;LysMCre) mice were injected with 1x $10^5$ 3LL or B16 tumor cells in the hindlimb. After 21 days, spleens were isolated from the mice, processed into single-cell suspensions and stained with Gr-1 and CD11b fluorescent antibodies. The percentage of Gr-1+CD11b+ cells was analyzed by flow cytometry (B). 2 hours prior to sacrifice, the tumor bearing mice were injected with BrdU and BrdU incorporation was measured in the Gr-1+CD11b+ cells by flow cytometry (C). * $p < 0.05$.

3A). Next we examined the role of C/EBPα in MDSC expansion under tumor conditions. We injected 3LL tumor cells subcutaneously (s.c.) into the flanks of *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ (CN) and *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$ (WT) litter mates for several weeks. Single cells suspensions from the spleens of mice with similar sized tumors were stained with anti-Gr-1 and anti-CD11b antibodies. Flow cytometry analysis revealed a significant increase in Gr1+CD11b+ MDSCs in tumor-bearing C/EBPα conditional null mice (CN) compared to littermate controls (WT) (Figure 4B).

*Conditional deletion of myeloid C/EBPα enhances MDSC proliferative capacity*

Given that C/EBPα inhibits proliferation in myeloid progenitors [73], we wondered if proliferation was greater in MDSCs from C/EBPα conditional null mice. Prior to sacrificing the mice, mice bearing similar sizes of tumors were injected intra-peritoneally (i.p.) with BrdU. Flow cytometry analysis revealed that BrdU incorporation in MDSCs from *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice was nearly double that of littermate control MDSCs (Figure 4C). These data suggest that C/EBPα inhibits MDSC expansion, likely through inhibition of cell proliferation, and tumor conditions resulted in an expansion of MDSCs through down regulation of C/EBPa in myeloid cells.

*Conditional deletion of myeloid C/EBPα increases myeloid progenitors*

Next, we attempted to determine the mechanism of C/EBPα in MDSC expansion. C/EBPα is known to inhibit proliferation and induce differentiation of myeloid progenitors [57]. Since MDSCs are immature myeloid cells, and deletion of C/EBPα led to an increase of MDSCs *in vivo*, it suggests a role of this transcription factor in myeloid cell maturation. To test the hypothesis, colony-forming unit (CFU) assays in semi-solid methylcellulose medium were performed. Single cell suspensions were prepared from bone marrow of *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice and *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$ littermate controls and cultured in methylcellulose for about a week. We observed a significant increase in the total number of colonies in bone marrow isolated from C/EBPα conditional null mice (Figure 5A,B). The number of multi-potential progenitor (CFU-GEMM), macrophage (CFU-M) and erythroid (BFU-E) progenitor colonies was also significantly greater than littermate controls (Figure 5B). Furthermore, in a similar CFU assay for GM (granulocyte-monocyte), M (monocyte) and G (granulocyte) progenitors, an increase in the total colony number and in the number and percentage of CFU-M progenitor colonies was observed (Figure 5C).

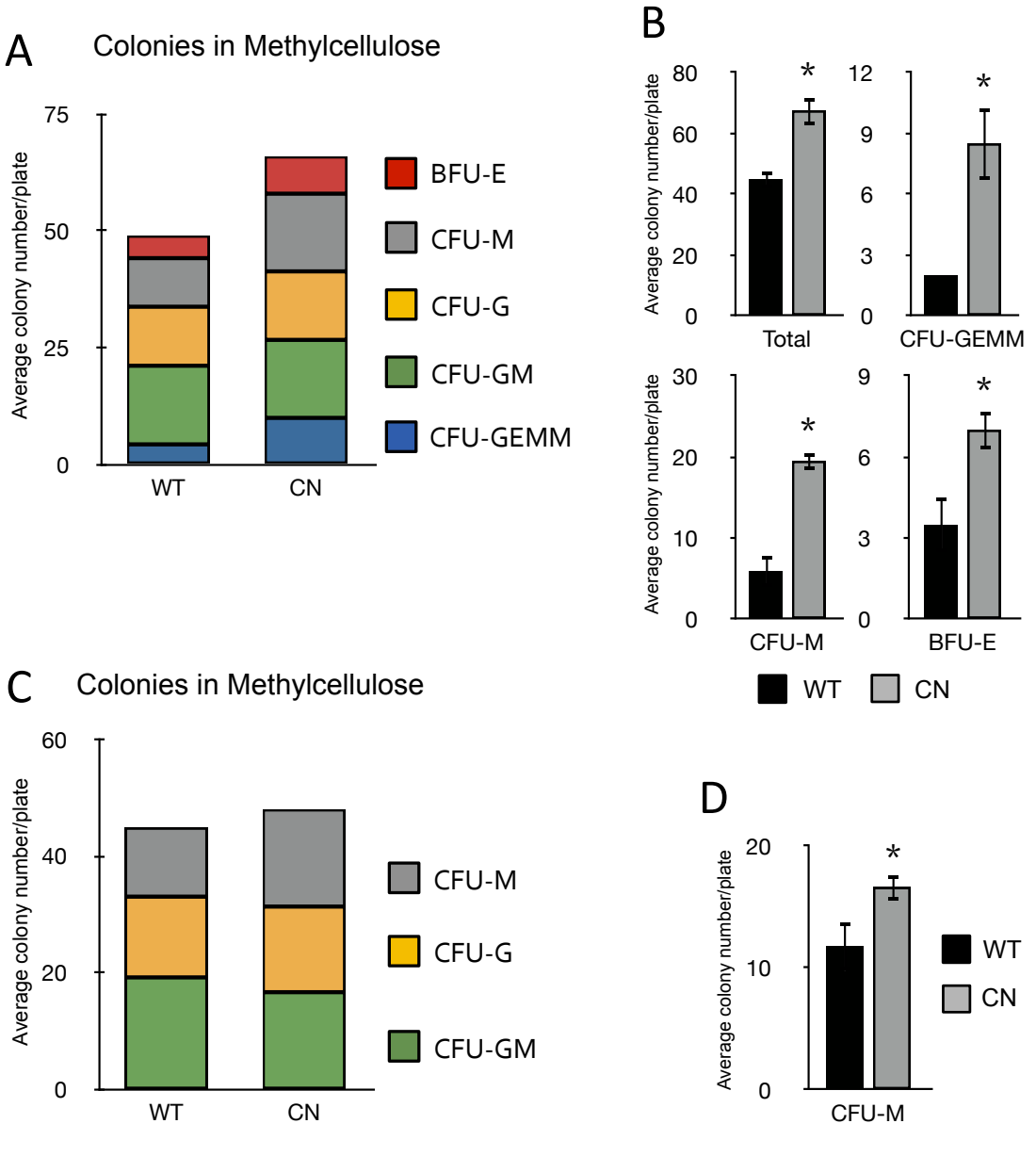


**Figure 5. Myeloid Progenitors are Increased in C/EBPα Conditional Null Mice.** Bone marrow was isolated from myeloid conditional null (*Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$, CN) mice and *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$ littermate controls (WT). Single cell suspensions were made and plated in MethoCult 3434 (A-B) or 3534 (C-D) semi-solid media. After 7-9 days incubation, colony types were evaluated and counted using inverted microscope. The number of individual colony types (A-D) and total colonies (B) was quantified. Bone marrow was pooled from 3 animals and experiments were repeated four times. * $p < 0.05$.

*Conditional deletion of myeloid C/EBPα decreases circulating monocytes*

We also analyzed the effect of myeloid-lineage C/EBPα ablation on circulating blood cells by performing complete blood counts (CBCs) on peripheral blood (Table 1). We found that the number of circulating monocytes was significantly decreased in *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice compared to controls. Other white blood cells were also decreased, although these differences did not reach statistical significance. Otherwise myeloid conditional null mice were not different from their littermate controls or from the normal range for their species, consistent with myeloid lineage-specific deletion of C/EBPα. Taken together, these findings show that deletion of C/EBPα in myeloid cells yields an increase in myeloid progenitors and an accompanying decrease in circulating mature cells.

| | WT | CN |
|---|---|---|
| **RBC (M/uL)** | 10.15 ± 1.49 | 11.10 ± 0.45 |
| **Hemoglobin (g/dL)** | 13.13 ± 1.86 | 13.87 ± 0.50 |
| **HCT (%)** | 42.27 ± 5.48 | 45.23 ± 1.40 |
| **Platlet (K/uL)** | 132.67 ± 64.50 | 341.33 ± 341.73 |
| **Total WBC (K/uL)** | 3.03 ± 1.78 | 2.89 ± 1.03 |
| **Lymphocyte (%)** | 61.74 ± 6.41 | 56.25 ± 6.08 |
| **Neutrophil (%)** | 24.83 ± 7.00 | 30.35 ± 5.90 |
| **Monocyte (%)** | 10.48 ± 1.47 | 10.33 ± 1.20 |
| **Eosinophils (%)** | 2.12 ± 1.20 | 2.14 ± 0.95 |
| **Basophils (%)** | 0.83 ± 0.20 | 0.72 ± 0.69 |
| **Lymphocyte (K/uL)** | 2.36 ± 0.23 | 1.66 ± 0.71 |
| **Neutrophil (K/uL)** | 1.23 ± 0.23 | 0.85 ± 0.26 |
| **Monocyte (K/uL)** | 0.45 ± 0.05 | 0.29 ± 0.09 |
| **Eosinophils (K/uL)** | 0.09 ± 0.05 | 0.06 ± 0.04 |
| **Basophils (K/uL)** | 0.04 ± 0.02 | 0.03 ± 0.02 |

**Table 1. Peripheral blood differential counts of myeloid conditional null mice and littermate WT mice.** Red blood cell **(**RBC), Hemoglobin (Hgb), Hematocrit (HCT), total white blood cell (WBC) and individual WBCs in peripheral blood were determined with an automated cell counter (HemaVet 960) in wild-type littermate *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$, (WT) and myeloid conditional null (*Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$, CN) mice. Shown are the numbers from 3 mice ± standard deviation. The experiment was repeated twice.

