Tumors and Ly6Chigh Monocytes

Fatemeh Zare

PhD Programme in Biomedicine

Programa de Doctorado en Biomedicina

Tumors and Ly6C^{high} Monocytes

Tumores y Monocitos Ly6C^{high}

Thesis submitted by - Memoria presentada por

Fatemeh Zare

To qualify for the Doctorate degree by - Para optar al grado de Doctor por la

University of Barcelona - Universidad de Barcelona

Departamento de Fisiologia y Inmunologia
Facultat de Biologia
Universidad de Barcelona

Programa de Doctorado de Biomedicina
Bienio 2014-2015

Memoria presentada por
Fatemeh Zare
Para optar al Grado de Doctora por la
Universidad de Barcelona

Director de Tesis
Dr. Antonio Celada Cotarelo
Catedratico de Inmunologia

Barcelona, Octubre de 2014

ACKNOWLEDGMENTS

My PhD has been a lengthy journey and many people have helped me along the way. Firstly I would like to acknowledge my supervisor for all his help and support throughout this time. Prof. Antonio Celada, thank you for encouraging me to work independently as a scientist and for providing me the opportunity to be a part of your group. Prof Roger Gomis, given that for you I was an external student, I wish to especially thank you for your constant support. Dr. Jordi Lloberas, thank you for your wonderful problem solving skills, especially under pressure, during those times when I showed up at your office door with a million questions. My gratitude also goes to Dr. Jaume Comas for teaching me flow cytometry, and developing the cell sorting protocol. I would like to extend my appreciation to all the members of the Macrophage Biology, both past and present, for their technical support and encouragement. Thank you to Juan A. calatayud for our rewarding collaboration and for the many discussions about our work. A million thanks to Dr. Gloria Sans, Dr. Selma Kuznetsova, Lorena V Strella, Juan Torres and Ester Ruiz that I could count on your help always. Many thanks to our lab technician Gemma López for her unending patience and help around the laboratory. I would like to thank my family and friends for their love and support during my studies. I am eternally grateful to my wonderful parents and sister who have always encouraged me in everything and who have been there not only for all the highs , but importantly for all the lows too. Without you I would never have gotten this far. This is for you.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

RESULTS

List of Tables

INTRODUCTION

RESULTS

ABBREVIATIONS

AP-1, activator protein 1

APC, antigen presenting cell

BMDCs, bone marrow-derived cells

BRCA, breast cancer susceptibility protein

CAT2, cationic amino acid transporter 2

CCL, CC-chemokine ligand

CCL, chemokines

CD, cluster of differentiation

CD62L, Cluster of Differentiation 62 ligand

CFU-GM, granulocyte-monocyte colony forming unit

cGAMP, cyclic-GMP-AMP

cGAS, cyclic-GMP-AMP synthase

CMP, common myeloid progenitor

COX-2, Cyclooxygenase-2

CpG, cytosine phosphodiester guanine

CRI, Cancer Related Inflammation

Csf1R, colony stimulating factor 1 receptor

CXCL, C-X-C motif chemokine ligand

CXCR, C-X-C chemokine receptor

DCs, dendritic cells

DNase, deoxyribonuclease

EAE, experimental autoimmune encephalomyelitis

FACS, subsequent fluorescence-activated cell sorting

Fas receptor, apoptosis antigen 1

Fc receptors, fragment crystallisable receptor

Fc-gamma receptors I (FcγR),

G-CFU, granulocyte colony-forming unit

G-CSF, granulocyte colony-stimulating factor

Glu, glutamic acid

GM-CFU, granulocyte/macrophage colony-forming unit

GM-CSF, granulocyte-macrophage colony-stimulating factor

GMP, granulocyte, macrophage progenitor

GPI, glycosylphosphatidylinositol

HIF-1, Hypoxia-inducible factor 1

HR, homologous recombination

HSC, haematopoietic stem cell

HSC, hematopoietic stem

HSC: hematopoietic stem cell

IDO, indoleamine 2,3-dioxygenase

IFI, interferon gamma-inducible protein

IFN, interferon

IFNGR, interferon-gamma receptor

IFNαR, interferon-α receptor

IL, interleukin

iNOS, inducible nitric oxide

IRF, interferon regulatory factor

IRF3, Interferon regulatory factor 3

ITAM, immunoreceptor tyrosine-based activation motifs

JAK1, janus-associated kinase 1

KO, knock-out

LN, lymph node

LPS, lipopolysaccharide

LY6C, lymphocyte antigen 6 complex, locus C

MACS, multistep magnetic-activated cell sorting

MAPK, mitogen-activated protein kinases

M-CFU, macrophage colony-forming unit

MCP-1, monocyte chemotactic protein 1

M-CSF, macrophage colony-stimulating factor

MDP, macrophage, DC progenitor

MDSCs, myeloid-derived suppressor cells

MHC, major histocompatibility complex

M-MDSC, mature myeloid-derived suppressor cells

MMPs, matrix metallopeptidase

MRC1, macrophage mannose receptor 1

mRNA, messenger ribonucleic acid

MSCs, mesenchymal stem cells

MyD88, myeloid differentiation primary response gene 88

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NK, natural killers

NKG2D, Killer cell lectin-like receptor subfamily K, member 1

NO, nitric oxide

NOS2, nitric oxide synthase

PBMCs, peripheral blood mononuclear cells

PDGF, platelet-derived growth factor

PG, prostaglandin

PMN-MDSCs, polymorphonuclear myeloid-derived suppressor cells

RANTES, regulated upon activation normal T cell expressed and presumably secreted

RNAse, ribonuclease

RNS, reactive nitrogen species

ROS, reactive oxygen species

STAT, signal transducer and activator of transcription

TAM, tumor-associated macrophage

TAMs, tumor associated macrophages

TANK, TRAF family member-associated NFKB activator

TBK1, TANK-binding kinase 1

TC-NER, transcription-coupled NER

TEMs, TIE2-expressing monocytes

TFEB, mammalian transcription factor EB

TGF-β, transforming growth factor beta

Th1, T helper cell 1

Th2, T helper cell 2

TLR, toll-like receptor

TNF, tumor necrosis factor

TNF-α, tumor necrosis factor-α

TRAIL, TNF-related apoptosis-inducing ligand

TRIF, TIR-domain-containing adapter-inducing interferon

VEGF, vascular endothelial growth factor

βFGF, basic fibroblast growth factor

1 INTRODUCTION

Introduction

1.1 Cancer

Cancer is a disease produced by a group of cells that multiply without control and in an autonomous manner, invade locally and at distance to other tissues. There are more than 200 types of cancers and the most common ones are the lung, breast and colorectal. Cancers arise in a multistep process that reflects dysregulation of oncogenes, tumor suppression and pro-apoptotic signals. Central to the development of cancer are genetic changes that endow these “cancer cells” with many of the hallmarks of cancer, such as self-sufficient growth and resistance to anti-growth and pro-death signals. Tumor promotion and progression are dependent on ancillary processes provided by cells of the tumor environment but that are not necessarily cancerous themselves.

Inflammation has long been associated with the development of cancer. In the last century, the question of whether the immune system positively or negatively controls the cancer progression has been a matter of debate. Accumulating data state that one of the physiologic functions of the immune system is to recognize and destroy transformed cells. However, some tumor cells are capable of evading recognition and destruction by the immune system. This review will discuss the reflexive relationship between cancer and inflammation with particular focus on how considering the role of $Ly6C^{high}$ monocytes may provide a logical framework for understanding the connection between the inflammatory response and cancer.

1.1.1 Cancer and the Immune System

To growth, cancer induces an inflammation and therefore is considered as an inflammatory disease. The types of cells of the immune system that are found infiltrating human malignancy are varied and consist of cells of the innate immune system (eg, macrophage, neutrophils, etc), as well as cells associated with an adaptive immune response (eg, T and B cells). Cancer-associated inflammation encompasses a number of components and processes that include the production of cytokines and chemokines, tissue remodeling, angiogenesis and infiltration of leukocytes.

The origins and progress of cancer immunology have been reviewed in depth, highlighting the development of ideas from Ehrlich and Medawar through to the cancer immune surveillance hypothesis of Burnet and into the era of cellular and molecular immunology (Blair and Cook, 2008). The goal behind many immunotherapeutic strategies is to tip the balance from tumor immune evasion to a productive anti-tumor response. Studies of the role of the cellular immune system in controlling cancer cells, promise to deliver not only fascinating insights into the immune system but also lay the foundation for future cellular immunotherapies.

1.1.2 Tumor Immunology

Tumor immunology is the study of interactions between the immune system and cancer cells (also called tumors or malignancies). It is also a growing field of research that aims to discover innovative cancer immunotherapies to treat and retard progression of this disease. An important role of the immune system is to identify and eliminate tumors. The main response of the immune system to tumors is to destroy the abnormal cells using killer T cells, sometimes with the assistance of helper T cells. Tumor antigens are presented on major histocompatibility complex class I (MHC I)

molecules of dendritic cells (DCs) and macrophages in a similar way to viral antigens (Kalupahana et al., 2005). This allows killer T cells to recognize the tumor cell as abnormal. Natural Killer (NK) cells also kill tumorous cells in a similar way, especially if the tumor cells have fewer MHC class I molecules on their surface than normal; this is a common phenomenon with tumors (Wang et al., 2011). Clearly, some tumors evade the immune system and go on to become cancers. Due to their process of dys-differentiation, tumor cells often have a reduced number of MHC class I molecules on their surface, thus avoiding detection by killer T cells (Wang et al., 2011). Some tumor cells also release products that inhibit the immune response.

1.1.3 Cancer Related Inflammation (CRI)

The association between cancer and inflammation dates back to Rudolf Virchow (1863) when he noticed the presence of leukocytes in neoplastic tissues (Balkwill and Mantovani, 2001). Several studies have identified two main pathways linking inflammation and cancer: one intrinsic and another extrinsic (Coussens and Werb, 2002). The first one includes genetic alterations that lead to inflammation and carcinogenesis, whereas the second one is characterized by microbial/viral infections or autoimmune diseases that trigger chronic inflammation in tissues associated with cancer development. Both pathways activate pivotal transcription factors of inflammatory mediators (e.g., nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription3 (STAT3), and Hypoxia-inducible factor1 (HIF-1)) and inflammatory cells (Hagemann et al., 2008; Grivennikov et al., 2010; Karin, 2009).

Inflammatory cells like DCs, macrophages, neutrophils etc. that are present in the tumor microenvironment either contribute to tumor progression or actively interfere with its development. It is now clear that the former takes precedence, largely because the tumor generally proceeds to establish

mechanisms responsible for its 'immune evasion' or escape from the immune intervention (Hanahan and Weinberg, 2011). Chronic inflammatory conditions have been observed in association with tumor incidence, tumor progression and detrimental prognosis in human cancer patients. It is still early to understand the molecular mechanisms of how and why tumors occur more frequently in an inflammatory microenvironment or in an inflammation-plagued host. The pro-inflammatory cytokines are found at the crossroad of this deregulation. Several of these cytokines are highly expressed in human cancers and alter the immune response in ways that are simultaneously beneficial to tumor growth (Lin and Karin, 2007). It is tempting to speculate that the observed derailing of antitumor immunity into an inflammatory response is at its core, a defensive strategy of the tumor, selected for independently of the tumor cell transformation. Alternatively, it might be the mere result of, and the default reaction to, the expression of transforming oncogenes within the tumor cell. The presence of mutant cell clones in an inflamed and regenerating tissue could simply be an unfortunate coincidence. Here, the tumor cell would take advantage of the improved cytokine mediated growth conditions for the nascent tumor, whereas the same cytokines inhibit the immune-mediated tumor surveillance and tumor cell elimination (Dunn et al., 2002).

Recent research has highlighted an important role for inflammation in cancer from the perspective that innate immune cells, such as macrophages, drive malignant progression through the production of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 (Rakoff-Nahoum, 2006).

1.2 Innate and adaptive immune response to tumors

Immune responses and inflammation are generally advantageous for the host, and may include suppressing growth of smaller tumors. Interestingly

however, inflammation can also promote neoplastic transformation and tumor progression.

Innate immunity represents the first line of defense of the body that recognizes immunogenic proteins, which are called antigens not present in the body. Adaptive immunity is a specific response to a particular tumor-associated antigen. Both innate and adaptive immune cells orchestrate an inflammatory environment that may function to either stimulate or inhibit cancer growth (see Table 1-1) (Lu et al., 2010). It is suggested that the inflammatory response found in many cancers is one of chronic inflammation, resulting in an environment rich in innate immune cells.

Stimulate Cancer Growth	Inhibit Cancer Growth
Innate immune cells	
Neutrophils	Dendritic cells
Macrophages (M2)	Macrophages (M1)
Myeloid-derived suppresor cells	
Adaptive immune cells	
Th2 CD4+ T cells	Cytotoxic CD8+ T cells
CD4+ T regulatory cells	Th1 CD4+ T cells
B lymphocytes	Th17 CD4+ T cells

Table 1-1. Innate and adaptive immune cells involved in regulating tumor growth (Lu et al., 2010).

The immune system works by discriminating self from non-self. Non-self is discriminated from self by fundamental differences in biochemistry, such as amino-acid differences, as well as the arrangement of carbohydrate residues on glycoproteins or the absence of methylated cytosine residues in DNA. These differences are detected by the numerous pattern receptors,

which are a hallmark of the innate immune system (O'Neill, 2008), including the Toll-like receptors. The activation of innate immunity leads to the efficient priming of adaptive immune responses mediated by B and T cells. These cells carry antigen receptors and, through education and cooperation, can distinguish self from non-self-antigen and trigger subsequent events. However, tumor cells are self in origin and their biochemistry and behavior differs only subtly from their healthy counterparts and thus, requires the detection of altered self. There is now a substantial body of data to show that innate and acquired immune responses to tumors do exist and that a multitude of immune cell types and their associated molecules are involved in detecting and eliminating tumors (Gajewski et al., 2006, 2013; Lowe et al., 2010).

The initiation of the anti-tumor immune response occurs when cells of the innate immune system become alerted of the presence of the growing tumor. Local tissue disruption, as a result of increased angiogenesis or tissue-invasive growth, induces pro-inflammatory molecules such as IL-1, tumor necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) or (IL-15), leading to the recruitment of innate immune cells (Coussens and Werb, 2002; Newton and Dixit, 2012). Dendritic cells may act as sentinel cells sensing tissue stress, damage and transformation. Their ability to uptake heat shock proteins and products of extracellular matrix breakdown promotes differentiation and subsequent cross-talk between DCs and NK cells. These last cells, macrophages, T cells and NK T cells are recruited to the site of "danger" and recognize molecules that can be induced on tumor cells upon cellular transformation, such as stress induced ligands for the activating receptor NKG2D (Smyth et al., 2005). In addition, MHC-tumor-associated peptide complexes or glycolipid-CD1 complexes expressed on developing tumor cells can be recognized by T cells and NKT cells, respectively (Smyth et al., 2002). These immune cells exert several effector mechanisms to eliminate tumor cells including the release of interferon γ (IFN-γ) and perforin or the expression of death receptor ligands such as TNF-

related apoptosis-inducing ligand (TRAIL). Secretion of IFN-γ controls tumor growth and amplifies immune responses through the production of chemokines. In return, chemokines attract more immune cells to the tumor site generating a positive feedback loop for tumor elimination. In the next phase tumor antigens released during tumor cell killing drive the development of tumor-specific adaptive immune responses. Recruited immature DCs become activated through the tumor cytokine milieu and uptake of tumor antigens and migrate to the lymph nodes, where they induce the activation of naïve tumor-specific $CD4^+$ and $CD8^+$ T cells (Sallusto et al., 2000). Once activated, tumor-specific T cells home to the tumor site to eliminate antigen-expressing tumor cells.