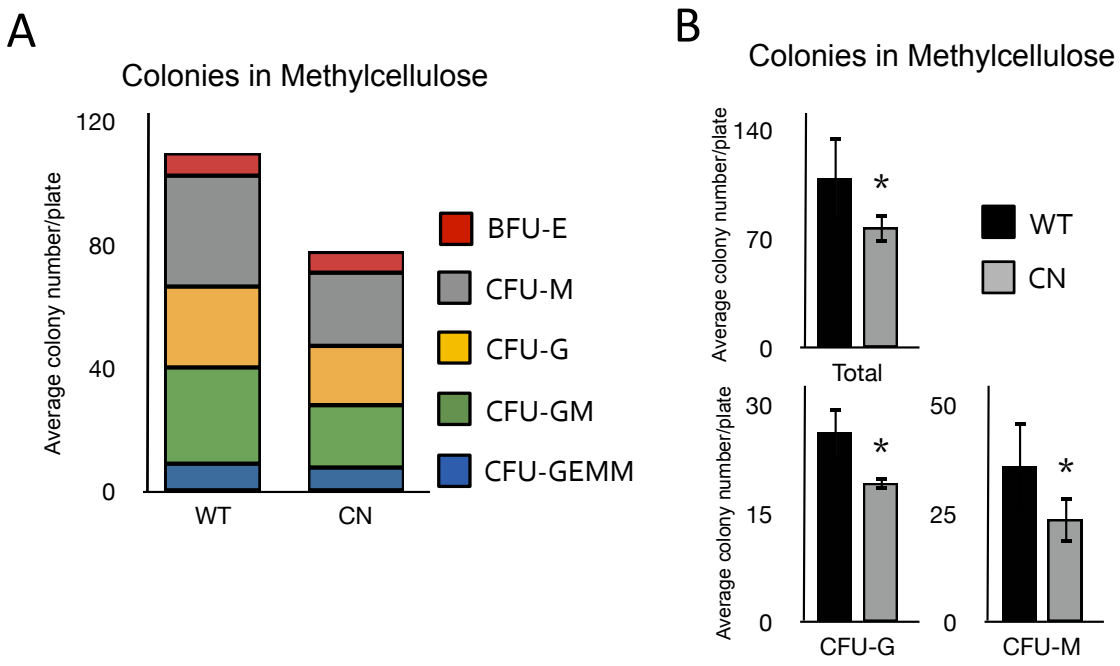


**Figure 6. Myeloid Progenitors are Reduced in tumor-bearing C/EBPα Conditional Null Mice.** Bone marrow was isolated from myeloid conditional null (*Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$, CN) mice and *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$ littermate controls (WT). Single cell suspensions were made and plated in MethoCult 3434 (A-B) semi-solid media. After 7-9 days incubation, colony types were evaluated and counted using inverted microscope. The number of individual colony types (A-B) and total colonies (B) was quantified. Bone marrow was pooled from 3 animals and experiments were repeated four times. * $p < 0.05$.

*Conditional deletion of myeloid C/EBPα decreases myeloid progenitors in tumor-bearing mice*

Methylcellulose CFU assays were also performed on bone from tumor-bearing mice in order to understand the effect of tumor conditions on myeloid progenitor proliferation and differentiation and the role C/EBPα plays in that process. We found that tumor conditions significantly reduced the number of total colonies (Figure 6A) as well as the number of myeloid progenitors (CFU-M and CFU-G) in *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice relative to control tumor-bearing mice. (Figure 6B) Intriguingly, there was no difference in the percentage of each individual colony type between wild-type and CN mice.

*Conditional deletion of myeloid C/EBPα increases circulating white blood cells in tumor-bearing mice*

Complete blood counts were also performed on tumor-bearing mice (Table 2). *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice had a significantly higher WBC count and significantly more eosinophils than wild-type mice. Additionally the percentage of lymphocytes was significantly decreased and the percentage of neutrophils was significantly increased in tumor-bearing C/EBPα conditional null mice. Furthermore, the monocyte percentage was decreased and the basophil percentage elevated in peripheral blood from tumor-bearing mice with a myeloid-lineage deletion of C/EBPα. Taken together, we found that deletion of C/EBPα in myeloid lineage cells resulted in decreased myeloid progenitor production and increased circulating white blood cells in tumor bearing mice.

| | WT | CN |
|---|---|---|
| **RBC (M/uL)** | 6.78 ± 0.64 | 7.31 ± 1.87 |
| **Hemoglobin (g/dL)** | 7.60 ± 1.14 | 8.10 ± 1.82 |
| **HCT (%)** | 30.93 ± 3.93 | 33.10 ± 6.11 |
| **Platlet (K/uL)** | 89.67 ± 63.69 | 87.33 ± 21.08 |
| **Total WBC (K/uL)** | 3.29 ± 3.24 | 5.32 ± 4.26 |
| **Lymphocyte (%)** | 69.24 ± 3.45 | 54.92 ± 10.66 |
| **Neutrophil (%)** | 18.02 ± 5.53 | 35.10 ± 12.95 |
| **Monocyte (%)** | 11.20 ± 2.74 | 7.53 ± 2.72 |
| **Eosinophils (%)** | 1.31 ± 0.46 | 2.07 ± 0.63 |
| **Basophils (%)** | 0.23 ± 0.06 | 0.38 ± 0.07 |
| **Lymphocyte (K/uL)** | 2.21 ± 2.08 | 2.63 ± 1.68 |
| **Neutrophil (K/uL)** | 0.71 ± 0.86 | 2.21 ± 2.33 |
| **Monocyte (K/uL)** | 0.32 ± 0.23 | 0.35 ± 0.20 |
| **Eosinophils (K/uL)** | 0.05 ± 0.06 | 0.10 ± 0.08 |
| **Basophils (K/uL)** | 0.00 ± 0.01 | 0.02 ± 0.02 |

**Table 2. Peripheral blood differential counts of myeloid conditional null mice and littermate WT tumor-bearing mice.** Red blood cell **(**RBC), Hemoglobin (Hgb), Hematocrit (HCT), total white blood cell (WBC) and individual WBCs in peripheral blood were determined with an automated cell counter (HemaVet 960) in wild-type littermate *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$, (WT) and myeloid conditional null (*Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$, CN) mice bearing large tumors. Shown are the numbers from 3 mice ± standard deviation. The experiment was repeated twice.

## Discussion

Based on the critical role of C/EBPα in myeloid differentiation [56], we hypothesized that C/EBPα plays a negative role in MDSC expansion. We found that C/EBPα expression is down-regulated in MDSCs from the spleens of tumor-bearing mice. Furthermore, C/EBPα expression was nearly undetectable in MDSCs from the tumor microenvironment. These results were mirrored and confirmed by our in vitro studies. Treatment of myeloid cells with tumor-conditioned media reduced C/EBPα mRNA in a time-dependent manner, suggesting that tumor-produced factors inhibit C/EBPα expression.

One such tumor-derived factor is TNF-α, which we found reduced C/EBPα expression in myeloid cells in a concentration-dependent manner. Others have shown that TNF-α decreases C/EBPα production by inhibiting the transcription and decreasing the stability of C/EBPα mRNA (Figure 7) [74]. TNF-α is an inflammatory cytokine produced by both tumor and host cells during tumor growth. It has been shown to recruit monocytes to the tumor as well as to promote tumor angiogenesis and impair immune surveillance [75]. Thus, TNF-α appears to participate in the recruitment of MDSCs from the bone marrow and spleen to the tumor, while at the same time down-regulating C/EBPα expression in MDSCs. Another long-range signal in the spleen could be IL-6, a cytokine produced by tumors or tumor-associated myeloid cells that is involved in MDSC expansion. IL-6 has been shown to inhibit C/EBPα transcription by activating MYC, which directly binds the CEBPA promoter and represses it (Figure 7) [76].

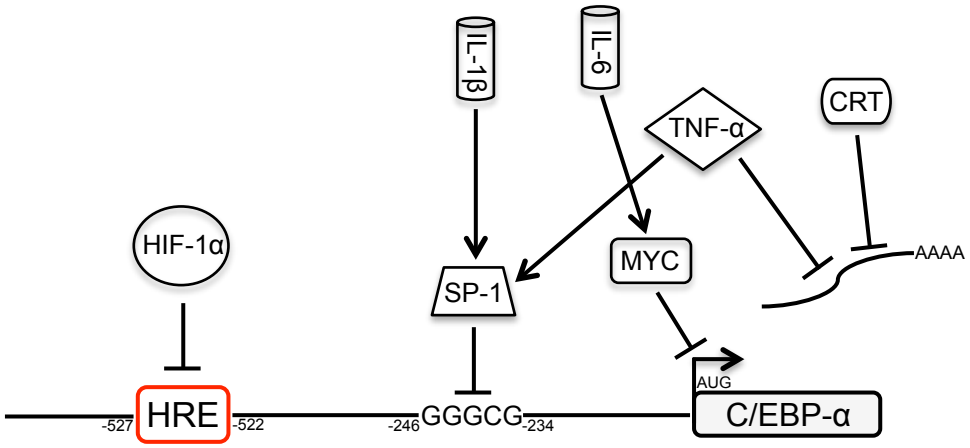


**Figure 7. Diagram of the C/EBPα promoter and known regulatory elements.** Soluble tumor-derived factors (TDFs) such as the inflammatory cytokines TNF-α, IL-1β and IL-6 are potential long-range signals responsible for C/EBPα downregulation in MDSCs. These signals activate the SP-1 transcription factor, which binds and inhibits the C/EBPα promoter. IL-6-induced Myc also represses C/EBPα transcription. Once in the tumor microenvironment, these TDFs and hypoxia, signaling through hypoxia-inducible factor (HIF-1α), which binds and represses a hypoxia-responsive element (HRE) in the C/EBPα promoter, might further reduce C/EBPα expression in MDSCs. Additionally, calreticulin (CRT) and TNF-α inhibit C/EBPα mRNA stability and translation, respectively.

We also found that hypoxia down-regulated C/EBPα expression in myeloid cells in a time-dependent manner. Hypoxia is a hallmark of solid tumors and is critically important in tumor growth and progression [77]. In addition to promoting tumor cell survival and angiogenesis, hypoxia signaling is involved in MDSC recruitment and activation. Hypoxia represses C/EBPα transcription in multiple cell types [78,79]. The C/EBPα promoter contains a hypoxia-responsive element (HRE), which is bound by hypoxia-inducible factor (HIF)-1 alpha and is essential for down-regulation of C/EBPα transcription in hypoxia (Figure 7) [78]. Additionally, calreticulin (CRT), a chaperone protein normally located in the endoplasmic reticulum, but is released into the extracellular matrix during hypoxia and other cancer-induced stresses binds C/EBPα mRNA and inhibits its translation [80]. In addition to hypoxia, other short-range signals that may down-regulate C/EBPα in the tumor microenvironment include the cytokines IL-1β and GM-CSF, which induce MDSC expansion. These cytokine signaling pathways (and TNF-α signaling) converge on the SP1 transcription factor, which is overexpressed in many cancers. SP1 directly binds the C/EBPα promoter represses it [81].

When mice with a conditional deletion of C/EBPα in myeloid-lineage cells were inoculated with tumor cells, we observed a significant increase in MDSC production in the spleens of those mice. C/EBPα regulates cell-cycle progression through many mechanisms including E2F inhibition [82]. We observed nearly a two-fold enhancement in the proliferative capacity of MDSCs isolated from tumor-bearing mice lacking C/EBPα in myeloid lineage cells.

Genetic deletion of C/EBPα results in an increase in immature myeloid cells and a complete lack of mature granulocytes [83]. Conditional deletion of C/EBPα in myeloid cells our system yielded similar results. We saw an increase in the number and percentage of myeloid progenitors in the bone marrow and a decrease in the number of circulating monocytes in the peripheral blood of these mice. We observed the opposite in tumor-bearing mice: C/EBPα conditional null mice had fewer myeloid progenitors in the bone marrow and more circulating white blood cells. Importantly, we didn't observe any difference in the relative percentages of the myeloid progenitors. Instead there were fewer total colonies and less of each individual progenitor in the bone marrow of C/EBPα conditional null tumor-bearing mice. Thus it appears that increased myeloid progenitor production in the bone marrow yielded greater MDSC expansion in tumor-bearing mice. Perhaps the increase in white blood cells observed in the peripheral blood of C/EBPα conditional null tumor-bearing mice resulted from increased MDSC production and expansion in those mice. Taken together, these data support the hypothesis that signals in the tumor microenvironment, including hypoxia and TNF-α secretion, down-regulate C/EBPα expression in myeloid lineage cells, resulting in MDSC proliferation and expansion.

# CHAPTER III

## C/EBPα REGULATES PRO-ANGIOGENIC FUNCTIONS OF MYELOID-DERIVED SUPRESSOR CELLS IN THE TUMOR MICROENVIRONMENT


### Abstract

MDSCs are capable of suppressing tumor immunity through multiple direct and indirect mechanisms on T-cells, dendritic cells and natural killer cells. MDSCs also directly promote tumor growth through mechanisms of tumor angiogenesis. Conditional deletion of C/EBPα in myeloid-lineage cells resulted in enhanced MDSC infiltration into tumors and increased tumor angiogenesis, resulting in accelerated tumor growth. Deletion of C/EBPα in MDSCs enhanced the pro-angiogenic and pro-tumorigenic behavior of these cells by upregulating the production of iNOS and nitric oxide, as well as MMP-9 and VEGF. However, myeloid lineage specific deletion of C/EBP$\alpha$ did not increase MDSC-mediated immune suppression.

## Introduction

Immune suppression and angiogenesis are hallmarks of cancer and are necessary for tumor growth and progression [84]. MDSCs posses both tumor-promoting functions; these cells have long been known to suppress the host immune response to cancer. MDSCs also directly promote tumor growth through mechanisms of tumor angiogenesis and vasculogenesis. They express higher levels of MMP-9, thereby increasing the bioavailability of VEGF [18] and promoting pericyte recruitment, which increases vascular stability. As such, MDSCs have been reported to mediate tumor refractoriness to anti-VEGF treatment by providing and alternative source of VEGF [39].

MDSCs from tumor-bearing mice have been shown to directly promote tumor angiogenesis and growth [18]. When MDSCs from mice with myeloid-lineage ablation of C/EBPα were combined with tumor cells in a co-injection study, tumor angiogenesis and growth was further increased. MDSCs promote tumor angiogenesis and tumor cell invasion through the production of MMP-9 and VEGF [18,40]. The expression of MMP-9 and VEGF was significantly increased in MDSCs purified from tumor-bearing C/EBPα conditional null mice.

The immune suppressive properties of MDSCs are largely due to arginase and iNOS activity. These enzymes metabolize L-arginine, thereby reducing its availability to T-cells, which require it for activation and proliferation. iNOS expression and activity was significantly greater in MDSCs isolated from tumor-bearing mice with a myeloid-lineage deletion of C/EBPα. However, we did not observe any difference in arginase expression between the two groups.

Additionally, when C/EBPα was deleted in MDSCs, we observed no change in their capacity to suppress antigen-specific T-cell proliferation. Taken together, these data suggest that in addition to inhibiting MDSC production, C/EBPα negatively regulates the pro-angiogenic functions of MDSCs in the tumor microenvironment.

## Materials and Methods

### *MDSC Isolation*

3LL lung cancer cells or B16 melanoma cells were subcutaneously implanted in C57Bl/6 mice for 3 weeks (3LL) or 30 days (B16). After sacrificing the mice, tumor tissues were removed and digested with collagenase A and hyaluronidase (both from Sigma Aldrich, St. Louis, MO) at 37°C overnight. Single cell suspensions were prepared from tumor and spleen tissues. MDSCs were isolated by sequential antibody labeling and separation by magnetic column, using anti-Gr1-PE (Cat. # 130-091-932) and anti-PE (Cat. # 130-48-801) multisort beads, followed by CD11b (Cat. # 130-093-634) MicroBeads (all from Miltenyi Biotech, Auburn, CA).

### *Quantitative RT-PCR*

RNA was isolated from cells using the RNeasy Kit from Qiagen (Cat. no. 74104, Valencia, CA), according to their protocol. cDNA was synthesized from 1 μg RNA with the iScript cDNA Synthesis Kit (Cat. # 170-8891, BioRad, Hercules, CA) according to the manufacturer's protocol. For quantitative real time PCR,

SsoFast EvaGreen Supermix (Cat. # 172-5201, BioRad, Hercules, CA) and a CFX96 or MyiQ machine (BioRad, Hercules, CA) were used. The primer sequences used for C/EBP-α are ACGGCGGGAACGCAACAACA and GAAGATGCCCCGCAGCGTGT, for iNOS (NOS2): ACCTTGTTCAGCTACGCCTT and CATTCCCAAATGTGCTTGTC, for VEGF-A: CCCGGGCCTCGGTTCCAGA and GCCTGGGACCACTTGGCATGG, for β-actin: GACAACGGCTCCGGCATGTGC and TGGCTGGGGTGTTGAAGGTC. After an initial denaturing (95°C for 2:00), 40 cycles of 5 s at 95°C and 9 s at 58.7°C were performed. Target and control reactions were performed in triplicate and the average Ct value was calculated. Relative changes in transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal control. Experiments were repeated three times.

*Cell lines*

32D myeloid cell line (# CRL-11346), Lewis lung cancer (3LL) cell line (# CRL-1642) and B16 melanoma (# CRL-6475) cell line (all murine, C57BL/6J mice) were purchased from ATCC (Manassas, VA). 32D cells were cultured in RPMI 1640 + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin () + 10% IL-3 Culture Supplement (Cat. # 354040, BD Biosciences, San Jose, CA) at 37°C, 5% $CO_2$. 3LL cells were grown in RPMI 1640 + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% $CO_2$. B16 cells were cultured in DMEM + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% $CO_2$.

*Mice*

C57BL/6J, LysMcre [69] and OT-1 [85] mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice with a floxed *Cebpa* gene, called *Cebpa*$^{flox/flox}$ [70], were a gift from Peter Johnson (NCI, Frederick, MD). The animals were housed in pathogen-free units at the Vanderbilt University Medical Center, in compliance with IACUC regulations. We generated mice with myeloid-specific deletion of *Cebpa* by breeding *Cebpa*$^{flox/flox}$ mice to *LysMCre* mice, which express Cre recombinase under the control of murine lysozyme M promoter. F1 LysMCre$^{+/-}$;*Cebpa*$^{fl/+}$ were then crossed to produce the genotypes used in the experiments. Mice used were between 6 and 10 weeks of age. The studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center and complied with all relevant institutional and federal guidelines and policies.

*Tumor allograft model*

B16 or 3LL cells (1 x $10^5$ cells), with or without Gr1+CD11b+ cells (1 x $10^4$ cells), were injected subcutaneously into the left flank of C57Bl/6 mice. The size of tumors was determined by measurement of tumor dimensions at 2–3 day intervals using calipers. The equation volume = length x $(width)^2$ x 0.5 was used to calculate tumor volume. Tumor samples taken at days 21 (3LL) or 30 (B16) post-injection were flash frozen in Tissue-Tek OCT (Sakura, Torrance, CA) and prepared for histology. Experiments were repeated at least three times.

*Single-cell sorting*

Single cell suspensions of splenocytes from tumor-bearing mice were stained with fluorescence-labeled Gr-1 (Cat. # 553126, monoclonal, rat) and CD11b (Cat. # 553311, monoclonal, rat) antibodies (BD Biosciences, San Jose, CA) for 15 minutes at 0°C. Gr-1+CD11b+ cells were sorted with a BD FACSAria cell sorter in the VA or VUMC Flow Cytometry Resource and collected for tumor growth experiments.

*Immunohistochemistry*

Cryo-preserved tumor sections were blocked with M.O.M. solution (Vector Laboratories, Burlingame, CA), incubated with antibodies to CD31 (BD Biosciences, San Jose, CA; 1:500 dilution) overnight (4° C), and visualized by using Texas Red-conjugated goat anti-rat 2nd antibody (Jackson Immuno Research Laboratories, West Grove, PA; 1:10000 dilution). Images were captured with a fluorescent microscope. Vascular density was calculated in 10 randomly selected fields and counted in a blinded manner. Average pixel density was measured with ImageJ software (http://rsb.info.nih.gov/ij/). These experiments were repeated 3 times.