Most likely, tumors circumvent either one or both arms of the immune system by employing multiple immune-evasive strategies. Several studies documented that tumors can either directly (tumor intrinsic mechanism) or indirectly (tumor extrinsic mechanism) impede anti-tumor immune responses. Tumor intrinsic mechanisms include: (a) modified expression level of MHC class I/II and co-stimulatory molecules (Marincola, 2000), (b) dysregulation of antigen processing (Seliger et al., 2000) and (c) low levels of tumor antigen expression (Spiotto et al., 2004). Indirect mechanisms are associated with: (a) tolerance of T cells to tumor-specific antigens resulting from anergy (Gajewski et al., 2006), (b) suppression of immune effector cells via immunosuppressive cytokines such as transforming growth factor beta (TGF-β) or interleukin 10 (IL-10) or via regulatory cells (Gabrilovich et al., 1998) and (c) secretion of soluble ligands that block lymphocyte activation (Groh et al., 2002).

1.3 Immune cells and anti-cancer therapies

Understanding the complexity of immunomodulation by tumors is important for the development of immunotherapy. Several articles in this focus

concentrate on how different immune cell populations function in, and are shaped by, the tumor micro-environment. Various strategies are being developed to enhance anti-tumor immune responses to overcome 'immune checkpoints'. Existing therapies are also being investigated for immune cell populations ability to induce an anti-tumor immune response, which could lead to the administration of combination immunotherapies that provide a more efficacious and enduring response. However, there are issues that remain to be understood. In particular, it is clear that there is variability in the ability of a tumor to induce an immune response and hence there is debate about the determinants of tumor immunogenicity. It will be important to resolve these issues in order to predict or modulate responses to immunotherapies.

Myeloid cells, such as dendritic cells (DCs) and macrophages, can drive potent anti-tumor immune responses. Gabrilovich and colleagues discuss how tumor cells inhibit the beneficial tumor suppressive effects of the myeloid system and instead promote the development of immunosuppressive myeloid cells, which favors tumor growth (Gabrilovich and Nagaraj, 2009). Restifo and colleagues focus on how effector T cells detect and destroy tumor cells (Restifo et al., 2012). *In vitro* proliferation and reinfusion of a patient's own tumor specific T cells has already shown success in treating some types of cancer. The type and distribution of immune cells and of the cytokines and chemokines that regulate them within the tumor microenvironment can determine the extent of immunomodulation and hence the prognosis of patients with cancer.

Macrophages have also been used to enhance the immune response or to potentiate chemotherapy specificity. Also, the delicate balance between M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages is a fundamental aspect in anti-cancer treatment. Several studies have shown that the activation of toll-like receptors (TLRs); for instance, TLR9, stimulates M1-polarized macrophage responses by inducing the activation of a pro-inflammatory program (Krieg, 2006). In general, the restoration of an M1

phenotype in tumor associated macrophages (TAMs) may provide a therapeutic benefit by promoting antitumor activities.

1.4 Cells in the tumor microenvironment

The tumor mass is undoubtedly a multifaceted show, where different cell types, including proliferating neoplastic cells, extracellular matrix produced by fibroblasts, a vascular network of endothelial cells, and immune-competent cells, interact with one to another continuously (Gajewski et al., 2013; Movahedi et al., 2010a). The composition and characteristics of the tumor microenvironment vary widely and is important in determining the anti-tumor immune response. For example, certain cells of the immune system, including natural killer cells, DCs and effector T cells, are capable of driving potent anti-tumor responses. A tissue microenvironment of developing tumor is comprised of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells (**Error! eference source not found.**) (Joyce and Pollard, 2009). It is a unique environment that emerges in the course of tumor progression as a result of its interactions with the host. It is created by and at all times shaped and dominated by the tumor, which orchestrates molecular and cellular events taking place in surrounding tissues. Immune cells present in the tumor include those mediating adaptive immunity, T-lymphocytes, DC and occasional B cells, as well as effectors of innate immunity, macrophages, polymorphonuclear leukocytes and rare NK cells (Whiteside, 2007).

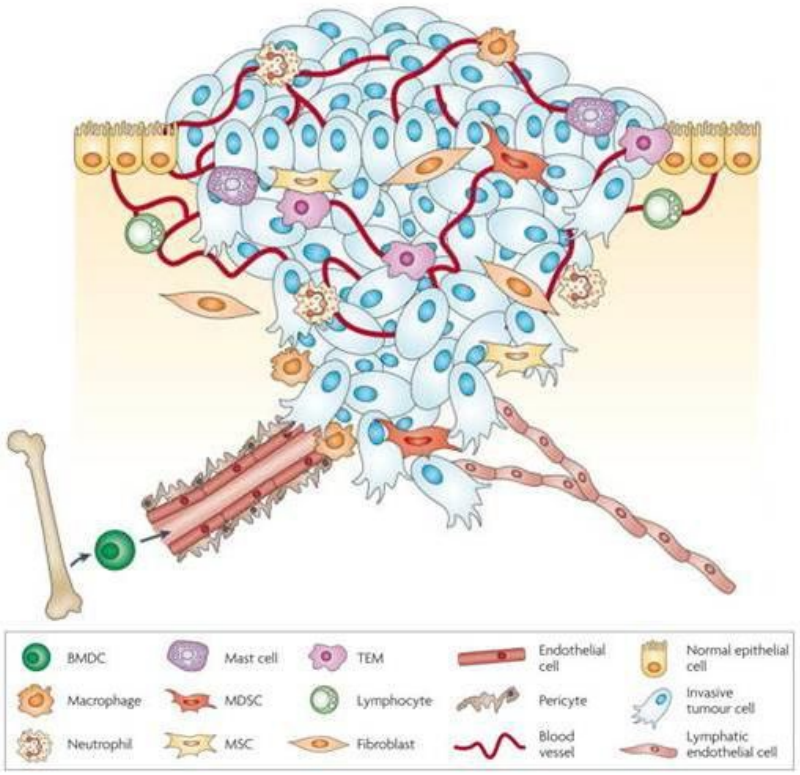

Figure 1-1. Tumor microenvironment. Complex microenvironment of primary tumors comprising numerous cells including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts and a variety of bone marrow-derived cells (BMDCs) including macrophages, myeloid-derived suppressor cells (MDSCs), TIE2-expressing monocytes (TEMs) and mesenchymal stem cells (MSCs) (Joyce and Pollard, 2009).

1.4.1 Inflammatory cell component of tumors

Tumor cells produce various cytokines and chemokines that attract leukocytes. The inflammatory component of a developing neoplasm may

include a diverse leukocyte population -for example, neutrophils, DCs, macrophages, eosinophils and mast cells, as well as lymphocytes- all of which are capable of producing an assorted array of cytokines, cytotoxic mediators including reactive oxygen species, serine and cysteine proteases, Matrix metallopeptidase 9 (MMPs) and membrane-perforating agents, and soluble mediators of cell killing, such as TNF-α, interleukins and interferons (IFNs).

1.4.2 Tumor-associated leukocytes

Tumor-associated leukocytes include macrophages, DCs, NK cells, neutrophils and mast cells. However, the major tumor-associated leukocytes present in tumors are macrophages, which in certain cases may account for as much as 50% of the tumor mass. Macrophage infiltration began very early during the pre-invasive stage of disease and increased progressively (Lin et al., 2001). There is increasing evidence that tumor associated macrophages (TAMs) express an immunosuppressive phenotype and display several pro-tumoral functions, including promotion of angiogenesis and matrix remodeling (Balkwill et al., 2005; Pollard, 2004). Although usually rare, DCs have been detected in several tumor types, but in tumors, these cells have been shown to express an immature phenotype and therefore to have low immune-stimulatory properties (Mantovani et al., 2002). Both DCs and macrophages have the ability to pick up tumor antigens for cross-presentation on MHC class I molecules (Ardavín et al., 2004). However, the phenotype of TAMs and intra-tumoral DCs has been suggested to promote tolerance through production of immune-suppressive factors rather than prime a protective immune response (Mantovani et al., 2002).

1.4.3 Macrophages

Macrophages are pleiotropic cells that participate in innate immune responses as well as initiating the acquired immune response. Macrophages were discovered in 1880 by Elie Metchnikoff (Tauber, 2003). These cells have multiple functions in other systems and control through the release of cytokines and other factors many different cells (Celada and Nathan, 1994). Macrophages are the terminally differentiated cell type and are made up of a number of subpopulations that are defined by anatomical location and phenotype, including specialized macrophages such as osteoclasts (bone), alveolar macrophages (lung), Kupffer cells (liver), marginal zone macrophages (spleen) and microglia (brain). These tissue macrophages seem to be derived from *in situ* stem cells. Also macrophages are generated in the bone marrow from the stem cells through a series of differentiations, which includes the monoblasts, promonoblasts as well as the monocytes (Hettinger et al., 2013). These cells in the blood survey any immunological problem and migrate to the inflammatory loci. The literature is replete with evidence of macrophage associated with different tumors in both, mice and humans (Ruffell et al., 2010; Steidl et al., 2010).

1.4.3.1 Tumor associated macrophages

TAMs are a significant component of inflammatory infiltrates in neoplastic tissues and are originated from blood monocytes that are recruited to the tumor site by several factors including macrophage colony-stimulating factor (M-CSF), GM-CSF, IL-3 and chemokines like CCL2. TAMs are one of the most important players in the inflammation and cancer arena and an important source of cytokines (Mantovani et al., 2008).

A better understanding of TAMs and other myeloid-derived tumor-infiltrating cells as pivotal players in the tumor microenvironment and as

sources of CRI could certainly shed new light on the mechanistic understanding and development of efficient anticancer therapies (Montovani et al., 2008).

1.4.3.1.1 TAMs phenotype

TAMs are phenotypically flexible and in response to different tumor micro-environmental signals they can switch from one functional phenotype to another (Hagemann et al., 2008; Stout et al., 2005).

Macrophages can be classified into M1 (pro-inflammatory) and M2 (anti-inflammatory) types (Grivennikov et al., 2010) M1 or “classically activated” macrophages, activated *in vitro* by IFN-γ and microbial products, express high levels of pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-12 or IL-23), major histocompatibility complex (MHC) molecules and inducible nitric oxide synthase (NOS2) and therefore activate type I T cell responses and are capable of killing pathogens and priming anti-tumor immune responses. By contrast, M2 or “alternatively” activated macrophages, which are induced *in vitro* by IL-4, IL-10 and IL-13, possess poor antigen presenting capacity, down-regulate MHC class II and IL-12 expression and show increased expression of the anti-inflammatory cytokine IL-10, scavenger receptor A, and arginase. Most TAMs are considered to have an M2 phenotype while suppressing inflammatory responses and Th1 adaptive immunity, scavenge debris and promoting tumor angiogenesis and tissue remodeling (Sica et al., 2008). However, most confirmed tumor-promoting cytokines are “M1 cytokines”, whereas IL-10, an M2 cytokines, may be tumor suppressive as shown in colorectal cancer (Lin and Karin, 2007). Furthermore, unlike T helper cell 1 (Th1) and Th2 cells, M1 and M2 macrophages are cells with high plasticity and their phenotype is defined by their gene expression prolife rather than by deterministic differentiation pathways and lineage choices. M2

macrophages appear to contribute to immune suppression through the production of IL-10 and TGF-β (Gajewski et al., 2013) (Figure 1-2).

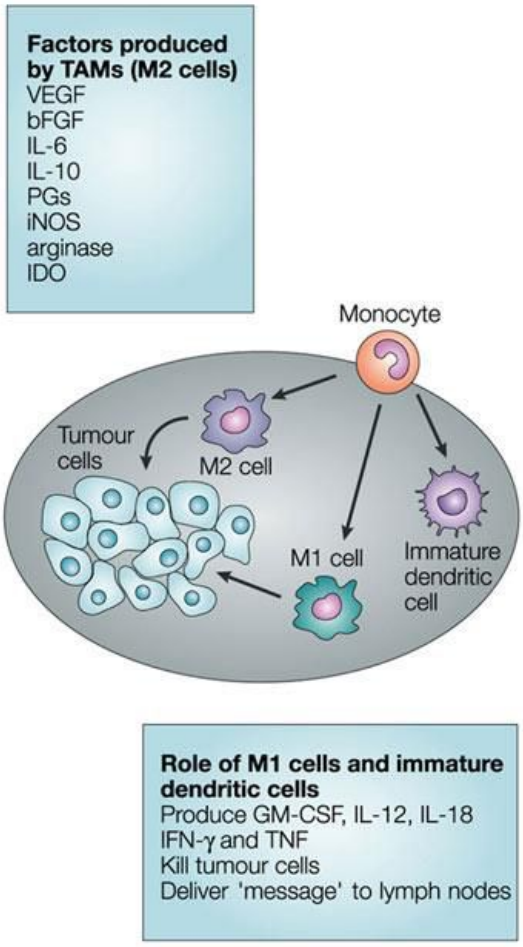

Figure 1-2 TAMS phenotype. Recruitment of blood-derived monocytes to tumor tissue depending on the present cytokines (colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) or IL-13) leads to differentiate either into macrophages or immature dendritic cells. Tumor macrophages can differentiate into M1 or M2 cells, which differ in their patterns of cytokine secretion and their functions. βFGF, basic fibroblast growth factor; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; iNOS, inducible nitric oxide; LN, lymph node; PG, prostaglandin; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor (Vakkila and Lotze, 2004).

In cancer, macrophage can either prevent the establishment and spread of cancer (pro-inflammatory) or support tumor growth (anti-inflammatory). These polar phenotypes are not expressed simultaneously but are regulated through decisive functional programs in response to different micro-environmental signals (Sica et al., 2008). It is generally accepted that TAMs resemble a modified M2 phenotype and their functional polarization is a result of the absence of M1 signals in the tumor like IFN-γ or bacterial components. Furthermore, M2 polarizing factors are highly presented and support their differentiation towards an IL-12^{low} IL-10^{high} phenotype. IL-10 secretion by TAMs promotes monocyte differentiation into macrophages and therefore avoids a differentiation to DCs (Allavena et al., 1998). An established gradient of IL-10 also influence incoming monocytes by their localization within the tumor. Sica and colleges observed that murine TAMs displayed a defective NF-κB activation in response to M1 signals confirming their low production of inflammatory cytokines (IL-12, IL-1β, TNF-α and IL-6) (Sica et al., 2000). Moreover, TAMs were poor producers of NO due to their low expression of NOS2 and secrete only low levels of reactive oxugen species (ROS) (Klimp et al., 2001). Genetic profiling of TAMs revealed an up-regulation of several M2-associated genes such as CD162, C-type lectin domains and heat shock proteins, therefore supporting their role as M2 macrophages (Biswas et al., 2006). Due to the high activity of IL-10 and defective NF-κB pathway, low levels of immune stimulatory cytokines like TNF-α, IL-1 and IL-12 are produced resulting in decreased anti-tumoral immune responses (Sica et al., 2000). TAMs are poor antigen presenting cells but showed high potential to suppress T cell activation and proliferation via TGF-β and IL-10 (Mantovani et al., 2002).

According to the predictions, it was recently confirmed that TAMs typically correlate with poor prognosis or faster tumor growth, in both mice and humans, and are indeed M2 or M2-like macrophages (Rauh et al., 2005;

Tjiu et al., 2008). It is well established that macrophages mediate tumor cell-killing via NO and TNF-α (Keller et al., 1990; Buhtoiarov et al., 2007), both of which are abundantly produced by M1 macrophages. Classically fully activated M1 TAMs can exert cytotoxic activity on tumor cells and elicit tumor-destructive reactions centered on the vessel walls (Mantovani et al., 2009). This makes them potent anti-tumor effectors. M2 macrophages, on the other hand, are poor producers of NO and TNF-α, and enhance tumor cell growth by expressing constitutively expressing the enzyme arginase-1, which competes with the NO-producing enzyme, NOS-2, for L-arginine to limit NO production in macrophages through the exhaustion of the finite L-arginine supply (Gordon and Martinez, 2010; Mills, 2001; Mosser and Edwards, 2008). It should be noticed that activated macrophages either by IFN-γ or IL-4, induce the expression of the transport system cationic amino acid transporter 2 (CAT2), releasing large amounts of arginine in the medium (Sans-Fons et al., 2013). M1 macrophage produce IL-12 not only skews towards an anti-tumor Th-1, cell-mediated adaptive response, it also stimulates the production of IFN-α from NK cells, which increases T cell proliferation for a robust T cell response (Trinchieri and Sher, 2007). M2 macrophages, on the other hand, produce IL-10 to skew to a humoral Th-2 response, which is less effective at killing tumor cells (Ding et al., 1993; Fiorentino et al., 1991; Beissert et al., 1995).

The phenotypes of TAM also differ between tumor regions. Recently, gene profiling data have shown that, although TAMs express many genes associated with the M2 macrophage phenotype (Movahedi et al., 2010; Ojalvo et al., 2009; Pucci et al., 2009), they also express M1 phenotype-associated genes (Van Ginderachter et al., 2006; Movahedi et al., 2010). This raises the possibility that there are distinct subpopulations of TAMs, with distinct functions (Lewis and Pollard, 2006). In MMTV-PyMT (Mouse mammary tumor virus which induce murine breast cancer tumors), higher numbers of TAMs were found at the margins of the tumor and the numbers decreased throughout the deeper stroma in the tumor. In the center of the tumor, TAMs

were found in association with blood vessels (Wyckoff et al., 2007). In breast cancer TAMs were equally distributed throughout the tissue whereas DCs were only present in the periphery (Allred et al., 1993). This indicates that TAMs reside within different tumor regions, and may have specialized functions depending on the micro-environmental influences of each region. TAMs that are capable of promoting tumor cell invasion show different gene expression signatures to the general TAM population (Ojalvo et al., 2009), suggesting that these invasive TAMs may have a unique phenotype as a result of their role in promoting tumor cell escape into the vasculature.

Another hallmark of TAMs is their tendency to accumulate into necrotic regions of the tumors that are characterized by low oxygen tension (Lewis and Murdoch, 2005). This preferential localization is regulated by tumor hypoxia, which induces the expression of HIF-1-dependent molecules (vascular endothelial growth factor (VEGF), C-X-C motif chemokine 12 (CXCL12) and its receptor C-X-C chemokine receptor type 4 (CXCR-4)) that modulate TAM migration in avascular regions (Talks et al., 2000; Schioppa et al., 2003). HIF-1 also regulates myeloid cell-mediated inflammation in hypoxic tissues (Cramer et al., 2003) and this link between hypoxia and innate immunity was confirmed recently, showing that HIF-1 is also regulated transcriptionally by NF-κB (Rius et al., 2008).

Macrophage phenotype and activation are regulated by three different pathways: (a) cytokine signaling pathway using janus-associated kinase (Jak)-STAT molecules, (b) microbial recognition receptors including toll-like receptors (TLRs) and (c) immune-receptors that signal via immunoreceptor tyrosine-based activation motifs (ITAM). M1 stimuli signals such as LPS and IFN-γ trigger TLR-4, IFN-α/β receptor or IFN-γ receptor, result in activation of the transcription factors NF-κB, activator protein 1 (AP-1), Interferon regulatory factor 3 (IRF3) and STAT1 and further to the transcription of M1 genes. In contrast, M2 stimuli such as IL-4/13, signal through the IL-4Rα receptor to activate STAT6, which regulates the expression of M2 genes.

Immune complexes trigger the Fc-gamma receptors I (FcγR), an ITAM-containing receptor, leading to activation of kinases (e.g. Syk and PI3K) and the expression of molecules like prostaglandin E and IL-10 (Hu et al., 2007) .

1.4.4 Monocytes

Monocytes are a heterogeneous population that represents 5-10% of peripheral blood mononuclear cells (PBMCs). They arise in the bone marrow, are released into the blood stream and enter tissues where they can give rise to macrophages or DCs (Gordon and Taylor, 2005; Taylor and Gordon, 2003; Yrlid et al., 2006).

Under steady state conditions in the mouse, about half of the circulating monocytes leave the blood stream each day, where these cells enter into different tissues of the body. In contrast to pro-monocytes, monocytes do not undergo cell division (Wyckoff et al., 2007). Dying monocytes are destroyed in the spleen (Muller, 2001). The life span of monocytes is still under debate, but it is thought that the half-life of monocytes in blood is relatively short: about three days in humans and one day in mice (Muller, 2001). The short half-life of monocytes in the blood has led to the idea that these cells may be continuously replenished in order to preserve tissue homeostasis (Ginhoux et al., 2006). In mice responding to an inflammatory challenge, the number of monocytes leaving the circulation per day is at least double. As with other immune cells, the immune function and differentiation of monocytes rely on communication with other cells through cell surface proteins.

Inflammation, trauma and immune stimuli all cause an increased recruitment of monocytes to peripheral tissues, aiding in host defense and tissue repair. The common features between mouse and human monocyte

subsets confirm a conserved system between species (Gordon and Taylor, 2005). Increased technical skill in isolating monocyte subsets, as well a better understanding of monocyte antigen composition will hopefully aid in a better understanding of the various physiological roles the monocytic lineage plays.

1.4.4.1 Monocyte heterogeneity

Monocytes develop from hematopoietic stem cells in the bone marrow via several commitment steps and intermediate progenitor stages that, in the prevalent model, pass through the common myeloid progenitor (CMP), the granulocyte/macrophage progenitor (GMP), and the macrophage/DC progenitor (MDP) stages (Taylor and Gordon, 2003). Each of these differentiation steps involves cell fate decisions that successively restrict developmental potential. During development, the origins of cells from the yolk sac that have macrophage-like phenotypes might be distinct from the origins of these cells in adults and after haematopoiesis properly begins in the fetal liver (Van Ginderachter et al., 2006). The mononuclear phagocyte cell line originates in the bone marrow as the granulocyte-monocyte colony forming unit (CFU-GM) which is a common committed cell for the granulocyte and monocyte-macrophage pathways. The cell is induced to differentiate into a monoblast by glycoprotein hormones called colony stimulating factors (Larco et al., 2004). Monoblasts differentiate into promonocytes, the first morphologically identifiable cell in the series (Roberti et al., 2011), and promonocytes differentiate into monocytes. The process of differentiation from the committed stem cell to the mature monocyte takes about six days, and the cells go through three to four cell divisions between the monoblast and monocyte stage. The mature monocyte is released from the bone marrow into the circulation within 24 hours. There is no evidence that monocytes are predestined for any particular tissue once they leave the bone marrow; rather, migration of monocytes into tissues appears to be a random phenomenon in

the absence of inflammation (Paik et al., 2004). Once in the tissues, monocytes do not re-enter the circulation; rather, they undergo transformation into tissue macrophages without further cell division (Roberti et al., 2011). The terminal stage of differentiation in the mononuclear phagocyte line is believed to be the multinucleated giant cell.

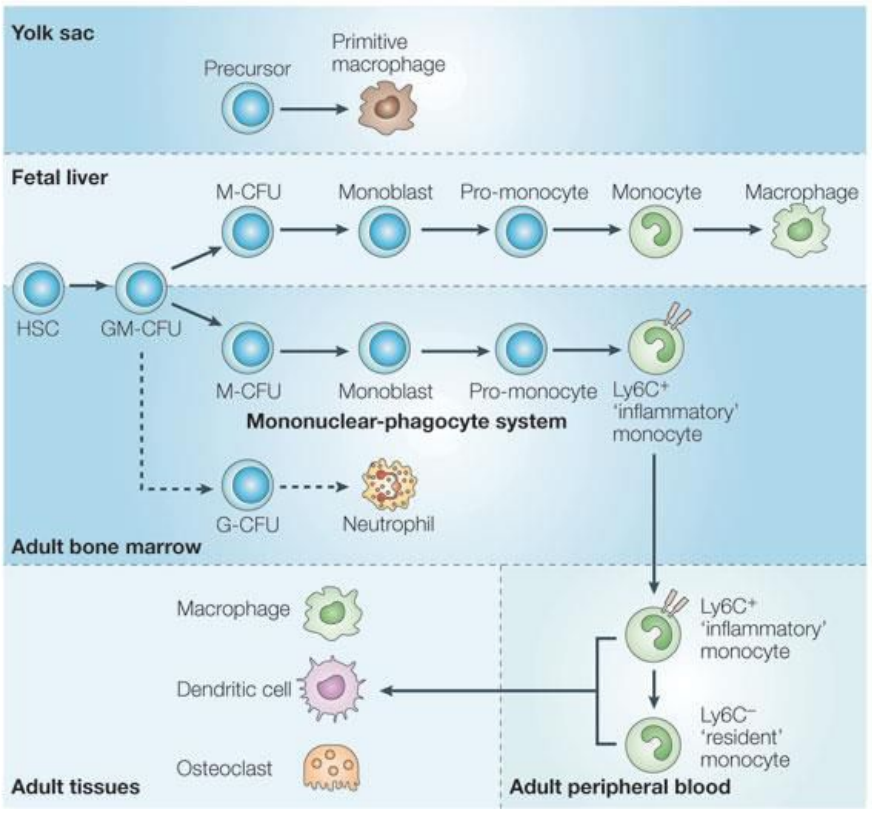

Figure 1-3. Monocytes heterogeneity. G-CFU, granulocyte colony-forming unit; GM-CFU, granulocyte/macrophage colony-forming unit; HSC, haematopoietic stem cell; M-CFU, macrophage colony-forming unit (Taylor and Gordon, 2003).

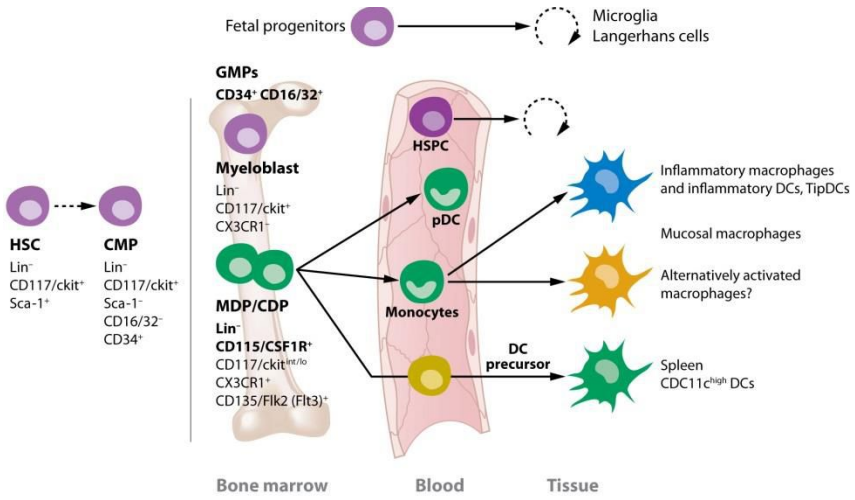

Figure 1-4. **Differentiation of the macrophage/DC progenitor and origin of macrophage and DC subsets.** (Auffray et al., 2009).

1.4.4.2 Monocytes sub populations

One of the most important hallmarks of monocytes is the morphological, phenotypic and functional heterogeneity. The identification of various subsets of monocytes has allowed, by studying mouse monocytes, researchers to address the *in vivo* relevance of human monocytes.

It has been proposed by Geissmann et al. that monocytes enter the bloodstream as at least two different phenotypic and functional subsets of circulating monocytes (Geissmann et al., 2010). These two distinct populations can be classified based on the expression of specific cell surface

markers as “inflammatory” monocytes and “resident” monocytes. Murine “inflammatory” monocytes are defined as $CCR2^{+}CX3CR1^{low}$ (CX3CR1; a chemokine receptor that is found on all monocytes) $Ly6C^{high}$, that home to inflamed tissue. In contrast, “resident” monocytes in mice are classified as $CCR2^{-}CX3CR^{high}Ly\text{-}6C^{low}$ that give rise to resident myeloid cells in non-inflamed tissues (Savino et al., 2012). Similar populations have been described in human monocytes: $CD14^{high}$ $CD16^{-}$ $CX3CR1^{low}$ $CCR2^{+}$ $CD62L^{+}$ monocytes more closely resemble the “inflammatory” murine sub population, whereas $CD14^{low}$ $CD16^{+}$ $CX3CR1^{high}$ $CCR2^{-}$ $CD62L^{-}$ cells are morphologically similar to the “resident” murine subpopulation (Geissmann et al., 2003). $CD14^{low}$ $CD16^{+}$ monocytes increase in number during infections (Ziegler-Heitbrock, 2007; Thieblemont et al., 1995) and are considered pro-inflammatory in phenotype (Belge et al., 2002). Subsequent studies have shown that the “inflammatory” and “resident” designations for monocyte subpopulations do not take into account the heterogeneity and phenotypic complexity of monocytes. In addition, a third subpopulation of monocytes with intermediate expression of Ly6C exists, and in human monocytes an “intermediate” $CD14^{high}$ $CD16^{+}$ population has also been identified (Sunderkötter et al., 2004) indicating a transition from $Ly6C^{high}$ to $Ly6C^{low}$ in the mouse and from $CD14^{high}$ to $CD14^{low}$ in humans. (Figure 1-5).

Inflammatory $Ly6C^{high}$ monocytes can differentiate into a variety of macrophages and DCs subtypes that can either activate or inhibit the immune response, depending on local or systemic cues received and the nature of encountered pathogen (Rose et al., 2012; Augier et al., 2010)

Ly6C ^{high} monocytes represent approximately 2–5% of circulating white blood cells in an uninfected mouse and when inflammation occurs they are rapidly recruited to sites of infection and inflammation (Hammond et al., 2014). CCR2 deficiency markedly reduces $Ly6C^{high}$ monocyte trafficking to sites of inflammation, indicating a crucial role for this chemokine receptor in release of these monocytes from bone marrow into blood.

In most cases of infection, the ensuing recruitment of Ly6C^{high} monocytes is more prominent and robust. $CX_3CR1^+Ly6C^{low}$ blood monocytes patrol blood vessels and enter non-inflamed tissues, whereas Ly6C^{high} monocytes selectively traffic to sites of inflammation (Lin et al., 2009).

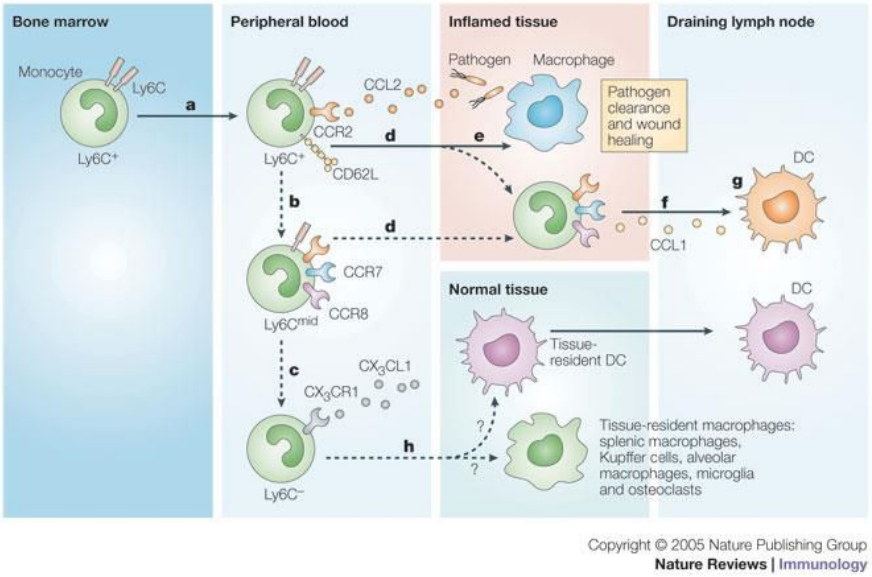

Figure 1-5 Monocyte sub populations. Ly6C+ bone-marrow monocytes after being released into the peripheral blood (a), are thought to adopt a Ly6Cmid phenotype (b), under steady-state conditions they form CX3CR1highCCR2-Ly6C- monocytes (c). Both Ly6C+ and Ly6Cmid monocytes recruit to inflammatory lesions in respond to pro-inflammatory cues (d) and mostly differentiate into macrophages (e). Some monocytes emigrate from the tissues to the draining lymph nodes (f) which there, these monocytes acquire dendritic cell (DC)-like characteristics (g). In the absence of inflammation, CX3CR1hiLy6C- monocytes enter the tissues and replenish the tissue-resident macrophage and DC populations (h). Solid arrows represent pathways that are supported by established data, whereas dashed arrows represent pathways that are indicated from a compilation of more recent data and speculation. CX3CL1: CX3C-chemokine ligand 1. CCR7/8: CC-chemokine receptor 7/8, CCL1/2: CC-chemokine ligand ½, CD62L: Cluster of Differentiation 62 ligand or L-Selectin (Taylor and Gordon, 2003).