*Enzyme activity*

NO production was measured as the product of $NO_3^-$ and $NO_2^-$ in culture media using a Nitrate/Nitrite Assay Kit (Alexsis Biochemicals, Farmingdale NY, Cat. # 850-001-KI01) according to manufacturer's instructions. Assay was

performed in triplicate and repeated twice.

*Suppression of antigen-specific T-cell proliferation*

MDSCs magnetically purified from the tumors of myeloid conditional null mice and wild-type littermates were cultured with various ratios with OT-1 [85] splenocytes that had been labeled with CFSE (Molecular Probes, Eugene, OR) and activated with ovalbumin (Thermo Fisher Scientific, Pittsburg, PA, Cat. # BP2535-5; 125 ug/mL). After 4 days in culture, CFSE fluorescence was measured by flow cytometry. The average number of cell divisions was calculated [86] and plotted. Assay was performed in triplicate and repeated twice.

*Statistical Analysis*

All data were averaged, analyzed using the Student's t test (one-sided; paired, equal variance) and were expressed as mean ± standard error across experiments.

# Results

*Conditional deletion of myeloid C/EBPα increases tumor growth*

C/EBPα has been identified as a tumor suppressor in multiple tissues based upon its role in inhibiting proliferation. Because of this and our finding that C/EBPα inhibits MDSC proliferation and expansion, we wanted to determine the role of myeloid C/EBPα in tumor growth. Toward that end, we injected *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ conditional null mice and *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$ littermates with 3LL tumors subcutaneously in the hindlimb. Tumor size was measured by caliper. We found that the tumor growth rate was significantly increased in C/EBPa myeloid conditional null mice compared to littermate controls (Figure 8A). We also inoculated mice with B16 melanoma cells and obtained similar results (Figure 8B). These results suggest a negative role for myeloid C/EBPα in tumor growth and progression.

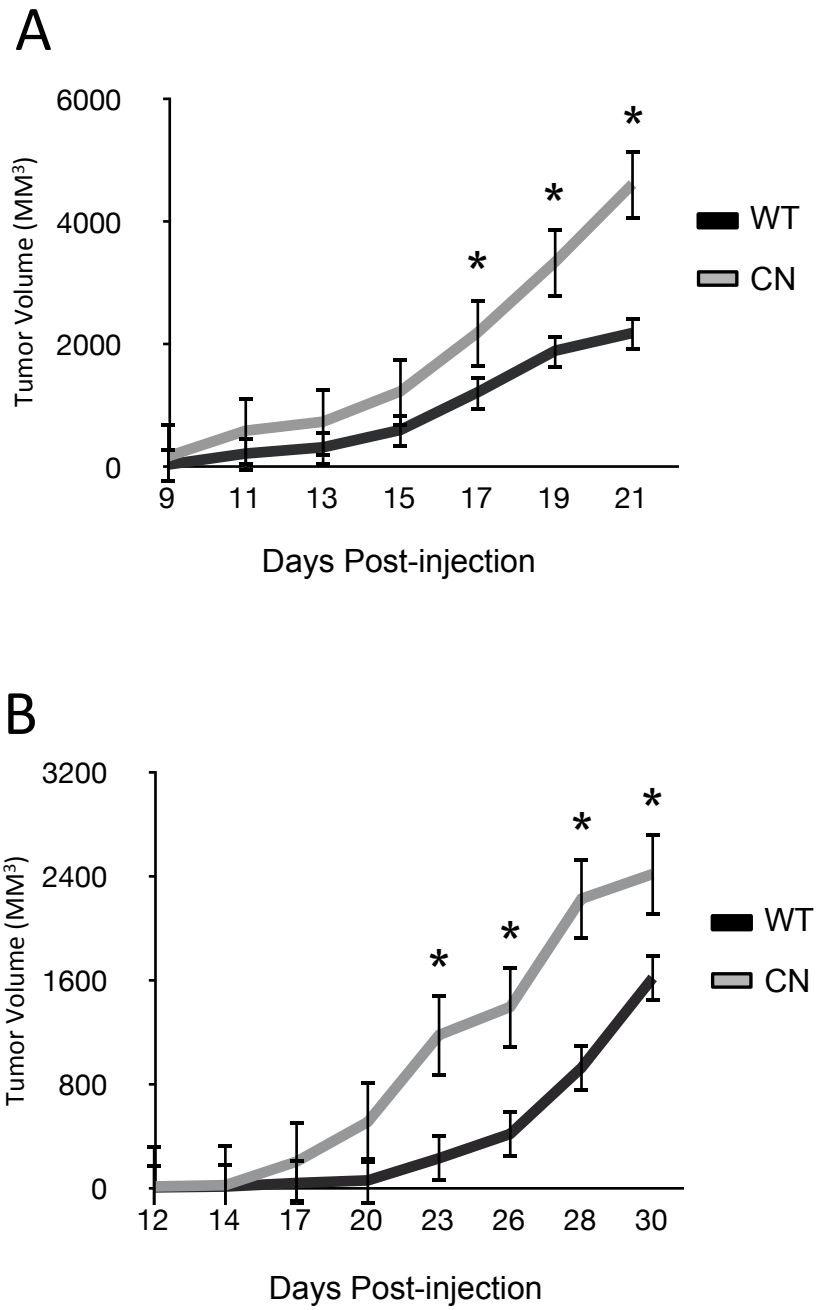


**Figure 8. Tumor growth is accelerated in C/EBPα conditional null mice.** (A) Wild-type (C/EBPα$^{flox/flox}$, WT) and myeloid conditional null (C/EBPα$^{flox/flox}$;LysMCre, CN) mice were injected with 1x $10^5$ 3LL (A) or B16 (B) tumor cells in the hindlimb. Tumor dimensions were measured every 2-3 days with caliper and tumor volume was calculated and plotted with time. * $p < 0.05$, n = 10 mice per group. The experiment was repeated 4 times.

*Tumors from C/EBPα myeloid conditional null mice have increased MDSC infiltration and vascularization*

MDSCs are known to infiltrate into tumors and modulate the tumor microenvironment to promote tumor progression [18]. We therefore compared MDSC infiltration in tumors harvested from *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ and *Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$ littermates 21 days after inoculation with 3LL tumor cells. We stained tumor sections with anti-Gr-1 antibody (Figure 9A) and Gr1+ cells were counted as a measure of MDSC infiltration. We observed a significant increase in MDSC infiltration into the tumors of C/EBPα conditional null mice relative to littermate controls (Figure 9B).

It is well established that MDSCs directly promote tumor growth through increased tumor angiogenesis [18]. We next analyzed tumor vascular density by staining tumor sections harvested from both groups with an anti-CD31 antibody (Figure 9C) and counted CD31+ blood vessels. A significantly higher blood vessel density was observed in the tumors grown in C/EBPα conditional null mice than in controls (Figure 9D).

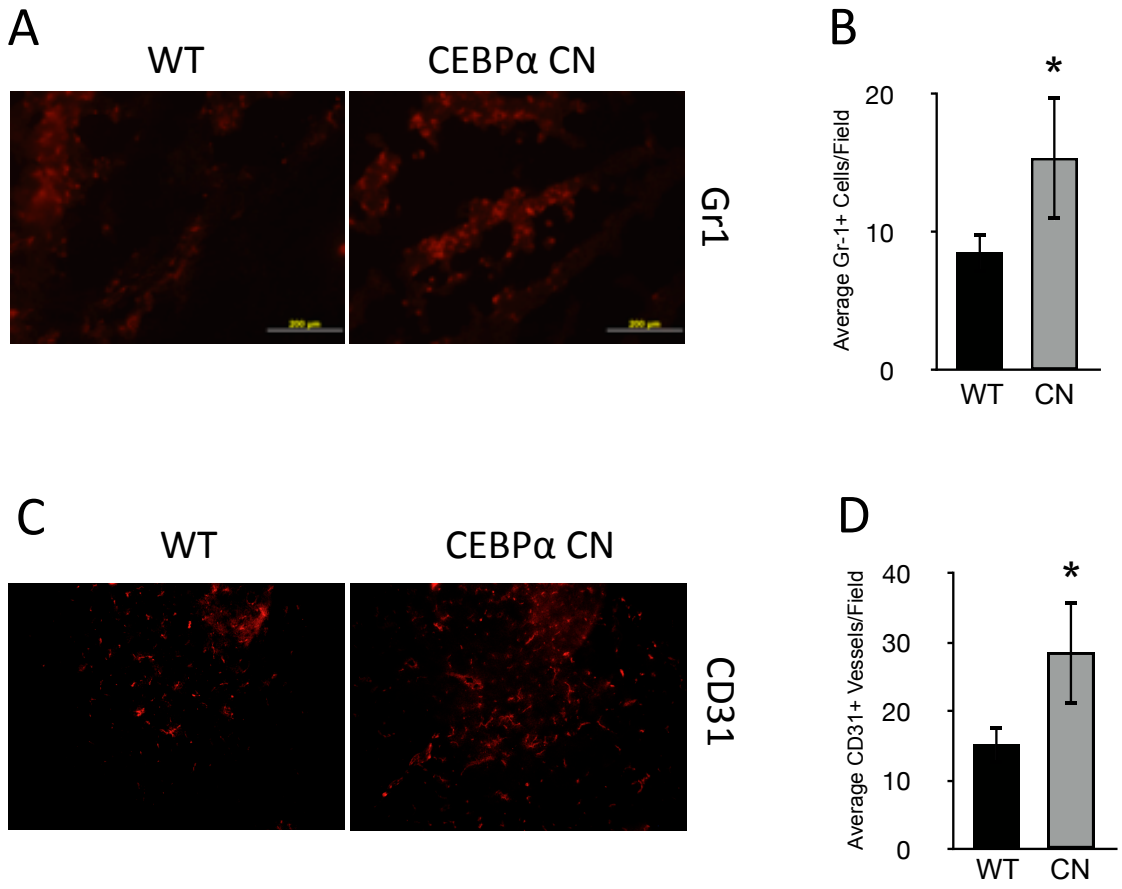


**Figure 9. Tumors from C/EBPα conditional null mice have increased MDSC infiltration and vascularity.** Size-matched tumors were harvested from myeloid conditional null (C/EBPα$^{flox/flox}$;LysMCre, C/EBPα CN) and control littermate mice (C/EBPα$^{flox/flox}$, WT), sectioned and stained with Gr-1 (A) or CD31 (C) fluorescent antibodies. Representative images are shown. The number Gr-1+ cells (B) and CD31+ vascular structures (D) were quantified in 10 randomly selected fields under microscopy. * $p < 0.05$.