1.4.4.3 Recruitment of monocytes to sites of inflammation

The extravasation of monocytes from the vascular lumen to the tissues involves a series of sequential molecular interactions between monocytes and endothelial cells. Selectins are cell-surface proteins on the monocyte that interact with glycoprotein ligands on the endothelial cells, allowing the monocyte to bind weakly and initiate the adhesion cascade. The rolling monocyte is then stimulated by chemokines or other chemotactic compounds to engage its surface integrins with counter-receptors expressed by endothelial cells. This results in firm adhesion of monocytes to the endothelium before they emigrate through the vessel wall. Monocytic integrins are activated by signals that are elicited by chemokines. This leads to the tight adhesion of monocytes to the vascular endothelium and the induction of active, cytoskeleton-driven migration (Kim et al., 2004). Chemokines are small secreted proteins that are produced by endothelial cells, leukocytes or stromal cells. Several chemokines can bind to transmembrane heparin sulfate proteoglycans on the luminal surface of vascular endothelial cells and be presented to leukocytes (Spillmann et al., 1998). When chemokines bind to these proteoglycans, the binding site for chemokine receptors remains exposed (Roscic-Mrkic et al., 2003). Thus, when a rolling monocyte encounters a chemokine bound to the endothelium, the chemokine interacts with a G protein coupled receptor expressed on the monocyte and elicits a rapid integrin-activation signal. Once the monocyte has adhered to the endothelium it may start to migrate through the vessel wall. For inflammatory and resident monocytes different mechanisms have been proposed. Because Ly6C^{high} monocytes express CCR2 a molecule well known to be involved in inflammatory monocyte recruitment (Imhof and Aurrand-Lions, 2004), it was proposed that Ly6C^{high} monocytes are rapidly recruited to sites of inflammation. In typical models of acute inflammation, Ly6C^{high} monocyte

recruitment is critically dependent on CCR2 (Boring et al., 1997; Merad et al., 2002), but not on CX3CR1 (Jung et al., 2000). In addition to CCR2, chemokine receptor 6 CCR6 binding to chemokine (C-C motif) ligand 20 (CCL20), is involved in the recruitment of Ly6C^{high} monocytes (León et al., 2007; Merad et al., 2004). Less is known about the trafficking and fate of Ly6C^{low} monocytes. In contrast to Ly6C^{high} monocytes, they migrate scarcely or not at all to inflamed tissue in mice, including the acutely inflamed peritoneum (Geissmann et al., 2003; Sunderkotter et al. , 2004), or to skin after intra-cutaneous injection of latex beads (Qu et al., 2004), administration of vaccine formulations (Le Borgne et al. , 2006), or epi-cutaneous UV exposure (Ginhoux et al., 2006). It has further been suggested that the Ly6C^{low} monocytes utilize CX3CR1 to migrate into non-inflamed tissue for replacing resident macrophages or DCs, given their higher surface expression of CX3CR1 and the CX3CL1-dependent trans-endothelial migration of human CD16^{+} monocytes *in vitro* (Ancuta et al., 2003; Geissmann et al. , 2003). However, the experimental evidence for the trafficking pattern and the potential role of Ly6C^{low} monocytes as precursors for resident tissue cells is rather limited.

1.4.4.4 Ly6C^{high} and Ly6C^{low} monocytes

As mentioned before, monocytes are thought to arise in the bone marrow as immature Ly6C^{high} cells and are released into the blood where it is thought, that as they mature, they down-regulate Ly6C expression, becoming Ly6C^{low} monocytes. It has been suggested that Ly6C^{high} monocytes can move bi-directionally between blood and bone marrow (Varol et al., 2007; Yrlid et al., 2006). Adoptively transferred Ly6C^{high} disappear from the blood in the absence of inflammation indicating that they may be converting to Ly6C^{low} (Geissmann et al., 2003). Following depletion of all blood monocytes, the returning circulating monocytes were predominantly Ly6C^{high} and after 5 days converted to Ly6C^{low} (Sunderkötter et al., 2004). In another study, monocytes

initially labelled as Ly6C^{high} had fully converted to Ly6C^{low} after 7 days during steady state (Tacke et al., 2006). Injection of Ly6C^{high} monocytes into the femoral bone cavity resulted in the isolation of graft-derived Ly6C^{low} monocytes in both, the bone and the blood of the recipient mice, implying that Ly6C^{high} monocytes act as precursors for Ly6C^{low} monocytes (Varol et al., 2007). It has concluded that Ly6C^{high} monocytes had differentiated into Ly6C^{low} monocytes, whereas the other studies have shown that distinct populations of monocytes are recruited from the blood. Auffray et al. explain the differentiation potential and functions of blood monocyte subsets during *Lm* infection and myocardial infarction. They claim that Ly6C^{high} monocytes are players in short term response to inflammation, whereas Ly6C^{low} monocytes are important for long-term healing (Auffray et al., 2009) (

Figure **1-6).**

Although all the data presented, the conversion of Ly6C^{high} to Ly6C^{low} monocytes has been questioned (Geissmann et al., 2010). Reduction of Ly6C^{high} monocytes does not always result in reduction of Ly6C^{low} monocytes indicating that Ly6C^{high} do not always convert into Ly6C^{low} (Scatizzi et al., 2006; Mildner et al., 2007). Ly6C^{high} monocytes are more migratory and typically recruited first to sites of tissue damage and inflammation, whereas Ly6C^{low} monocytes generally arrive later (Geissmann et al., 2003; Geissmann et al., 2008). Ly6C^{high} monocytes have been shown to be pro-inflammatory in nature (Ginhoux et al., 2006; Sunderkötter et al., 2004; Xu et al., 2005).

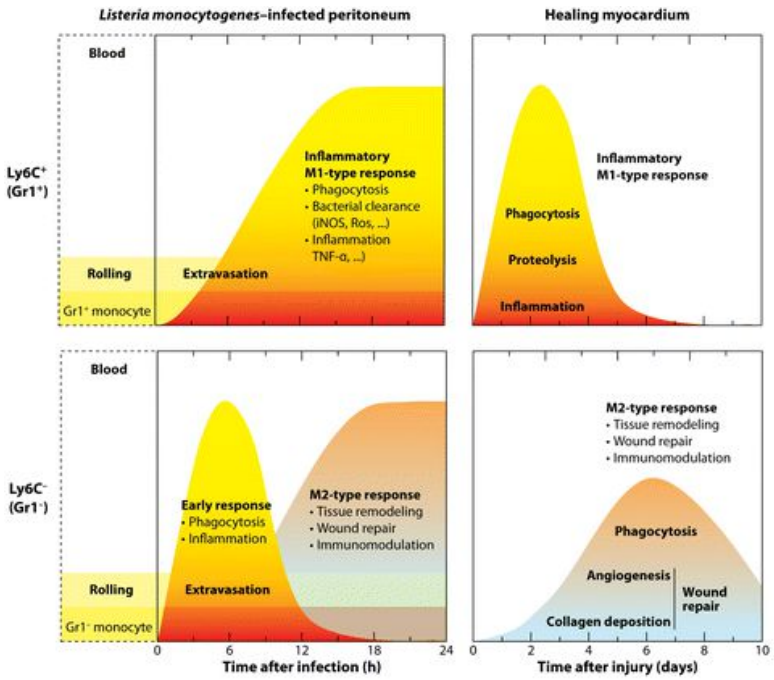

Figure 1-6. Ly6C^{high} and Ly6C^{low} Monocytes role in inflammation. Differentiation potential and effector functions of blood monocyte subsets during Listeria monocytogenes infection and myocardial infarction (Auffray et al., 2009).

Increasing evidences indicates that Ly6C^{low} monocytes also have inflammatory properties and can secrete inflammatory cytokines (Auffray et al., 2007). In humans CD14^{low} CD16^{+} monocytes were found to patrol the blood vessel endothelium after adoptive transfer into mice. Conversely, in atherosclerotic lesions and liver injury, Ly6C^{high} monocytes were recruited early and displayed pro-inflammatory functions (Robbins et al., 2012; Karlmark et al., 2009). This was also seen in a model of myocardial infarction, where Ly6C^{high} monocytes dominated the early phase of recruitment and displayed inflammatory, phagocytic and proteolytic functions, while Ly6C^{low} monocytes infiltrated later and expressed VEGF (vascular endothelial growth

factor) and initiated wound healing (Nahrendorf et al., 2007). In skeletal muscle injury, Ly6C^{high} monocytes were recruited first. In this study, Ly6C^{high} monocytes expressed pro-inflammatory cytokines, such as IL-1β and TNF-α, whereas Ly6C^{low} monocytes expressed anti-inflammatory cytokines, such as IL-10 and TGFβ (Arnold et al., 2007). Similar findings were also seen in a murine model of kidney injury, where it was proposed that Ly6C^{high} monocytes converted into trophic Ly6C^{low} monocytes (Lin et al., 2009).

Ly6C^{high} monocytes were found to accumulate in the central nervous system prior to the onset of experimental autoimmune encephalomyelitis (EAE) and West Nile virus encephalitis (King et al., 2009; Getts et al., 2008). In the EAE model, these monocytes were pro-inflammatory in phenotype, secreting TNF-α and producing NOS2. In this model, Ly6C^{low} monocytes up-regulated IL-12, IL-23 and IL-6 gene expression, indicating that they were also somewhat pro-inflammatory in phenotype, although they were far less numerous and not as thoroughly examined (King et al., 2009). However, in another study, a subset of Ly6C^{high} monocytes was shown to suppress T cell responses in EAE (Zhu et al., 2007). In spinal cord injury, adoptively transferred Ly6C^{high} monocytes contributed to the resolution of inflammation and initiation of tissue repair (Shechter et al., 2009), whereas in this case, Ly6C^{low} monocytes appeared to be pro-inflammatory in phenotype (Donnelly et al., 2011).

1.4.4.5 Monocytes differentiation to macrophages

The last step in becoming a macrophage occurs when the monocytes extra-vase out of the bloodstream and into tissues to become a fully matured macrophage. It has been demonstrated that resident macrophages can arise from Ly6C^{low} monocytes in the circulation, and that this pattern of migration and differentiation is not significantly disturbed during an infection (Geissmann et al., 2003). Inflammatory macrophages, on the other hand, seem to

differentiate from the CCR2^{-}expressing Ly6C^{high} monocytes, only arising during inflammation (Geissmann et al., 2003). Immunologically, resident macrophages are the first defenders of the innate immune system against pathogens through direct phagocytosis and/or bactericidal reactive nitrogen species (RNS) release (Stefater III et al., 2011). Thereafter, inflammatory macrophages, arising from patrolling monocytes extra-vase into the inflamed tissue, sustain the inflammation until the pathogen is eliminated (Stefater III et al., 2011; Auffray et al., 2007).

Given the above results, it is possible that Ly6C^{high} monocyte subpopulations are capable of initiating inflammatory responses.

1.4.4.6 Monocytes recruitment to tumors

In current studies, the roles of specific monocytes subsets in tumor progression, the molecular mechanisms for their impacts and their infiltration to tumors as TAMs have not been elucidated . Blood monocytes increase in patients with tumors compared to normal individuals. TAMs originate from blood monocytes recruited at the tumor site by molecules produced by neoplastic and by stromal cells (Pollard, 2004) (Figure **1**-**7**). The chemokine CCL2 (also known as MCP1), earlier described as a tumor-derived chemotactic factor, is the main player in this process (Allavena et al., 2008; Pollard, 2004). In experimental and human studies its levels correlate with TAM abundance in many tumors, such as ovarian, breast and pancreatic cancer (Sunderkötter et al., 2004). CCL2-producing tumors recruit increased numbers of monocytes into the tumor mass, whereas tumors that do not produce CCL2, monocytes are found mainly in the peri-tumoural regions (Zhang et al., 1997). It has been proposed that recruitment of monocytes via CCL2 can have dual effects. In a melanoma model, low-level secretion of CCL2 promoted tumor formation, whereas high-level CCL2 secretion resulted in considerable TAM infiltration and tumor destruction (Nesbit et al., 2001).

TAM themselves produce CCL2, suggesting the action of an amplification loop and anti-CCL2 antibodies combined with other drugs have been considered as an anti-tumor strategy (Colombo and Mantovani, 2005). Other chemokines involved in monocyte recruitment are CCL5 (Chemokine (C-C motif) ligand 5), CCL7 (Chemokine (C-C motif) ligand 7), CXCL8 (chemokine (C-X-C motif) ligand 8), and CXCL12 (Chemokine (C-X-C motif) ligand 12), as well as cytokines such as VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor) and the growth factor M-CSF (Balkwill, 2004; Allavena et al., 1998). Moreover, monocytes could be attracted by fibronectin, fibrinogen and other factors produced during the cleavage of extracellular matrix proteins induced by macrophage and/ or tumor cell-derived proteases (Denardo et al., 2008).

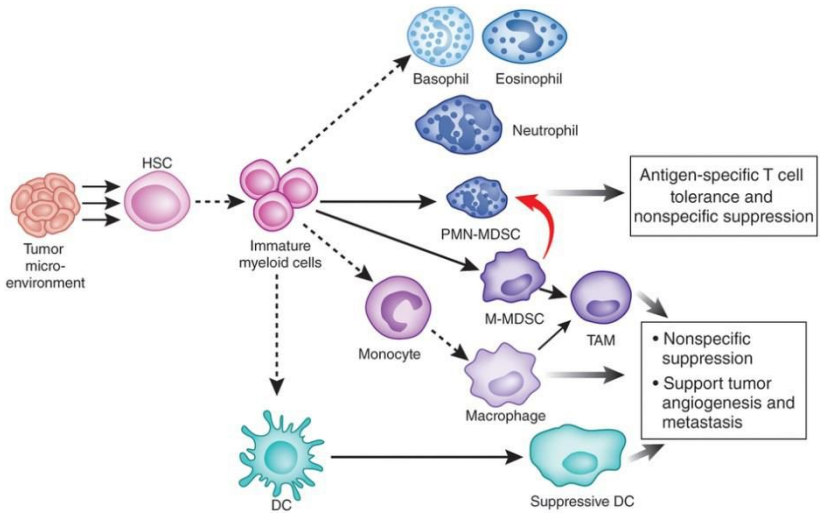

Figure 1-7 **Monocytes differentiation in tumors.** Dashed lines indicate the normal developmental pathway of immature myeloid precursor cells, which differentiate into DCs, monocytes-macrophages and granulocytes (basophils, eosinophils and neutrophils) in non-tumor-bearing hosts. Solid lines indicate the aberrant pathways of myeloid cell development in tumor-bearing hosts. New data (thick red line) suggest that a large proportion of PMN-MDSCs emerge from the M-MDSC pool. HSC: hematopoietic stem cell; TAM: tumor-associated macrophage; PMN-MDSCs: polymorphonuclear myeloid-derived suppressor cells; M-MDSC: mature myeloid-derived suppressor cells (Wynn et al., 2013).

After a short transitory passage in the blood, monocytes attach to the endothelium, roll and through diapedeses move into extravascular tissues, where they differentiate into macrophages and DCs.