*MDSCs from C/EBPα conditional null mice have increased capacity to promote tumor growth*

We observed increased myeloid progenitor production in mice with a myeloid-lineage deletion of C/EBPα. Upon inoculation of those mice with tumor cells, we observed greater MDSC expansion and infiltration into the tumors, increased tumor angiogenesis and accelerated tumor growth. We sought to determine if MDSCs were responsible for increased tumor angiogenesis and accelerated tumor growth in the conditional null animals. Additionally, we wanted to establish whether that effect was due solely to greater MDSC expansion in those mice. We next performed a reconstitution experiment where Gr1+CD11b+ cells were sorted from the spleens of *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ (CN) and wild-type (*Cebpa*$^{flox/flox}$;*LysMCre*$^{-/-}$, WT) tumor-bearing littermates by FACS. We consistently achieved greater than 95% purity. Next, 1 x $10^4$ purified Gr1+CD11b+ cells were mixed with 1 x $10^5$ 3LL tumor cells (1:10 ratio) and co-injected subcutaneously into the flanks of C57BL/6 mice. C57BL/6 mice were also injected with tumor cells alone and tumor cells mixed with MDSCs isolated from wild-type mice as controls. Tumor size was measured with a caliper and tumor volume was calculated. As expected, the tumor growth rate was greater when tumor cells were co-injected with Gr1+CD11b+ MDSCs from wild-type tumor-bearing mice compared to tumor cells alone (Figure 10). Interestingly, when mice were injected with tumor cells mixed with Gr1+CD11b+ MDSCs from tumor-bearing *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice, tumor growth was further accelerated (Figure 10). This led us to conclude that MDSCs contributed to the

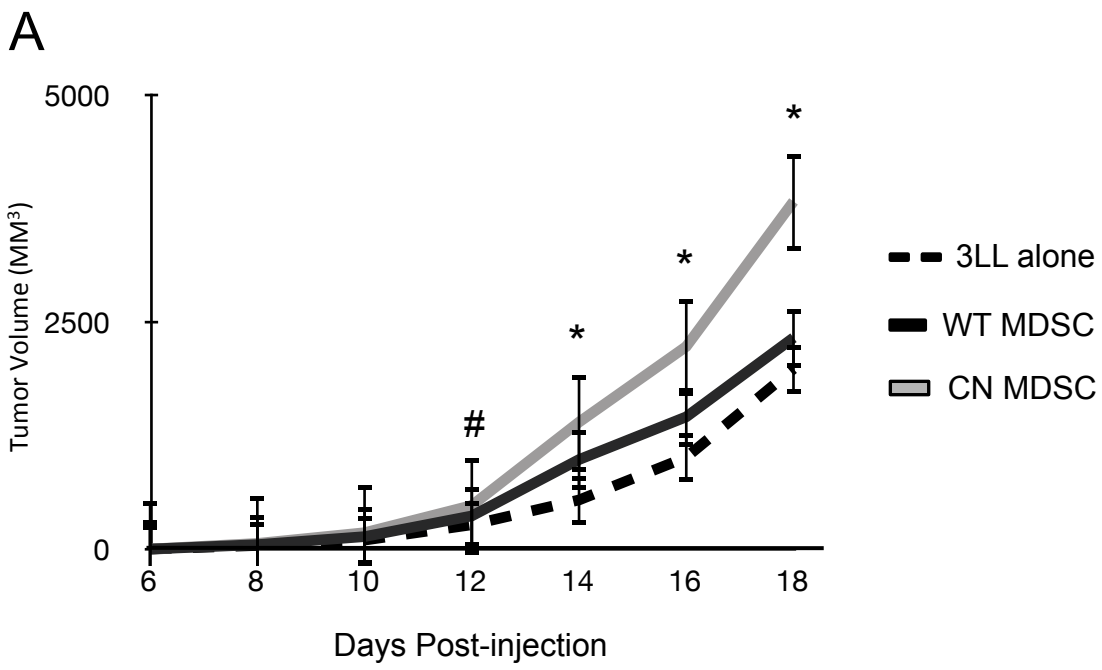


**Figure 10. MDSCs from C/EBPα conditional null mice promote tumor growth and angiogenesis.** Wild-type (C/EBPα$^{flox/flox}$, WT) and myeloid conditional null (C/EBPα$^{flox/flox}$;LysMCre, CN) mice were injected with 1x $10^5$ 3LL tumor cells in the hindlimb. After 21 days, Gr-1+CD11b+ cells were purified from the spleens by FACS. Wild-type C57BL/6 mice were injected with 1x $10^5$ 3LL tumor cells or 3LL cells combined with 1x $10^4$ Gr-1+CD11b+ cells from wild type (WT MDSC) or C/EBPα conditional null mice (CN MDSC). Tumor dimensions were measured every 2-3 days with caliper and tumor volume was calculated and plotted with time. * $p < 0.05$ (CN MDSC vs. WT MDSC), # $p < 0.05$ (CN MDSC vs. WT MDSC and 3LL alone).

increased angiogenesis and accelerated tumor growth we observed in *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice. Since equal numbers of MDSCs were injected with tumor cells, the only difference was whether C/EBPα had been deleted in the MDSCs. We hypothesized that conditional C/EBPα deletion in *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice had somehow enhanced MDSC tumor-promoting properties within the tumor microenvironment.

*MDSCs from C/EBPα conditional null mice have increased capacity to promote tumor angiogenesis*

Next we sought to determine if the accelerated tumor growth we observed in mice injected with MDSCs from *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice was due to increased tumor angiogenesis. After an analysis of tumor vascularity by staining the tumor sections with CD31 antibody, we observed a significant increase in vascular density in the tumors co-injected with Gr1+CD11b+ MDSCs from *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice compared with controls (Figure 11B). Furthermore, the blood vessels in tumors from mice co-injected with Gr1+CD11b+ MDSCs from tumor-bearing myeloid conditional null mice were bigger and had larger lumens (Figure 11A). These data collectively support dual roles of C/EBPα in MDSC biology; it negatively regulates MDSC expansion, as well as the pro-angiogenic and pro-tumor activities of MDSCs.

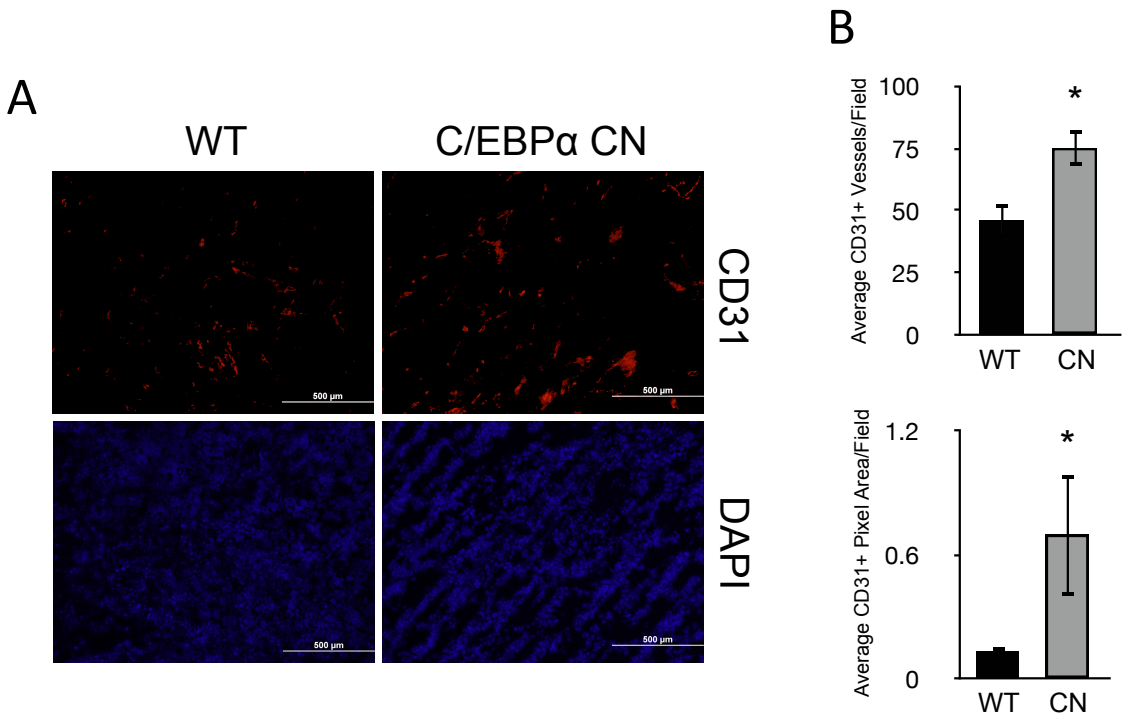


**Figure 11. MDSCs from C/EBPα conditional null mice promote tumor angiogenesis.** Wild-type (C/EBPα$^{flox/flox}$, WT) and myeloid conditional null (C/EBPα$^{flox/flox}$;LysMCre, C/EBPα CN) mice were injected with 1x $10^5$ 3LL tumor cells in the hindlimb. After 21 days, Gr-1+CD11b+ cells were purified from the spleens by FACS. Wild-type C57BL/6 mice were injected with 1x $10^5$ 3LL tumor cells or 3LL cells combined with 1x $10^4$ Gr-1+CD11b+ cells from wild type or C/EBPα conditional null mice. Tumors were harvested; sections were cut and stained with CD31 fluorescent antibody. Representative images are shown (A). The number CD31+ vascular structures were quantified in 5 randomly selected fields under microscopy (B, upper). CD31+ vascular area was quantified in 5 randomly selected fields as average pixel area using ImageJ (B, lower). * $p < 0.05$.