In mice, inflammatory macrophages (with an M1 phenotype) are derived from "inflammatory" $CCR2^{+}CX3CR1^{low}Ly6C^{high}$ monocytes. On the other hand, "resident" $CCR2^{-}CX3CR1^{high}Ly\text{-}6C^{low}$ monocytes, resemble M2 macrophages (Movahedi et al., 2010a) (they express low levels of pro-inflammatory cytokines and high levels of anti-inflammatory molecules such as IL-10 and transforming growth factor-β). In cancer, it is generally accepted that after recruitment to tumor $Ly\text{-}6C^{low}$ monocytes differentiate more readily into M2-like TAMs and $Ly6C^{high}$ monocytes cells to M1-like TAMs (Movahedi et al., 2010; Guo et al., 2013; Priceman et al., 2010). It is clear that these two interpretations are not mutually exclusive: early recruited $CX3CR1^{high}Ly6C^{high}$

macrophages might undergo a reprogramming to CX3CR1^{high}Ly6C^{low} M2-type cells similar to those obtained *in vitro* following prolonged exposure to LPS, but the changing tumor microenvironment might at later stages promote the preferential recruitment of CX3CR1^{high}Ly6C^{low} macrophages from the blood. In any case, addressing the mechanistic bases of these complex population dynamics *in vivo* will be extremely challenging (Jacquelin et al., 2013).

Although it has been reported that monocytes proliferate within tumors to generate TAMs, it is still unclear whether Ly6C^{high} "inflammatory monocytes" or Ly6C^{low} "resident monocytes", are the primary source of TAMs in mice. Therefore, TAMs originate from myeloid progenitors in response to tumor-secreted soluble factors, although the origin of TAMs in human cancer remains unclear.

Interestingly, in BALB/c mammary adenocarcinoma model TS/A (a metastasizing mouse cell line), the relative percentages of these distinct myeloid subpopulations dramatically changed as tumors progressed. Within the TAM compartment, the percentage of Ly6C^{intermidiet} TAMs decreased, whereas the Ly6C^{low}MHCII^{low} TAMs subset, became gradually more prominent, reaching up to 60% of the myeloid tumor infiltrate in large tumors. Because the amount of tumor-infiltrating CD11b^{+} cells increased as tumors progressed, MHCII^{low} TAMs also strongly accumulated in absolute numbers, to a much greater extent than MHCII^{high} TAMs (Movahedi et al., 2010b).

1.5 Monocytes generation

The isolation of murine monocytes from peripheral blood remains impractical due to the large numbers of donor mice required to obtain significant blood volume and cell yield. Classic murine monocyte enrichment

approaches make use of density gradient centrifugation (Francke et al., 2011) with subsequent fluorescence-activated cell sorting (FACS) (Heil et al., 2002) or multistep magnetic-activated cell sorting (MACS) (Houthuys et al., 2010; Zhu et al., 2007). Unfortunately, in addition to being difficult, such isolation methods can alter the biological activity of the cells (Seeger et al., 2007; Auffray et al., 2009), a major problem for applications and experimental reproducibility. The difficulty of obtaining pure monocyte cultures greatly impedes progress in many research fields.

Here, we aim to overcome these obstacles by describing both a novel time and cost efficient approach for isolating large amounts of murine monocytes from native murine bone marrow suspensions cultured on ultra-low attachment surfaces. We further confirm that these cells display the appropriate phenotypic and functional properties, particularly with regard to downstream compatibility and their complex role *in vivo*.

In culture, monocytes differentiate into macrophages upon exposure to M-CSF (Stanley et al., 1997), and into DCs upon exposure to GM-CSF and IL-4 (Sallusto et al., 2000; Sallusto and Lanzavecchia, 1994). However, *in vivo*, the situation is different because monocytes are under the constant influence of the local microenvironment, whose tremendous variety is reflected in the heterogeneity of these cells (Burke et al., 2002). Apart from the influence of cytokine, the endothelium plays a pivotal role in the differentiation of monocytes. The adhesion molecules can influence the functional activity of macrophages (Xaus et al., 2001). In fact, cultured monocytes on un-stimulated monolayers of human umbilical vein endothelial cells (grown on a collagenous matrix), diapedesis into the subendothelial collagen layer. A proportion of these monocytes “reverse transmigrate” and become DCs, and those that remained differentiated into macrophages (Randolph et al., 2002).

1.6 Animal models

There are several animal models to study cancer. Each of these models comes with inherent advantages and disadvantages. Different models may represent distinct aspects or subtypes of this heterogeneous disease and studying one model will never be adequate to encompass the whole disease. My research has focused on the mouse cancer models because we desired a model that will give us the ability to study tumor formation, progression and therapy response *in vivo,* with special emphasis on the role of inflammation in the development of tumors. Furthermore, the mouse is amenable to genetic manipulation. Hence, there cannot be only one model for cancer but rather a myriad of models, each being unique to a different subtype or a particular aspect of the disease. There are many mouse tumor models that have been generated over the past twenty years, which among them we chose the MDA-MB-231 breast cancer and the C26 colon carcinoma.

1.7 Objectives

1. Evaluate the migratory capacity $CCR2^{+}$ $Ly6C^{high}$ $CD11b^{+}$ *in vitro* generated monocytes to the tumor in mice cancer models.
2. Demonstrate that the migration of $Ly6C^{high}$ *in vitro* generated monocytes in peripheral blood will modulate the growth rate of tumors in mice cancer models.
3. Demonstrate that recruitment of $Ly6C^{high}$ monocytes alter the balance of pro-inflammatory/anti-inflammatory pool of macrophages in the tumor.
4. Establish the modulatory impact of infiltrated $Ly6C^{high}$ monocytes on mice cancer progression.

2 MATERIALS AND METHODS

Materials and methods

2.1 Mouse strains

Female BALB/C mice and female STAT6 knockout mice and control wild type were purchased from Jackson Laboratory (Bar Harbor, Maine USA). Nude BALB/C female were obtained from Charles River Laboratories (Wilmington, MA). Mice were kept in the animal facility of Parc Scientific Barcelona under germ free conditions. All experiments were performed according to University of Barcelona animal experimental ethics committee guidelines approved by the Generalitat de Catalunya. Mice were maintained at a constant temperature (22ºC) and on a 12 hours light: 12 hours dark cycle.

2.2 Cell lines and culture

2.2.1 MDA-MB-231 cell line

The breast cancer cell line MDA-MB-231/ luciferase (Mbalaviele et al., 1996), was kindly provided by Dr. Roger Gomis (Institute for Research in Biomedicine, Barcelona). Cells were cultivated in high glucose DMEM (Sigma, St Louis, MO) containing 10% heat-inactivated fetal bovine serum (Sigma), 2% glutamine (Invitrogen) 1% Penicillin/Streptomycin (Sigma). Tumor cells were harvested for passage by washing the monolayer with PBS, followed by 3 to 4 minutes of exposure to Trypsin, 0.5 mM EDTA (Sigma) diluted in PBS

and injection in mice were performed after 3 passages. For cell freezing, cell suspensions were centrifuged (1200 rpm, 10 min, at room temperatue) and the pellet was re-suspended in freezing medium (DMEM, 10% FBS, 5% Dimethyl sulfoxide (Sigma) at a concentration of $5x10^6$ cells/ml. Cells were immediately aliquoted in cryovials and placed in freezing containers. After 24 hours at -80℃ vials were transferred to liquid nitrogen for long term storage.

2.2.2 C26 cell line

C26 colon carcinoma cell line has been established *in-vitro* from the colon-26 tumor of female mice. This tumor was induced in BALB/C mice by single rectal application of N-Nitroso-N-Methylurethan (NMU) (Gali-Muhtasib et al., 2008).This cell line was a generous gift from Dr.Maria Busqets (University of Barcelona , Spain). Cells were cultivated in high glucose DMEM (Sigma, St Louis, MO) containing 10% heat-inactivated FBS (Sigma) and 1% Penicillin/Streptomycin (Sigma). Tumor cells were harvested for passage by washing the monolayer with PBS, followed by 3 to 4 minutes of exposure to Trypsin, 0.5 mM EDTA (Sigma) diluted in PBS. Injection of cells in mice was performed after 3 passages. For cell freezing, cell suspensions were centrifuged (1200 rpm, 10 min, RT) and the pellet was re-suspended in freezing medium (DMEM, 10% FBS, 5% Dimethyl sulfoxide (Sigma) at a concentration of 5×10^6 cells/ml and immediately aliquoted in cryovials and placed in freezing containers. After 24 hours at -80℃ vials were transferred to liquid nitrogen for long term storage.

2.3 Mouse bone marrow-derived Ly6C^{high} monocytes

2.3.1 Generation of bone marrow-derived Ly6C^{high} monocytes

Mice were killed by cervical dislocation and both femurs were dissected free for adherent tissue. The ends of the bones were cut off and the bone marrow cells were harvested by flushing marrow cavities of femurs and tibiae.. Cells were suspended by vigorous pipetting. To obtain Ly6C^{high} monocytes, 20 x 10^6 cells were cultured in plastic tissue culture dishes of 60 cm^2 (Lab-Tek 4030,Miles laboratories, Inc., Napervile, IL) in DMEM rich in glucose (Sigma, ST Louis, MO) containing 25% fetal bovine serum (FBS) (Sigma), 20 % of L-cell conditioned media as a source of M-CSF, 1% Penicillin/Streptomycin (Sigma) and other growth factors (Protocol under patent).. Cells were incubated at 37℃ in humidified 5% CO_2 atmosphere. Cells were grown to reach a statement of sub-confluence and after 7 days a heterogeneous population was observed, consisting of attached and floating cells, which form aggregate. Ly6C$^+$ monocytes form part of a floating population and for this reason only cells in suspension were used from the total culture and characterized.

Generation of bone marrow-derived Ly6C^{high} monocytes from STAT6 wild type and knockout BALB/C mice was carried out in the same manner as mentioned above.

2.3.2 Enrichment of Ly6C^{high} monocytes by cell sorting

Enriched Ly6C^{high} monocytes were obtained from total bone marrow culture after 7 days of differentiation with specific growth factor by a positively

isolation with a sorting technique. Cell suspensions were recovered from total culture, centrifuged and re-suspended in 1 ml of sorting media containing, DMEM 10% FBS (PAA laboratories) and 4% EDTA (ethylenediaminetetraacetic acid, Sigma). Cells were counted by an hemocytometer and the necessary volume of sorting media was added to reach the density of $20x10^6$ cell/ml suspension. Before sorting procedure, blocking of Fc-γ receptors was accomplished by incubating the cells with the antibody CD16/CD32 (BD Pharmigen), at a concentration of 1:200 for 15 minutes at 4°C. Then, Ly6C marker labeling was performed by FITC-conjugated anti-Ly6C (BD Pharmigen) at a 1:200 concentration for 30 minutes at 4°C. Isotype control was made with the corresponding rat IgM, K monoclonal immunoglobulin isotype control (BD Pharmigen). $Ly6C^{high}$ monocytes purification was accomplished using MoFlo Systems sorter (Bechman Coulter, Inc., Spain). Only the higher fraction of Ly6C positive population was sorted. $Ly6C^{high}$ monocytes purity reached > 98%. To control the purity of the sorting, 10^6 sorted cells were labeled with CD11b, CD11c, CCR2, CD62L, F4/80 and CX3CR1 (Table 2.1) extracellular markers and were analyzed by GalliosTM flow cytometry (Bechman Coulter, Inc).

2.3.3 *In vitro* activation of $Ly6C^{high}$ monocytes with INF-γ or IL-4

In some experiments, after sorting a suspension of $Ly6C^{high}$ monocytes ($10x10^6$)in sorting media were treated with recombinant IL-4 (R&D SYSTEMS) (10ng/ml) or recombinant INF-γ (20μg/ml) (Thermo SCIENTIFIC) for 30 minutes with total media at 37°C. After that, cells were centrifuged and cell pellet was washed with PBS.

2.3.4 Labeling Ly6C^{high} monocytes with DiR tracking dye

To obtain *in vivo* images with the IVIS technology, in some experiments, sorted Ly6C^{high} monocytes were labeled with DiR (Invitrogen, Paisley, UK). 10x10^6 Ly6C^{high} monocytes in PBS suspension were labelled with DIR by incubating in a 1:1 proportion with DIR 10uM solution diluted with PBS. Cells were mixed immediately and incubated for 10 minutes at 37ºC. Cells were washed 3 times with 5ml of PBS at 37ºC in order to eliminate the excess of unbound tracer. Afterward, diluted cells in PBS were kept on ice until injection.

2.3.5 Labeling of Ly6C^{high} monocytes with PKH26 tracking dye

To proceed to *in vivo* imaging by IVIS after injection in mice and for histology, in some experiments, sorted Ly6C^{high} monocytes were labeled with PKH26 (Sigma Chemical Co, St Louis, MO, USA) (Invitrogen). 20x10^6 sorted Ly6C^{high} monocytes were washed once in serum-free medium (RPMI-1640) and suspended in 2 ml of diluent solution C (included in the PKH26 labelling kit). Then, 8 µl of PKH26 in 2ml diluent C was added and mixed, and cells were incubated for 10 minutes at room temperature in the dark. The staining reaction was halted by addition of an equal volume (2 ml) of medium supplemented with 10% FBS. The mixture was centrifuged and cells were washed once with PBS and re-suspended in PBS. Afterward diluted cells in PBS were kept on ice until *in vivo* injection.

2.4 Animal models

2.4.1 MDA-MB-231 breast cancer model

Eight week old female nude BALB/C mice were injected unilaterally with $5x10^5$ MDA-MB-231 cells in 50µl of 1:1 Matrigel (BD bioscience)/PBS1% into the fourth abdominal fat pad by subcutaneous injection at the base of the nipple. Tumor growth was monitored externally using Vernier calipers 3 times weekly during the entire experiment to develop the tumor growth curves. When tumor volume was around 1500 mm^3, mice were divided into the 2 groups of control and treatment such that the mean tumor size and the numbers of larger or smaller tumors were equal in each group. Treatment group received 3 times injection of $2x10^6$ DiR or PKH26 labeled bone marrow-derived $Ly6C^{high}$ monocytes intra-cardiac, between days 50 to 70 after tumor cells inoculation, while control group was injected with PBS. Migration of $Ly6C^{high}$ monocytes was monitored daily by acquiring *in vivo* IVIS (Advanced Molecular Vision, Inc. Grantham, UK) images daily to tracked labeled monocytes. At day 75 after tumor cells injection, mice were sacrificed by CO2 asphyxiation, weighed, and subjected to necropsy. The volume and weight of xenograft tumors were recorded. Breast tumors were imaged by IVIS and samples for histology and RNA extraction were collected.

2.4.2 C26 colon carcinoma model

For tumor inoculation, after 3 passages, $1x10^6$ C26 colon carcinoma cells were diluted in 100ul PBS and subcutaneously inoculated in the dorsal region of the neck to eight weeks old female BALB/C mice. Non-tumor-bearing (Non-TB) control mice were injected with 100 µl PBS. Standard rodent chow and water *ad libitum* were freely accessible. C26-inoculated mice

exhibited extensive carcass weight loss and high mortality. Carcass weight was calculated as total body weight and was measured daily after C26-inoculation. Depending on each experimental design, $2x10^6$ *in vitro* generated $Ly6C^{high}$ monocytes were injected intravenously at different time-points after tumor cells inoculation. Tumor-bearing control mice were injected with 100 μl of PBS intravenously. In some experiments, survival rate was evaluated measuring the number of dead mice in each time-point. Except from survival measuring experiments, mice were sacrificed by CO2 asphyxiation in certain days in each experiment, weighed, and subjected to necropsy. The volume and weight of tumors were recorded. Tumor tissue samples for histology and RNA extraction were collected.