*C/EBPα negatively regulates angiogenic gene expression in MDSCs.*

MDSCs infiltrate into tumors and modulate the tumor microenvironment through production of growth factors and cytokines. Based on our findings that C/EBPα negatively regulates the angiogenic and tumor-promoting activities of MDSCs (Figure 11), we measured the expression of genes involved in MDSC-mediated immune suppression and angiogenesis. MDSCs were purified from the spleens of tumor-bearing mice by magnetic cell sorting. We found that C/EBPα ablation resulted in increased the expression of inducible nitric oxide synthase (iNOS), with no effect on arginase 1 expression (Figure 12A). The production of nitric oxide, a mediator critical for immune suppressive functions of MDSCs as well as angiogenesis, was also significantly increased in *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ (CN) MDSCs (Figure 12B). Additionally, we found that levels of MMP-9 and vascular endothelial growth factor A (VEGF-A), two important angiogenic mediators associated with MDSCs, were also significantly elevated in MDSCs from *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice. Furthermore, overexpression of C/EBPα in 32D cells resulted in decreased iNOS and VEGF-A expression when compared to vector control (Figure 12C). These data provide molecular evidence supporting a negative role of C/EBPα in the pro-angiogenic properties of MDSCs by inhibiting angiogenic gene expression.

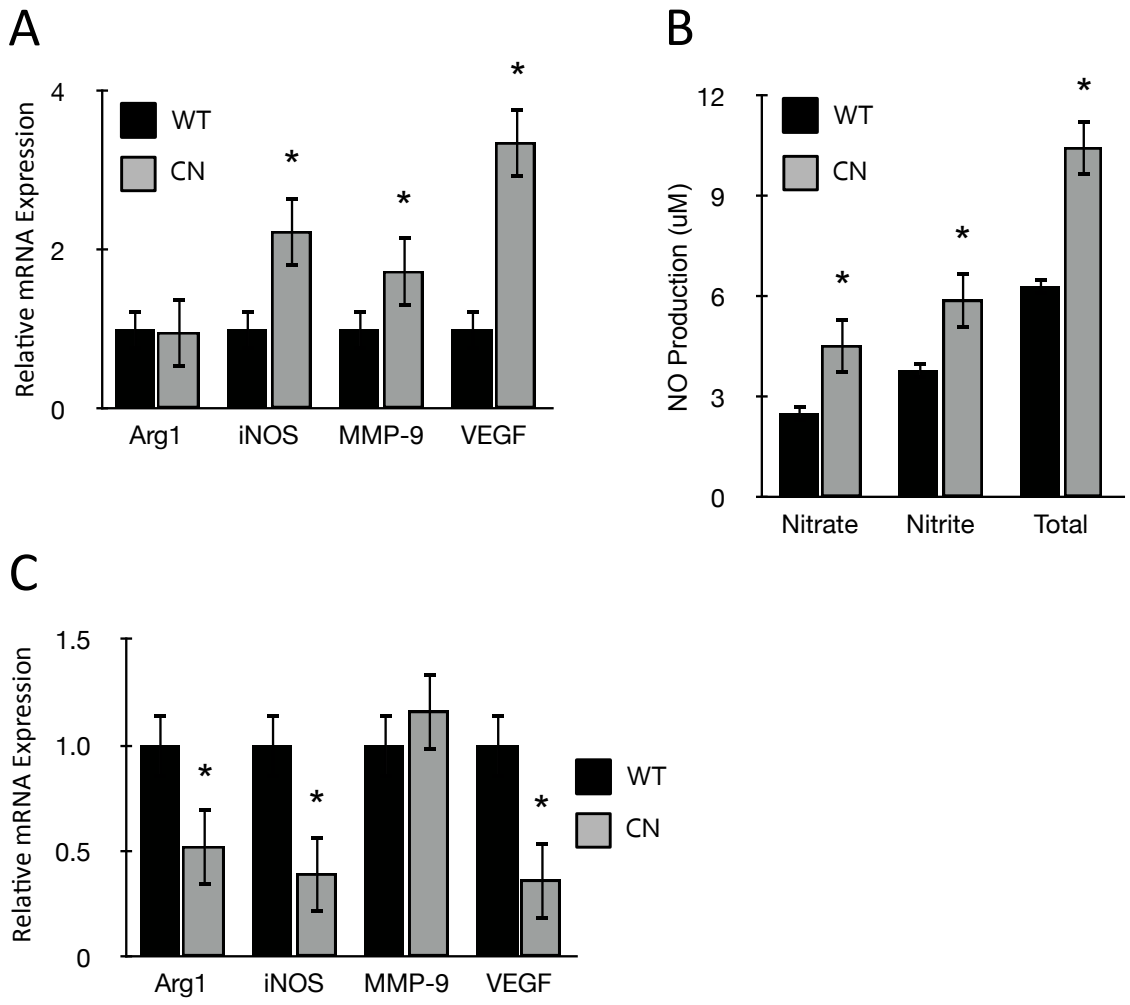


**Figure 12. C/EBPα negatively regulates angiogenic gene expression.** Gr-1+CD11b+ MDSCs were magnetically purified from spleens of C/EBPα conditional null (CN) and WT littermate 3LL tumor bearing mice (n= 5 mice pooled together per group). RNA was isolated and arginase 1 (Arg1), inducible nitric oxide synthase 1 (iNOS), matrix metalloproteinase 9 (MMP-9) and VEGF-A expression was measured by real time PCR (A). Gr-1+CD11b+ MDSCs from tumor bearing mice were cultured for 3 days. Nitrate and nitrite production was measured in the culture supernatants with Nitrate/Nitrite Assay Kit and total NO production was calculated (B). 32D cells were transfected with a C/EBPα expression plasmid or vector control. RNA was isolated and arginase (Arg1), inducible nitric oxide synthase 1 (iNOS), matrix metalloproteinase 9 (MMP-9) and VEGF-A expression was measured by real time PCR (C). *$p < 0.05$. The experiment was done in triplicate and repeated 3 times.

*C/EBPα does not play a major role in MDSC-mediated immune suppression*

Given the ability of MDSCs to suppress anti-tumor immune responses and our gene expression data, we wondered if C/EBPα regulates the immune-suppressive function of MDSCs. We therefore performed an immune suppression assay. MDSCs were purified from the tumors of *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ (CN) mice and littermate controls and cultured with activated splenocytes that had been labeled with CFSE. After a few days in culture, we quantified CFSE dilution on a flow cytometer as a measure of proliferation. We observed no difference in the ability to inhibit antigen-specific T-cell proliferation between MDSDs from *Cebpa*$^{flox/flox}$;*LysMCre*$^{+/+}$ mice and littermate controls (Figure 13). We concluded that C/EBPα does not play a major role in MDSC-mediated immune suppression.

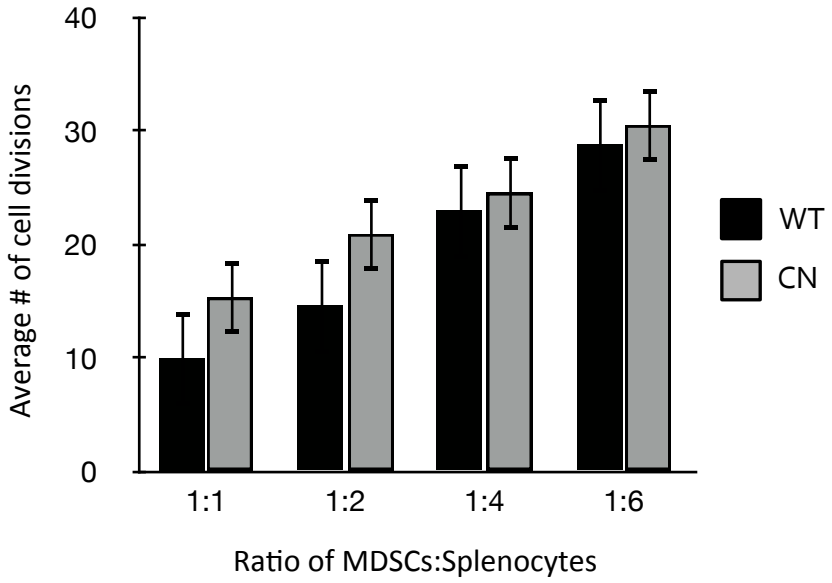


**Figure 13. C/EBPα does not play a major role in MDSC-mediated immune suppression.** Gr-1+CD11b+ MDSCs were magnetically purified from spleens of C/EBPα conditional null (CN) and WT littermate 3LL tumor bearing mice (n=5 mice pooled together per group). OT-1 splenocytes labeled with CFSE and stimulated with ovalbumin were cultured with the purified MDSCs. CFSE fluorescence was measured and the number of cell divisions was calculated. Differences between WT and CN were not significant. This experiment was repeated twice.

## Discussion

MDSCs infiltrate into tumors where they promote tumor angiogenesis and suppress the host immune response to cancer. MDSCs from tumor-bearing mice have been shown to directly promote tumor angiogenesis and growth [18]. We observed that tumor growth was significantly accelerated in mice harboring a conditional deletion of C/EBPα in myeloid-lineage cells. Furthermore, the tumors had greater MDSC infiltration and increased vascular density. When MDSCs from mice with myeloid-lineage ablation of C/EBPα were combined with tumor cells in a co-injection study, tumor angiogenesis and growth was further increased.

MDSCs promote tumor angiogenesis and tumor cell invasion through the production of MMP-9 and VEGF [18,40]. We found that the expression of MMP-9 and VEGF was significantly increased in MDSCs purified from tumor-bearing C/EBPα conditional null mice. The immune suppressive properties of MDSCs are largely due to arginase and iNOS activity. Although we did not observe any difference in arginase expression, we observed that iNOS expression and activity was significantly greater in MDSCs isolated from tumor-bearing mice with a myeloid-lineage deletion of C/EBPα. In addition mediating the immune suppressive properties of MDSCs, NO also regulates angiogenesis [87].