2.5 Experimental analysis

2.5.1 Isolation of single cell suspension from tumor tissue

Tumor bearing mice were sacrificed by dislocation of the neck and tumor was excised using sterile forceps. Subsequently, tumors were cut into small pieces and digested in 10 ml digestion buffer (0.5 mg/ml hyaluronidase type V, Sigma, 0.5 mg/ml collagenase B, Roche) for 30 min at 37°C. Single cell suspension was obtained by mincing the digested tumor through a 70 μm pore cell strainer. Cells were collected in a 50 ml Falcon tube and washed with PBS (1500 rpm, 10 min, 4°C). Pellet was re-suspended in 8 ml of PBS and filtered through a 40 μm pore strainer. Live cells were enriched by centrifugation and diluted in PBS and kept on ice to proceed the staining for FACS analysis.

2.5.2 Flow cytometry assay (FACS)

To analyze the expression of cell surface molecules 10^6 cells from cultured bone marrow, sorted Ly6C^{high} monocytes or single cell suspension obtained from tumor digestion were re-suspended in 100 µl FACS buffer (PBS, 1% 0.5 mM EDTA). To block Fc-γ receptors a 1:200 dilution of CD16/CD32 antibody was used. Cells were incubated for 15 min at 4°C. Appropriate antibodies (Table 2.1) were added and cells were further incubated for 30 min at 4°C in the dark. After staining, all samples were washed in FACS buffer to remove unbound antibodies. Samples were analyzed by Gallios™ flow cytometer and data was analyzed using flowjo software. For the analysis, dead cells were excluded by their small size and low level of granularity.

Antibodies	Reference	Commercial	Concentration
PACIFIC BLUE-conjugated anti-CD11c	117322	Biolegend	1:50
APC-conjugated anti-CCR2	FAB5538A	RD Systems	1:50
PE-conjugated anti-CD11b	557397	BD Pharmingen	1:100
APC-conjugated anti-CD11b	17-0112	eBiosciences	1:500
PE-conjugated anti-LY-6C	560562	BD Pharmingen	1:1000
FITC-conjugated anti-Ly-6C	553104	BD Pharmingen	1:50
PE/CY7-conjugated anti-CX3CR1	126516	Biolegend	1:200
ALEXA FLUOR 700-conjugated anti-CD68	MCA1957A700T	AbD serotec	1:500
BRILLIANT VIOLET 421-conjugated anti-F4/80	123132	Biolegend	1:200
PE/CY7-conjugated anti-CD45	25045182	eBiosciences	1:1000
ALEXA FLUOR 700 -conjugated anti-CD45R (B220)	21270044	eBiosciences	1:50
FITC-conjugated anti-CD3	100203	Biolegend	1:50
ALEXA FLUOR 700-conjugated anti-CD62L	56-0621	eBiosciences	1:50
CD16/CD32 (Fcy III/II R)	553142	BD Pharmingen	1:20
ALEXA FLUOR 488ANTI-RABBIT IgG (H+L)	A21441	Invitrogen	1:200
Anti-Mouse F4/80	14-4801-81	eBiosciences	1:150

Table 2.1 Antibodies used in the study.

2.5.3 Isolation of total RNA from tumor tissue

Collected tumors after sacrifice, were kept in RNAlater™ (GIAGEN®. Valencia, CA, USA) solution at -80ºC until RNA extraction, to assure RNA stability. Tumor tissue was ruptured with T 10 basic Ultra-Turrax (IKA®-Werke GmbH & Co. KG. Staufen, Germany) in TRIzol® solution from Invitrogen (Chomczynski and Sacchi, 1987). Homogenized samples were incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. For sample homogenization, 200µl of chloroform (Fluka analytical) per 1 mL of TRIzol reagent was added. Samples were centrifuged at 12000 rpm for 15 minutes at 4ºC. The mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the upper phase, which was removed and placed into a new tube with 1:1 volume of 70% ethanol (Applichem Panreac) diluted in water. For RNA isolation, the previous solution was added in columns from PureLink® RNA Mini Kit Ambion (Alcobendas, Madrid) where RNA extraction was performed according to manufacture protocol.

2.5.4 Real-Time (RT-PCR)

RNA concentration was determined by Nano Drop® (ND-1000 spectrophotometer, BioLab,GA, USA) and 1 µg of total RNA was reverse transcribed with a mix of M-MLV reverse transcriptase RNase Hminus (GibcoBRL), oligo $(dT)_{15}$ primer (Life Technologies), Transcription optimized 5X buffer (Promega) and PCR nucleotide from Promega Corporation, as described by the manufacture. For quantitative PCR analysis SYBER green PCR Core Reagents (Biosystems) and the ABI Prism 7900 Detection System

was used from Applied Biosystems (Arlington Heights, IL). The relative quantification of gene expression was performed as described in the SYBER Green manufacture manual using L14 and *β*-actin as housekeeping genes. The threshold cycle (C_T) was defined as the cycle number at which the fluorescent corresponding to the amplified PCR product is detected. The PCR arbitrary units of each gene were defined as the mRNA levels normalized to the housekeeping genes expression level in each sample. The primers used to amplify cDNA for real-time monitoring are listed in Table 2.2.

Gene	Forward primer	Reverse primer
TGF-B	5′TGGAGCAACATGTGGAACTC3′	5′GTCAGCAGCCGGTTACCA3′
Arginase1	5′TTGCGAGACGTAGACCCTGG3′	5′CAAAGCTCAGGTGAATCGGC3′
TNF-α	5′CCAGACCCTCACACTCAGATC3′	5′CACTTGGTGGTTTGCTACGAC3′
IL-6	5′TCCTCTCTGCAAGAGACTTCCATCC3′	5′GGGAAGGCCGTGGTTGTCACC3′
MRC	5′CGCCCACCAGAGCCCACAAC3′	5′TGCTCGCCAGCTCTCCACCT3′
IL1β	5′TGGGCCTCAAAGGAAAGAAT3′	5′CAGGCTTGTGCTCTGCTTGT3′
COX2	5′CCACTTCAAGGGAGTCTGGA3′	5′AGTCATCTGCTACGGGAGGA3′
MMP9	5′CTTCTCTGGACGTCAAATGTG3′	5′AGAAGAATTTGCCATGGCAG3′
L14	5′TCCCAGGCTGTTAACGCGGT3′	5′GCGCTGGCTGAATGCTCTG3′
B-ACTIN	5′ACTATTGGCAACGAGCGGTTC3′	5′AAGGAAGGCTGGAAAAGAGCC3′

Table 2.2. Primers used in the study.

2.5.5 *In vivo* imaging

For obtaining *in vivo* images of tracking Ly6C^{high} monocytes, animals injected with labeled cells were anesthetized with Isoflurane (Baxter, USA) (Xie Z. et al., 2007) and placed in the scanning machine from Xenogen–IVIS® Spectrum. Matched controls (age and strain matched animals) were imaged on the same scan to provide reliable and objective representation of the background and/or auto-fluorescence signals. After the acquisition, image

processing was realized using the Living Image ® Software (PerkinElmer Inc. Waltham, MA). IVIS calibration was realized before each image acquisition to avoid background. To determine the specific signal on a tested animal, the same anatomic location was analyzed on a proper negative control animal. Therefore, images displays were set up so the fluorescence did not come from control region of interest.

2.5.5.1 Bioluminescence Imaging

Noninvasive, whole-body imaging was performed to monitor luciferase-expressing MDA-MB-231/luc using Xenogen–IVIS ® Spectrum (). Before analysis, mice were injected i.v. with 100 µL luciferin (45 mg/mL in PBS, Synchem OHG, Felsberg/Altenburg, Germany) and anesthetized with 2–4% isofluran. Photon emission was measured over an integration time of 1 second and recorded as pseudo-color images that were quantified using Living Image Software (PerkinElmer Inc. Waltham, MA).

2.5.6 Immunohistochemistry analysis

Extracted tumors were kept in PBS paraformaldehyde 4% (Sigma) overnight at 4°C (not exceed the incubation time). Samples were then transferred to PBS 30% Sacrose (Aplichem Panreac), 0,2% Sodium azide (Sigma) and incubated overnight at 4°C. Afterward tumors were moved to freezing blocks filled with OCT freezing media (Sakura Olympus) placed on dry ice and after freezing, stored at -80°C, at least 24 hours. Serial cuts were performed in 30µm using Leica Cryotome™ and stored at 4°C (preferentially -20°C). For staining slides were at first incubated for 30 minutes in PBS 0,1M

glycine (Sigma), 5 minutes in PBS 0,2%Triton (Sigma) and 2 hours in blocking buffer (4:3000 anti-CD16/CD32 (Table 2.1), PBS1%, BSA(Sigma) 3%, 0,2%Triton). After blocking, step slides were washed for 5 times in Perm buffer (PBS 0,2%Triton). For primary antibody, staining slides were incubated overnight at 4°C in 1:150 of anti-Mouse F4/80 (eBioscience) (Table 2.1) in blocking buffer. After washing, slides were incubated for 1hour at room temperature with a1:200 dilution of ALEXA647-CHICKEN α-RAT IgG (Invitrogen) in blocking solution. For nuclei staining, a 1:1000 concentration of DAPI (Life Technologies) in Perm buffer was incubated for 10 minutes and washed. The images were acquired with Leica TCS SP5 Confocal microscope and analyzed by ImageJ software.

2.6 Statistical analysis

The differences between groups were calculated using unpaired Student's t-test. Differences in survival between groups of mice were calculated using log-rank test. Values of $P < 0.05$ were considered to be statistically significant.

3 RESULTS

Results

3.1 *In vitro* generation of Ly6C^{high} monocytes from mice bone marrow

Monocytes originate from hematopoietic stem (HSC) cells in the bone marrow, are released into the blood stream and enter tissues where they can give rise to macrophages or DCs. Murine “inflammatory” monocytes, which are defined as CCR2^{+}CX3CR1^{low}Ly6C^{high}, can differentiate into a variety of macrophages and DCs subtypes. They have been shown to traffic to sites of inflammation playing a physiologic role in animal models of infection (King et al., 2009).

The isolation of murine monocytes from peripheral blood in physiological conditions remains impractical since they represent around 2–5% of circulating white blood cells. Therefore, large numbers of donor mice are required to obtain a significant blood volume and cell yield. The classic murine monocyte enrichment approaches requires the use of density gradient centrifugation (Berthold, 1981) with subsequent fluorescence-activated cell sorting (FACS) or multistep magnetic-activated cell sorting (MACS) (Francke et al., 2011). Unfortunately, in addition to being difficult, such isolation methods can alter the biological activity of the cells (Auffray et al., 2009), a major problem for downstream applications and experimental reproducibility. The difficulty of obtaining pure monocyte cultures greatly impedes progress in many research fields.

Due to the importance of Ly6C^{high} monocytes in inflammatory process as described in the introduction, the generation and isolation of high numbers of these cells *in vitro* allowed us to study their role in cancer.

In order to generate Ly6C^{high} monocytes, bone marrow of mice was extracted and cultured in a growth factor cocktail (protocol described previously in our laboratory and under patent). After 7 days, population of floating cells, forming aggregates (Figure3-1 B), were recovered from total culture and labeled by FITC-conjugated anti-Ly6C and PE-conjugated anti-CD11b. Afterward Ly6C^{high} population was stablished and sorted using MoFlo Systems sorter (Bechman Coulter, INC. Spain). The analysis of forward and side scatter characteristics by flow cytometry shows two populations of cells in the total culture: a population of 76% of Ly6C positive and 24 % of Ly6C negative, which both are almost 100% positive for CD11b. The Ly6C positive population can be separated in three subpopulations: 30 to 40% of Ly6C^{high}, 40% of Ly6C^{low} and 20 to 30% of Ly6C^{int} (Figure3-1 A). These subpopulations correspond with the three different states of Ly6C monocytes subsets in the blood, as described by different authors previously (Getts et al., 2008; Lin et al., 2009).

As explained before, Ly6C^{high} monocytes are the subtype of monocytes that are believed to have the ability to migrate in the blood stream and recruit to the site of inflammation and play important role in modulation of inflammation. So to study the role of monocytes in cancer, we decided to enrich just the Ly6C^{high} subpopulation from the various populations of monocytes in culture. Therefore, using a Moflo flow cytometer machine we sorted the pure population of Ly6C^{high} monocytes stained with FITC-conjugated anti-Ly6C (Figure3-1 A). After sorting 99% of cells were Ly6C^{high} monocytes.

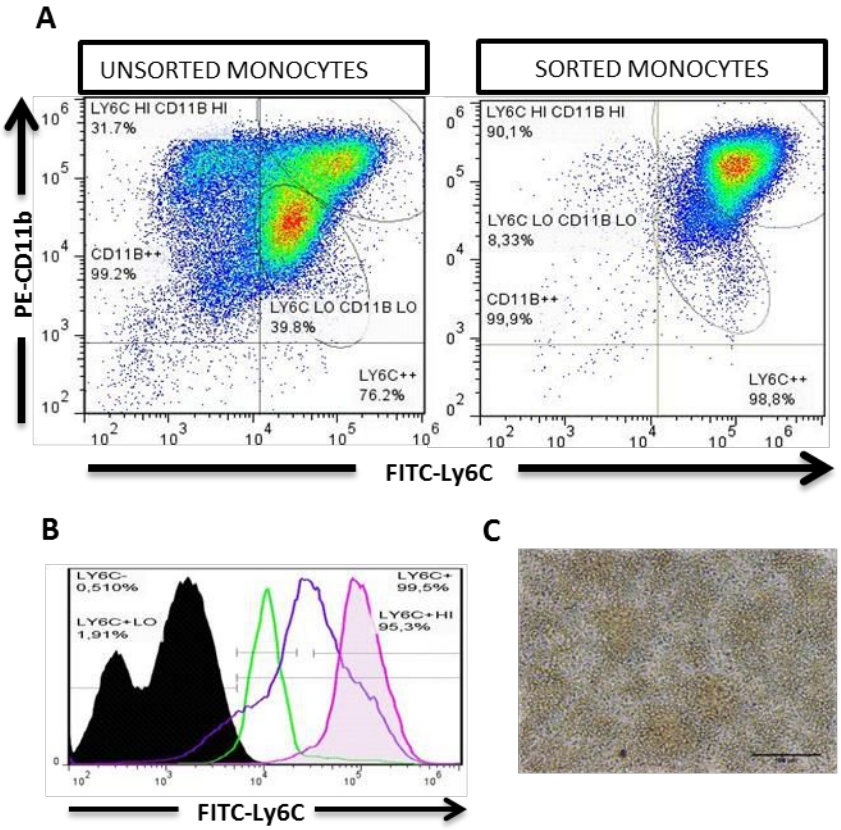

Figure 3-1. In vitro generation of Ly6C^{high} monocytes. The cells of mice bone marrow cultured with growth factor cocktail and after 7 days were stained with FITC-conjugated anti-Ly6C and PE-conjugated anti-CD11b antibody. A) Enrichment of pure Ly6C^{high} monocytes using a Moflo flow cytometer machine. B) Percentage of different populations of Ly6C monocytes in culture. C) Microscope image of cultured bone marrow populations of floating cells, forming aggregates. Results are representative of five different experiments.

3.1.1 Characterization of enriched Ly6C^{high} monocytes generated *in vitro*

In order to characterize the phenotype of *in vitro* cultured monocytes in our growth cocktail combination, the cells after 7 days of culture, were stained with several cellular markers. Surface protein markers defined by monoclonal antibodies provided an efficient tool to define various populations of monocytes and macrophages. Among these markers we used F4/80 (known as mature mouse macrophage marker), CCR2 (a marker well known to be involved in inflammatory monocyte recruitment) (Imhof and Aurrand-Lions, 2004), CD62L (L-selectin) (a cell adhesion molecule, which is involved in homing of lymphocytes) (Yang et al., 2011), CX3CR1 (expressed by monocytes and is a chemokine and adhesion molecule) and CD11c (a marker of macrophage differentiation).