Intriguingly, when we overexpressed C/EBPα in 32D myeloid cells, arginase expression was significantly reduced. This apparent discrepancy may be due to differences between primary cells and cell lines. Though a literature search yielded no reports of C/EBPα regulating arginase expression, the arginase 2

promoter does contain a predicted C/EBPα binding site. Additionally we observed no effect on MMP-9 expression when C/EBPα was overexpressed in 32D cells despite a modest but significant increase in its expression in MDSCs isolated C/EBPα conditional null mice. Although the human, mouse and rat MMP-9 promoters have predicted C/EBPα binding sites, there are no reports of C/EBPα inhibiting MMP-9 expression.

Regardless, the overexpression data did confirm our observations regarding iNOS and VEGF expression in C/EBPα null MDSCs. C/EBPα overproduction inhibited iNOS and VEGFA expression in the 32D myeloid cell line. The human iNOS and VEGFA promoters both contain confirmed C/EBPα binding sites. Furthermore, macrophages isolated from tumor-bearing mice display reduced C/EBPα binding to the iNOS promoter [88]. C/EBPα is also a negative regulator of VEGFA expression in the heart [89].

In order to rule out a role for C/EBPα in immune suppressive properties of MDSCs, we compared the ability to suppress T-cell proliferation between MDSCs purified from C/EBPα conditional null mice and littermate controls. We observed no difference between the groups and therefore, concluded that C/EBPα does not play a major role in MDSC-mediated immune suppression. The results suggest that, in addition to inhibiting MDSC expansion, C/EBPα also functions as a negative regulator of MDSC activation and function. Through the ability to repress iNOS and VEGF expression and NO production, C/EBPα inhibits the promotion of tumor angiogenesis in MDSCs.

# CHAPTER IV

# PRELIMINARY DATA

## Materials and Methods

### *Single-cell sorting*

Single cell suspensions of splenocytes from tumor-free mice were stained with fluorescence-labeled Gr-1 (Cat. # 553126, monoclonal, rat) and CD11b (Cat. # 553311, monoclonal, rat) antibodies (BD Biosciences, San Jose, CA) for 15 minutes at 0°C. Gr-1+CD11b+ cells were sorted with a BD FACSAria cell sorter in the VA or VUMC Flow Cytometry Resource and collected for tumor growth experiments.

### *Cell lines*

Lewis lung cancer (3LL) cell line (# CRL-1642) and B16 melanoma (# CRL-6475) cell line (all murine, C57BL/6J mice) were purchased from ATCC (Manassas, VA). 3LL cells were grown in RPMI 1640 + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% $CO_2$.

### *Mice*

C57BL/6J and LysMcre [69] mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice with a floxed *Cebpa* gene, called *Cebpa*$^{flox/flox}$ [70], were a gift from Peter Johnson (NCI, Frederick, MD). The animals were housed in

pathogen-free units at the Vanderbilt University Medical Center, in compliance with IACUC regulations. We generated mice with myeloid-specific deletion of *Cebpa* by breeding *Cebpa*$^{flox/flox}$ mice to *LysMCre* mice, which express Cre recombinase under the control of murine lysozyme M promoter. F1 LysMCre$^{+/-}$;*Cebpa*$^{fl/+}$ were then crossed to produce the genotypes used in the experiments. Mice used were between 6 and 10 weeks of age. The studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center and complied with all relevant institutional and federal guidelines and policies.

*Tumor isograft model*

3LL cells (1 x $10^5$ cells), with or without Gr1+CD11b+ cells (1 x $10^4$ cells), were injected subcutaneously into the left flank of C57Bl/6 mice. The size of tumors was determined by measurement of tumor dimensions at 2–3 day intervals using calipers. The equation volume = length x $(width)^2$ x 0.5 was used to calculate tumor volume. Experiments were repeated at least three times.

*Statistical Analysis*

All data were averaged, analyzed using the Student's t test (one-sided; paired, equal variance) and were expressed as mean ± standard error across experiments.

# Results

*Gr-1+/CD11b+ cells from tumor-free C/EBPα conditional null mice promote tumor growth.*

Our finding that C/EBPa inhibits pro-angiogenic gene expression led us to wonder whether MDSCs isolated from tumor-free CN mice might also promote tumor growth. To that end, Gr-1+/CD11b+ cells were isolated from tumor-free C/EBPα conditional null mice by FACS. Since they were purified from normal mice, i.e. mice that did not have tumors, these Gr-1+/CD11b+ cells are not technically MDSCs [25]. Intriguingly however, when mixed with 3LL tumor cells (1:10) and injected into wild-type C57Bl/6 mice, they behaved liked MDSCs and tumor growth was significantly accelerated (Figure 14). This finding was obtained in a pilot experiment that must be repeated with control Gr-1+/CD11b+ cells from wild-type tumor free mice. With that caveat, this is the first instance to our knowledge of Gr-1+/CD11b+ cells from tumor-free mice phenotypically mimicking MDSCs. This data hints that C/EBPα may be key regulator of MDSCs biology.

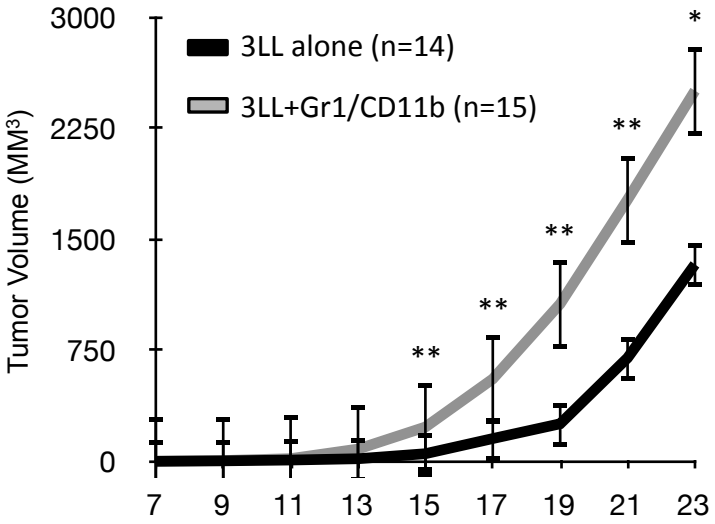


**Figure 14. Gr-1+/CD11b+ cells from tumor-free C/EBPα conditional null mice promote tumor growth.** FACS-purified Gr-1+/CD11b+ cells from the spleens of C/EBPα conditional null mice were mixed with 1x $10^5$ 3LL tumor cells (1:10) and injected s.c. into the flanks of C57BL/6 mice (3LL+Gr1/CD11b). C57Bl/6 mice were injected with 1x $10^5$ 3LL tumor cells as a control (3LL alone). Tumor dimensions were measured every 2-3 days with caliper and tumor volume was calculated and plotted with time. * $p < 0.05$, ** $p < 0.01$.

# CHAPTER V

# CONCLUSION

## Summary

In this work, we sought to elucidate the role of C/EBPα in regulating myeloid-derived suppressor cell expansion and the pro-tumor activities of these cells. In Chapter II, we present evidence that C/EBPα is a negative regulator of MDSC expansion through its ability to inhibit proliferation. Deletion of myeloid C/EBPα in mice yielded an increase in myeloid progenitors, accompanied by a reduction in circulating monocytes. Upon inoculation with tumor cells, splenic MDSC production was enhanced nearly two-fold in mice lacking C/EBPα in myeloid lineage cells.

In Chapter III, we give evidence that C/EBPα is a negative regulator of the pro-angiogenic properties of MDSCs. Conditional deletion of C/EBPα in myeloid lineage cells enhanced MDSC infiltration and tumor vascularization, resulting in markedly accelerated tumor growth in those mice. In an experiment where equal numbers of MDSCs were injected with tumor cells into mice, C/EBPα ablation resulted in an enhancement in the pro-tumor MDSC phenotype: tumor growth and tumor angiogenesis was significantly greater. In order to understand the mechanism behind our observation, we measured the expression genes involved in MDSC-mediated immune suppression and angiogenesis. We found that C/EBPα deletion resulted in upregulation of MMP-9, VEGF and iNOS expression,

as well as increased NO production. Additionally, overexpression of C/EBPα in myeloid cells caused a significant reduction in iNOS and VEGF expression. We did not, however, observe any difference in the ability to suppress T-cell proliferation. Given that NO can also regulate angiogenesis, we concluded that C/EBPα does not play a major role in MDSC-mediated immune suppression.

The process of MDSC production begins in the bone marrow, where tumor-produced or tumor-induced expansion signals initiate MDSC expansion. Many of the growth factors that normally induce myeloid progenitors to proliferate and mature, including granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF), also serve as expansion signals for MDSCs in the bone marrow and spleen. Inflammatory molecules and cytokines such as IL-1β, IL-6 and $PGE_2$ are also involved in MDSC expansion. These signals activate the JAK-STAT pathway and the STAT-3 transcription factor in particular. Interestingly, many of the receptors for these signals are C/EBPα target genes, including GM-CSFR, M-CSFR, G-CSFR and IL-6R, suggesting that C/EBPα initially may be required in the MDSC pathway.

Tumor and stromal cells also secrete chemokines that recruit MDSCS from the bone marrow, spleen and liver to the tumor. This process involves multiple pathways, including CCL2-CCR2 and SDF-1-CXCR4 interactions. TNFα is also involved in MDSC recruitment and our data suggests that it may participate in down-regulation of C/EBPα in the spleen and tumor. Once MDSCs are recruited and infiltrate into tumors, numerous signals within the tumor microenvironment

activate MDSCs. Hypoxia signaling through HIF-1α is indispensible for MDSC activation. Other signals for MDSC activation include interferon-γ, IL-1B and stem cell factor. The hypoxic and inflammatory microenvironment strongly inhibits C/EBPα expression, allowing for increased MDSC proliferation and resulting in the up-regulation of MMP-9, NO and VEGFA production.

The ability of MDSCs to promote tumor growth and metastasis has made uncovering the regulators of MDSC expansion and function a priority. While we have a good understanding of the molecules crucial for MDSC biology, much less is known about the negative regulation of MDSCs. A recent study determined that a micro-RNA, miR-223, suppresses accumulation of tumor-induced CD11b+Gr1+ MDSCs [90]. This coincides with the data presented here, as miR-223 is a known C/EBPα target gene; C/EBPα up-regulates miR-223 expression during granulocyte differentiation [91]. Another recent article identified interferon (IFN) regulatory factor 8 (IRF-8) as a potential inhibitor of MDSC production and demonstrated that modulation of IRF-8 levels in tumor-induced CD11b+Gr-1+ cells can significantly abrogate their pro-tumorigenic behavior [92]. IRF-8 is a hematopoietic transcription factor that facilitates myeloid differentiation [93]. Interestingly, forced IRF-8 expression in a model of CML, BCR/ABL-transformed myeloid cells, specifically restored C/EBPα expression [94]. To our knowledge, this is the only report linking IRF-8 and C/EBPα expression and more work is needed to determine the specific role of these transcription factors in inhibiting MDSC expansion and activation.

A recent study indicated a critical role for C/EBPβ in the immune suppressive functions of MDSCs [29]. Marigo et al. provided evidence that hematopoietic deletion of C/EBPβ, a member of the same family of proteins as C/EBPα, led to a decrease in the number of MDSCs and a loss in the tolerance promoting activities of MDSCs, accompanied by a difference in the differentiation of myeloid cells [95]. Additionally, C/EBPδ regulates VEGFC signaling in lymphangiogenesis through HIF-1α [96]. These findings, along with our findings, point to important roles for this family of transcription factors in MDSC biology.

An emerging model proposes that a shift from 'steady-state' hematopoiesis driven by transcription factors such as C/EBPα to transcription factors that promote inflammatory 'emergency granulopoiesis,' including C/EBPβ is necessary for MDSC maturation [97]. C/EBPα appears to function as an inhibitor of this process. Notably, C/EBPα activates the protease that cleaves full-length C/EBPβ to generate LIP (liver-enriched transcriptional inhibitory protein), a dominant-negative transcription inhibiting isoform [98]. Therefore, tumor-induced down-regulation of C/EBPα may be a required step in MDSC maturation and/or activation. This would simultaneously release the brakes on proliferation and activate angiogenesis-inducing VEGF and NO production. Additionally, reduced LIP conversion allows the active isoform of C/EBPβ critical for immune suppression to predominate.

Therapeutic targeting of MDSCs in cancer and other pathological conditions is an area of intense and ongoing research. Recently proposed strategies include promoting myeloid-cell differentiation, inhibiting MDSC expansion, inhibiting

MDSC function and elimination of MDSCs [99]. Our work suggests that restoring C/EBPα expression in MDSCs may promote their differentiation while inhibiting MDSC expansion and function. The validity of this approach has already been demonstrated in the myeloid leukemia field, where reintroduction of C/EBPα in CD34+ leukemic cells impaired their self-renewal capacity and enhanced myeloid differentiation [100]. More promising is a recent report that nanoparticles were used to efficiently deliver C/EBPα to mesynchymal stem cells [101]. If successful, restoring C/EBPα expression could lead to fewer MDSCs in the tumor microenvironment due decreased MDSC proliferation. It could also result in MDSC differentiation into mature, functional immune cells able to recognize and destroy the tumor. Furthermore, it might also result in increased LIP generation and decreased C/EBPβ activity. When C/EBPβ was conditionally deleted in the hematopoietic system, the percentage of MDSCs was decreased [102]. This was accompanied by reduced arginase and NO production and the inability to suppress immune responses. Thus, it may be possible to reduce MDSC-mediated immune suppression and angiogenesis, while increasing immune surveillance by reactivating or re-expressing C/EBPα in MDSCs.

Taken together, our findings reveal dual negative roles for C/EBPα in the expansion and pro-angiogenic gene expression in MDSCs. Our work suggests that therapy aimed at restoring C/EBPa expression in MDSCs may be promising and effective in the treatment of solid tumors.

## Future Directions

Our finding that C/EBPa inhibits pro-angiogenic gene expression led us to wonder whether Gr-1+/CD11b+ isolated from tumor-free C/EBPα conditional null mice might also promote tumor growth. To that end, wild-type C57Bl/6 mice were injected with either Gr-1+/CD11b+ cells isolated from tumor-free C/EBPα conditional null mice mixed with 3LL tumor cells (1:10) or tumor cells alone. Intriguingly, we found that Gr-1+/CD11b+ cells from tumor-free CN mice, when mixed with tumor cells, promote tumor growth and tumor growth was significantly accelerated in those mice. Thus, conditionally deleting C/EBPα in myeloid cells from tumor-free mice produces Gr-1+/CD11b+ cells that phenotypically mimic MDSCs in tumor-bearing mice.

Our preliminary data suggests that C/EBPα may be a key regulator of MDSC biology. However, this finding was obtained in a pilot experiment and must be confirmed with control Gr-1+/CD11b+ cells from wild-type tumor-free mice. If C/EBPα is a key regulator of MDSC biology, it is expected that conditional C/EBPα deletion in Gr-1+/CD11b+ cells might phenocopy the pro-tumor activities of MDSCs. To confirm this, tumor size and growth rate could be compared between wild-type mice injected with tumor cells alone, tumor cells mixed with Gr-1+/CD11b+ cells from tumor-free wild-type or C/EBPα conditional null mice, and tumor cells mixed with MDSCs from tumor-bearing wild-type or C/EBPα conditional null mice. The number and percentage of MDSCs in tumor tissues could be measured with flow cytometry and compared between the experimental

groups. It would be particularly interesting to compare the tumor-promoting phenotype of Gr-1+/CD11b+ cells from tumor-free C/EBPα conditional null mice with that of MDSCs from wild-type and null tumor-bearing mice. Additionally, MDSC infiltration and vascular density could be measured in tumor sections from those mice to determine if Gr-1+/CD11b+ cells from C/EBPα null mice phenocopy the ability of MDSCs to infiltrate into tumors and promote tumor angiogenesis. Finally iNOS, MMP-9 and VEGF expression could be measured by real-time PCR in Gr-1+/CD11b+ cells isolated from C/EBPα null mice and compared with MDSCs from tumor-bearing mice.

We hypothesize that the mechanism behind the ability of Gr-1+/CD11b+ cells to promote tumor growth is similar to that of MDSCs lacking C/EBPα, namely the capacity of C/EBPα to inhibit MDSC expansion and negatively regulate MMP-9 and VEGF expression in MDSCs. As in MDSCs with C/EBPα ablation, Gr-1+/CD11b+ cells with reduced C/EBPα expression are expected to have increased proliferative capacity and greater expression of MMP-9 and VEGF. To test this, one could measure and compare BrdU incorporation between MDSCs from wild-type and C/EBPα null tumor bearing and tumor-free mice. We could also compare MMP-9 activity by zymography and assay the net effect of MMP-9 and VEGF expression by performing a VEGF ELISA on tumor tissues from wild-type and C/EBPα null mice.

To determine whether increased VEGF bioavailability in the C/EBPα null tumors is responsible for increased tumor growth, *in vitro* angiogenic assays could be performed with a VEGF inhibitor. MDSCs isolated from tumor tissues of

wild type or C/EBPα null mice are cultivated overnight. Endothelial cells, (HUVECs) are cultivated in the conditioned media from the MDSCs with or without a VEGF inhibitor or neutralizing antibody on Matrigel, allowing vascular tube structures to form. Branch points are counted at various intervals. One could also perform Boyden chamber migration assays to assess the ability of endothelial cells to migrate towards wild type and null MDSCs. For this assay, MDSCs are isolated from the tumor tissues of wild type and C/EBPα myeloid null mice and cultivated overnight. Then, HUVECs are placed in the top of a Boyden chamber with or without a VEGF inhibitor or neutralizing antibody and allowed to migrate toward the bottom chamber containing the MDSCs for 3.5 hours.

Recent studies have reported that C/EBPα expression is reduced in a number of human cancers [78,103-109]. Additionally, mutations in the C/EBPα gene and disruptions in its function have been observed in a majority of acute myeloid leukemias [43,110-112], a cancer of myeloid lineage blood cells characterized by the aberrant accumulation of immature myeloid cells. Loss of C/EBPα function in myeloid progenitor cells results in a block of differentiation and deregulated proliferation, which are characteristic features of AML [113]. The similarity between AML cells and MDSCs and the observation of C/EBPα down-regulation in human cancers suggests that C/EBPα expression may be reduced in MDSCs from human cancer patients. Humans lack the Gr-1 gene, making MDSCs more difficult to define phenotypically human cancer patients. As a result, studies have reported many different markers for human MDSCs. However, the consensus is that human MDSCs express the myeloid markers CD11b and CD33 with no or

minimal expression of myeloid maturation markers [27]. It would be interesting to measure C/EBPα expression and protein levels in MDSCs purified from the peripheral blood of human cancer patients and compare it to tumor-free individuals.

Given that C/EBPα is a transcription factor, it would be interesting to determine if it regulates the expression of known MDSC genes/cytokines. This could be investigated with 2 approaches. First, a cytokine array could be performed comparing MDSCs isolated from WT and null tumor bearing mice and the results could be confirmed with real-time PCR. Additionally, C/EBPα is regulated by and/or regulates the expression of a number of genes that have been demonstrated to play a role in MDSC biology, including IL-23, IRF-8, NF-κB, STAT-1, STAT-3 and TGF-β. The expression of these genes could be measured by real-time PCR and compared between MDSCs from wild-type and conditional null mice. C/EBPα could also be overexpressed in myeloid cell lines and gene expression measured and compared to vector control. This approach may even be possible in MDSCs as technology allowing their isolation and culture improves.

## Final remarks

In conclusion, this body of work established the important role of C/EBPα in MDSC biology and cancer growth and progression. It identified C/EBPα as a negative regulator of both MDSC expansion and MDSC function. C/EBPα suppresses MDSC production by inhibiting proliferation in myeloid progenitors and MDSCs. As a transcription factor, it inhibits pro-angiogenic gene expression in MDSCs. Therapeutic activation of this pathway in cancer could promote MDSC differentiation and decreased angiogenesis, resulting in delayed or halted tumor progression.

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