The cultured cells presented the following phenotype: Ly6C^{high}CD11b^{+}CCR2^{+}CD62L^{+}F4/80^{+} (Figure3-2 B). To characterize the phenotype of Ly6C^{high} monocytes after sorting the Ly6C^{high}CD11b^{+} population was stained with APC-conjugated anti-CCR2, ALEXA700-conjugated anti-CD62L, APC-conjugated anti-F4/80, PECy7-conjugated anti-CX3CR1 and P.BLUE-conjugated anti-CD11c (Figure3-2 C). The results of at least 3 different experiments showed that the population of bone marrow generated Ly6C^{high} CD11b^{+} was around 65% CCR2^{+}, 80% CD62L^{+}, 60 % F4/80^{+}, 35% CX3CR1^{+} and 1% CD11c^{+}.

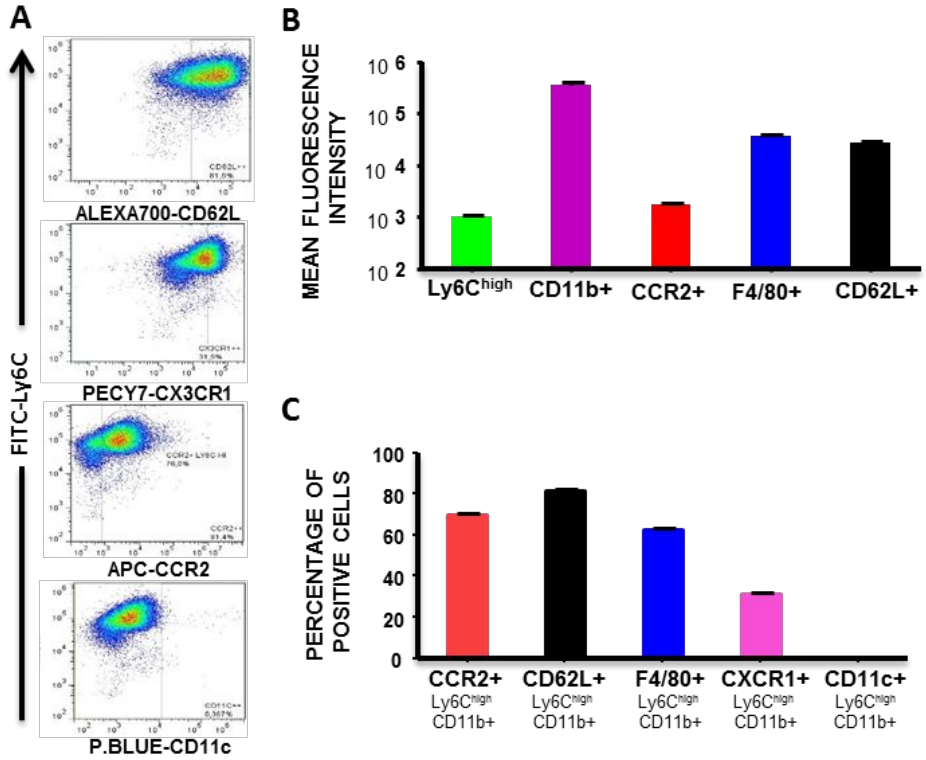

Figure 3-2 Characterization of *in vitro* generated Ly6C^{high} monocytes .After 7 days of culture, the in-vitro cultured monocytes in our growth cocktail combination were stained with FITC-conjugated anti-Ly6C. After sorting of the Ly6C^{high} monocytes, cells were stained with surface markers to characterize their phenotype. A) Forward and side scatter gating by flow cytometry Galios. B) Mean fluorescent intensity of Ly6C, CD11b, CCR2, F4/80 and CD62L in *in vitro* cultured monocytes. C) Percentage of positive cells: CCR2, F4/80 and CD62L, CX3CR1 and CD11c in Ly6ChighCD11b+ sorted population.

3.1.2 Staining of Ly6C^{high} monocytes

Ly6C^{high} monocytes are known to have the ability to migrate from the blood stream to inflamed tissues. Since our hypothesis is that the *in vitro* generated Ly6C^{high} monocytes are also capable to migrate to the site of inflammation after injection into blood stream, a proper method of staining is required to enable us the tracking of injected cells *in-vivo* and meanwhile observation of homing and recruitment of these cells in this case to tumoral tissue. We chose two different dyes for Ly6C^{high} monocytes staining: PKH26 and DiR.

PKH26 is a lipophilic dye with long aliphatic tails that binds irreversibly into the lipid regions of the cell membrane. PKH26 fluoresces in the yellow-orange region of the spectrum and has been found to be useful for *in vitro* and *in-vivo* cell tracking applications in a wide variety of systems (Ude et al., 2012). The appearance of labeled cells may vary from bright and uniform to punctate or patchy, depending on the cell type being labeled.

Therefore, in order to track the recruitment of Ly6C^{high} monocytes in vivo, in tumor histology experiments Ly6C^{high} monocytes were labeled in-vitro with PKH26 dye In Figure 3-3 A, a microscope image of stained Ly6C^{high} monocytes with PKH26, just after staining is shown. Additionally labeled Ly6C^{high} monocytes with PKH26 were monitored after injection in the blood stream with IVIS machine,. As shown in Figure 3-3 C, when PKH26 labeled Ly6C^{high} monocytes were injected intravenously, a significant increase in fluorescent intensity ($p<0.05$) was observed when compared to the mice injected with PBS as control.

DiR (1,1´-Dioctadecyl-3,3,3´,3´-Tetramethylindotricarbocyanine Iodide) is a colorant with weakly fluorescent in aqueous phase, but highly fluorescent and photo-stable when incorporated into membranes or bound to lipophilic biomolecules. These optical characteristics make them ideal for staining the cytoplasmic membrane of cells. Once applied to cells, these dyes diffuse laterally within the plasma membrane, resulting in staining of the entire cell. In Figure 3-3 B, FACS analysis of stained cells with DiR comparing to unstained Ly6C^{high} monocytes is shown. This fluorescent has excitation and emission maxima near infrared region, where many tissues are optically transparent. Therefore , when DiR labeled Ly6C^{high} monocytes were injected intravenous in the blood stream of the mice, higher increase in fluorescent intensity was obtained when compared to control mice ($p<0.01$) (Figure 3-3 D). Also, less background of auto-florescence was observed comparing to the mice injected with Ly6C^{high} monocytes PKH26 labeled (the number of injected cells in both experiments have been equal). We could conclude that for *in vivo* tracking Ly6C^{high} monocytes, DiR have advantages comparing to PHK26. On the other hand, DiR has some disadvantages, for example is not visible with microscope, so it cannot be used for histology.

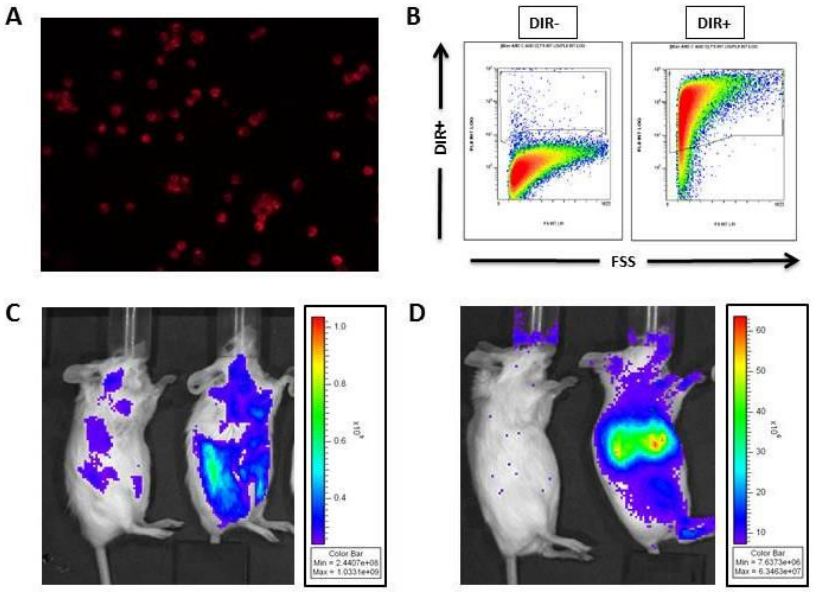

Figure 3-3 Staining of Ly6C^{high} monocytes with PKH26 and DIR. A) Microscope image of Ly6C^{high} monocytes dyed with PKH26 in-vitro (10x). B) Facs analysis of Ly6C^{high} monocytes dyed with DiR. C) In-vivo IVIS image of BLAB/C mice, on the right mice is injected IV with 2x10^{6} Ly6C^{high} monocytes labeled with PKH26 and mice on the left with PBS. (Image is taken at day 1 after injection). D) In-vivo IVIS image of BLAB/C mice, mice on the right is injected IV with 2x10^{6} Ly6C^{high} monocytes labeled with DiR and mice on the left with PBS. (Image is taken at day 1 after monocytes injection).

3.2 Role of Ly6C^{high} monocytes in immunodeficient MDA-MB-231 breast cancer model

3.2.1 Establishment of the breast tumor animal model to study the role of Ly6C^{high} monocytes

The primary step to study the role of Ly6C^{high} monocytes in breast cancer was to establish the tumor growth rate in this animal model. To achieve that we injected 5x10^5 MDA-MB-231 breast cancer cells in the mammary fat pad of the nude BALB/C and monitored the growth rate of the tumors, measuring external edges of the tumor by calipers to obtain the tumor growth rate. In order to study the impact of Ly6C^{high} monocytes, we also need to know for how long they will circulate in the blood stream after injection. By analyzing the *in vivo* images taken with IVIS machine after intra-cardiac injection of DiR labeled Ly6C^{high} monocytes daily, we observed that these cells remained in blood circulation at least for 7 days (

Figure **3-4**).

Next step was to determine the proper time for injection of Ly6C^{high} monocytes during tumor growth. Therefore, we performed several experiments of injecting Ly6C^{high} monocytes during different stages of tumor growth and also single or multiple injections of monocytes were tested to observe the modulatory effect of Ly6C^{high} monocytes. The results suggest that when breast tumors reach around 1500mm^3, multiple injections of Ly6C^{high} monocytes have larger impact on modulation of tumor growth (see results chapter 3.2.4).

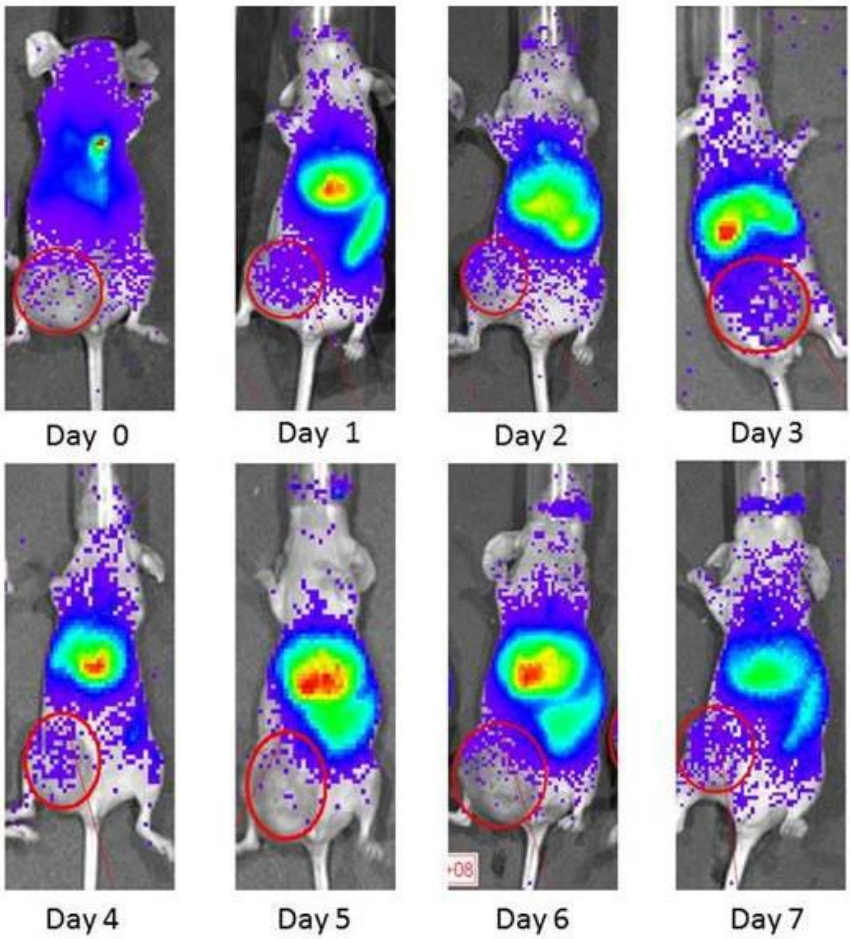

Figure 3-4. Circulation of Ly6C^{high} monocytes in-vivo. *In vivo* IVIS images of MDA-MB-231 tumor-bearing nude BALB/C injected with DiR labeled Ly6C^{high} monocytes in days 0 to 7 after monocytes injection.

3.2.2 Tracking the migration of *in vitro* generated Ly6C^{high} monocytes to breast cancer tumors

To evaluate the migratory capacity of *in vitro* generated Ly6C^{high} monocytes, after staining with "DiR", cells were injected intra-cardiac in nude-BALB/C mice bearing breast tumor of around 2000mm^3. Control group were mice with the same tumor size injected with PBS. IVIS images were taken immediately after injection, and at day one and two after injection. As shown before in

Figure **3-4** Ly6C^{high} monocytes circulate in the blood. To avoid auto-florescent of other parts of the body, in this experiment we captured IVIS images only of the tumor region. The image shows a significant increase in florescent signal of DiR dye indicating a specific migration of Ly6C^{high} monocytes to breast tumor.

Additionally, comparing to day1 after monocytes injection, more Ly6C^{high} monocytes accumulate into tumor at day 2 (Figure 3-5 A). However, analysis of mean florescent intensity taken by IVIS image did not show a significant difference. In this experiment mice were sacrificed at day 2 after injection of Ly6C^{high} monocytes or PBS and tumors were collected and IVIS image was taken. Data demonstrates specific migration of Ly6C^{high} monocytes just to tumor since as you can see in Figure 3-5 B tibia of the tumor-bearing mice injected with Ly6C^{high} monocytes shows no increase in florescent signal. This means that Ly6C^{high} monocytes mainly migrate to the site of inflammation that in this model is the tumor. The mean florescent intensity of Ly6C^{high} injected in the tumor gained IVIS image was significantly higher than the control.

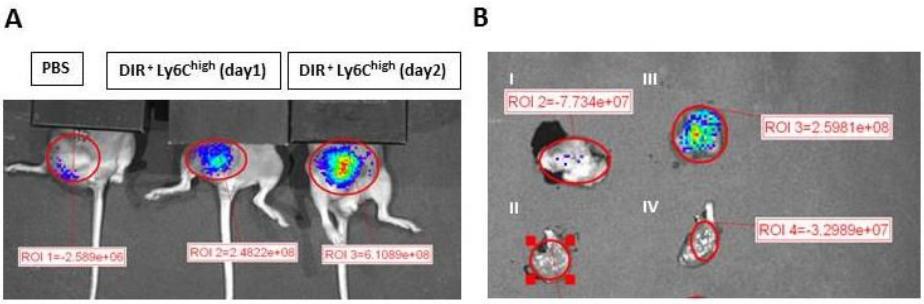

Figure 3-5. Specific migration of *in vitro* generated Ly6C^{high} monocytes to breast cancer tumor. A) *In-vivo* IVIS image of MDA-MB-231 breast tumors injected with of DiR^{+} Ly6C^{high} cells at days 1 and 3 after injection of monocytes and comparing to PBS injected in MDA-MB-231 breast tumor-bearing nude BALB/c. B) *In-vivo* IVIS image of: I) breast tumor of PBS injected mice (day 75 after MDA-MB-231 injection and day 2 after PBS injection). II) Tibia of PBS injected mice. III) Breast tumor of DiR^{+} Ly6C^{high} injected mice (day 75 after MDA-MB-231 injection and day 2 after of DiR^{+} Ly6C^{high} injection). IV) Tibia of DiR^{+} Ly6C^{high} injected mice.

3.2.3 Recruitment of *in vitro* generated Ly6C^{high} monocytes to breast tumor

In addition to observe the specific migration of *in vitro* generated Ly6C^{high} monocytes to breast tumor, which was proved by IVIS images, we were interested to see the recruitment of these cells to the tumor site in tissue sections by histology. Therefore, after staining with "PKH26", Ly6C^{high} monocytes were injected intra-cardiac in nude-BALB/C mice bearing breast tumor of around 2000mm^3. Control group were mice with the same tumor size injected with PBS. Mice were sacrificed two days after cell injection and harvested tumors were cryopreserved. Using the immunohistochemistry protocol described in materials and methods, the cryo-sections of tumors from both groups were labeled with surface marker for macrophages F4/80. Confocal images shows PKH26+ recruited cells to tumors, which some co-localize with F4/80^{+} cells.

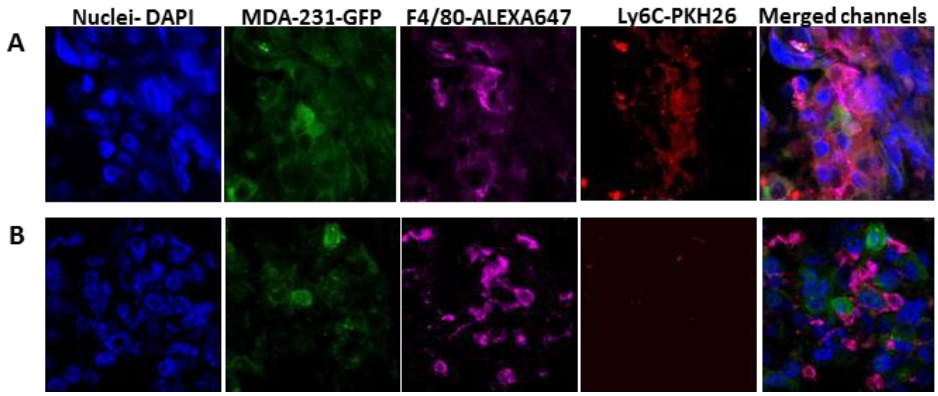

Figure 3-6 Confocal image of breast tumor cryo-sections to track recruitment of Ly6C^{high} monocytes in the tumor. Nude-BALB/C mice bearing breast tumors were injected intra-cardiac with PKH26^{+} Ly6C^{high} monocytes or with PBS. Cryo-sections of tumors from both groups were labeled with the surface marker for macrophages F4/80. A) Confocal microscope image of PKH26^{+} Ly6C^{high} monocytes injected. B) Confocal microscope image of PBS injected. Nuclei stained with DAPI in blue, MDA-MB-231 cancer cells transfected with GFP in green, macrophages labeled with F4/80-ALEXA647 in purple and PKH26^{+} Ly6C^{high} recruited cells in red.

3.2.4 Modulatory role of Ly6C^{high} monocytes on breast cancer

To study the modulatory role of *in vitro* generated Ly6C^{high} monocytes on the growth of breast tumor in nude BALB/c, mice were injected with 5x10^{5} MDA-MB-231 cells in the mammary fat pad and tumor volume was monitored weekly. When tumors reached approximately 1500mm^{3} in volume mice were randomly divided to two groups. One group received 3 times intra-cardiac injection of Ly6C^{high} monocytes (each time 2x10^{6} cells) during days 50 to 70 after tumor cell injection and the other group was injected with PBS in each time point. Data analyses of breast cancer volume shows that Ly6C^{high} injected tumors have smaller size compared to control group.

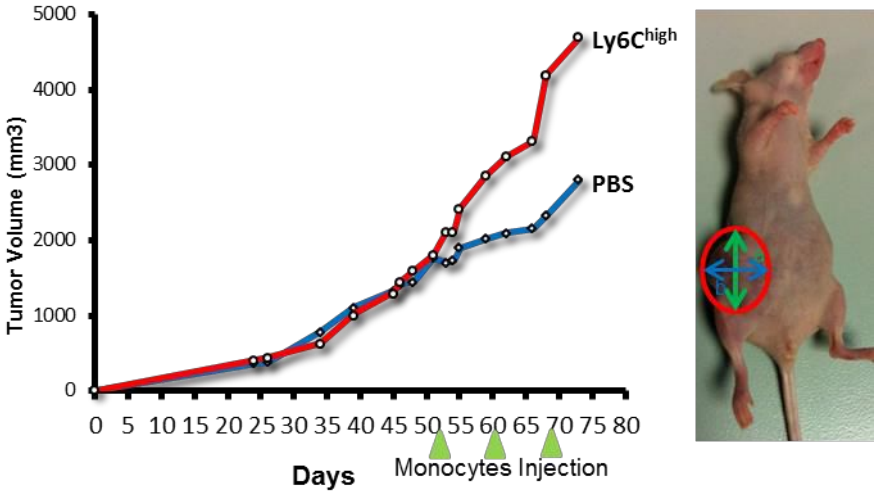

Figure 3-7. Modulatory role of Ly6C^{high} monocytes on breast tumor volume. Nude BALB/c were injected with $5x10^5$ MDA-MB-231 cells in the mammary fat pad and tumor volume was monitored weekly by measurement of external edges of tumor using Vernier calipers and with this formula $(4/3\pi(a+b/2)^3)$. One group received 3 times intra-cardiac injection of Ly6C^{high} monocytes during days 50 to 70 after tumor cell injection (each time $2x10^6$ cells) and the other group was injected with PBS. Difference between the tumor volume of two groups was significant ($P<0.05$) (n=4).

3.2.5 Role of Ly6C^{high} monocytes on the breast tumor pro-inflammatory and anti-inflammatory gene expression

Breast tumors from the two groups described in part 3.2.4 were harvested at day 75 after tumor cell injection. From the whole tumor mRNA was extracted and expression of some pro-inflammatory and anti-inflammatory genes was measured by Real-time PCR.

Mice injected with Ly6C^{high} monocytes present lower expression of anti-inflammatory genes such as mannose receptor, arginase-1 and *cox2* (Figure3-9). On the contrary, *tnfα* was highly expressed in mice where Ly6C^{high} was injected. Meanwhile, other pro-inflammatory genes like *il1β* and *il6* are expressed equally in both groups.

Additionally, we determine the expression of TGF-β, which is a growth factor for tumor cells secreted by TAMs and tumor cells (Gupta et al., 2014). Also, MMP9, that is involved in the breakdown of extracellular matrix and angiogenesis helping the tumor growth was determined (Lu et al., 2012). The expression of both, TGF-β and MMP9 was decreased in the tumors obtained from Ly6C^{high} injected mice, relative to ones obtained from the control group.

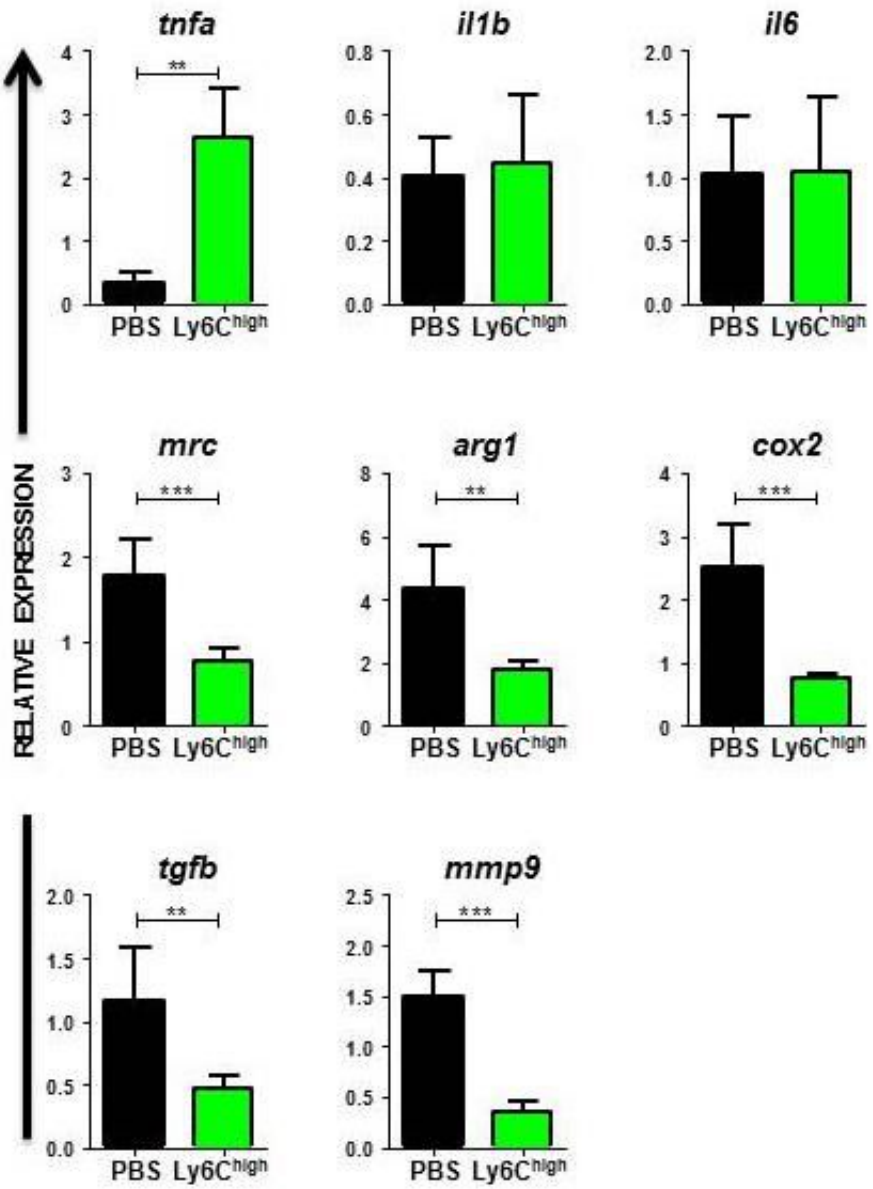

Figure 3-8 Effect of Ly6C^{high} monocytes on the gene expression of breast tumor. Nude BALB/C mice carrying a breast tumor were inoculated 3 times with Ly6C^{high} monocytes during days 50 to 70 after tumor cell inoculation. Control group received PBS. At day 75 tumors were harvested mRNA was extracted and gene expressions was measured by Real-time PCR. The result represents the means ± SEM of two independent experiments with 4 mice per group of study. (*P<0.05, ** P<0.001, *** P<0.0001)

3.2.6 Modulatory role on breast cancer of Ly6C^{high} monocytes generated from STAT6$^{-/-}$ KO mice

It is known that STAT6 in response to cytokines and growth factors, is phosphorylates and trans-locates to the cell nucleus, where it acts as transcriptional activator. This protein plays a central role in the IL-4 responses (Kis et al., 2011). In the STAT6 knockout (KO) mice the involvement of this protein in Th2 responses has been demonstrated (Hebenstreit et al., 2006). Moreover, in macrophages, IL-4 induces an anti-inflammatory phenotype and therefore STAT6 is crucial to express the M2 phenotype.

In the next experiments we wanted to see whether the generation of Ly6C^{high} cells from STAT6 knockout mice will lead to activate this monocytes toward infiltration to tumor in a pro-inflammatory phenotype direction. For these experiments we extracted bone marrow of STAT6$^{-/-}$ mice to generate Ly6C^{high} monocytes and cells were injected in tumor-bearing mice exactly in the same conditions as explained in part 3.2.4.

We observed that the breast tumor grows less when injected with Ly6C^{high} monocytes generated from STAT6$^{-/-}$ mice, suggesting that deletion of the STAT6 gene play a role in tumor growth mediated by macrophages. It is possible that the absence of STA6 enhances pro-inflammatory polarization of Ly6C^{high} monocytes.

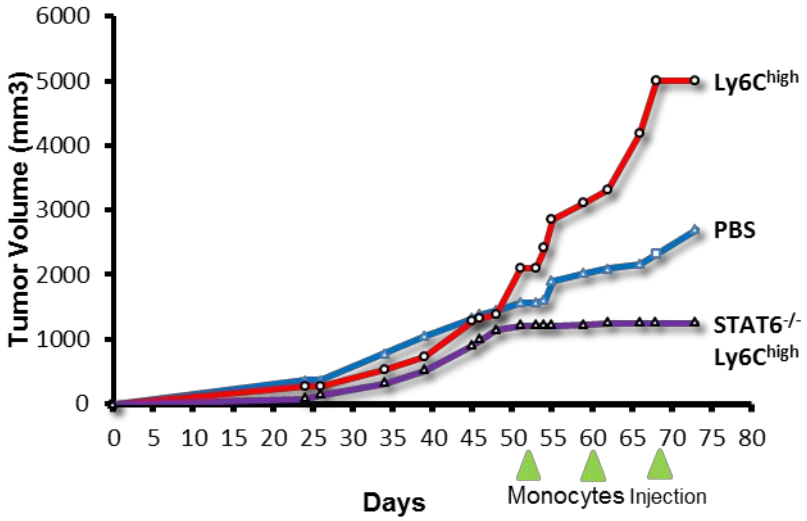

Figure 3-9 Modulatory role of Ly6C^{high} monocytes generated from STAT6$^{-/-}$ mice on breast tumor volume. Nude BALB/C mice carrying a breast tumor were inoculated 3 times with Ly6C^{high} monocytes from STAT6 KO mice or from control during days 50 to 70 after tumor cell inoculation. Control group received PBS. Tumor volume was monitored weekly by measurement of external edges of tumor using Vernier calipers. Difference between the tumor volume of the group injected with Ly6Chigh monocytes generated from STAT6$^{-/-}$ mice and group of PBS was significant ($P<0.05$) (n=4).

3.3 The role of Ly6C^{high} monocytes in C26 colon carcinoma model

Immune-deficient mice engrafted with human cells like MDA-MB-231 have provided an exciting alternative for the study of human tumor biology. However, there are a number of limitations in such humanized mouse models. First, the immune system is not functional in this animal model and second, since we inject murine derived cells in our model, an allo-reaction between human and murine tissues can be produced. For these reason, our results

could be influenced by factors rather than the micro-ambient of normal tumor development.

For these reasons we decided to change our experimental model to an homologous system, using the same strain of mice for both generation of Ly6C^{high} monocytes and also as a cancer animal model. Therefore, we chose another animal model of cancer, to study the role of Ly6C^{high} monocytes in cancer,.which is C26 colon carcinoma model.

This tumor was induced in BALB/C mice by a chemical mutagenesis. The subcutaneous inoculation of this tumor cell line in BALB/C mice, causes severe cachexia and rapid growth of a tumor, which leads to mortality (Murphy et al., 2012).

3.3.1 Establishment of C26 colon carcinoma model

The first step to examine the role of *in vitro* generated Ly6C^{high} monocytes on C26 colon carcinoma, is to establish the cancer animal model. For this aim, we inoculated 1x10^6 C26 cancer cells subcutaneously behind the neck of 20 female BALB/C, 8 weeks old. Body weight was measured daily to obtain the rate of weight lost, since this cancer induces sever cachexia. As a control, to monitor the body weight loss, 8 female BALB/C 8 weeks old from the same origin were picked randomly and also weighed daily.

During the tumor growth at days 5, 6, 7, 9 and 11, each day 4 mice were sacrificed and the tumor volume and weigh were measured. At day 11 the last remaining group of mice were sacrificed and the percentage of weight loss was measured and compared to non-bearing tumor mice. As shown in Figure 3-11 C, by day 11 C26 tumor-bearing mice had lost almost 15 % of initial body weight. This difference in body weight comparing to control group was significant ($p<0.0001$).

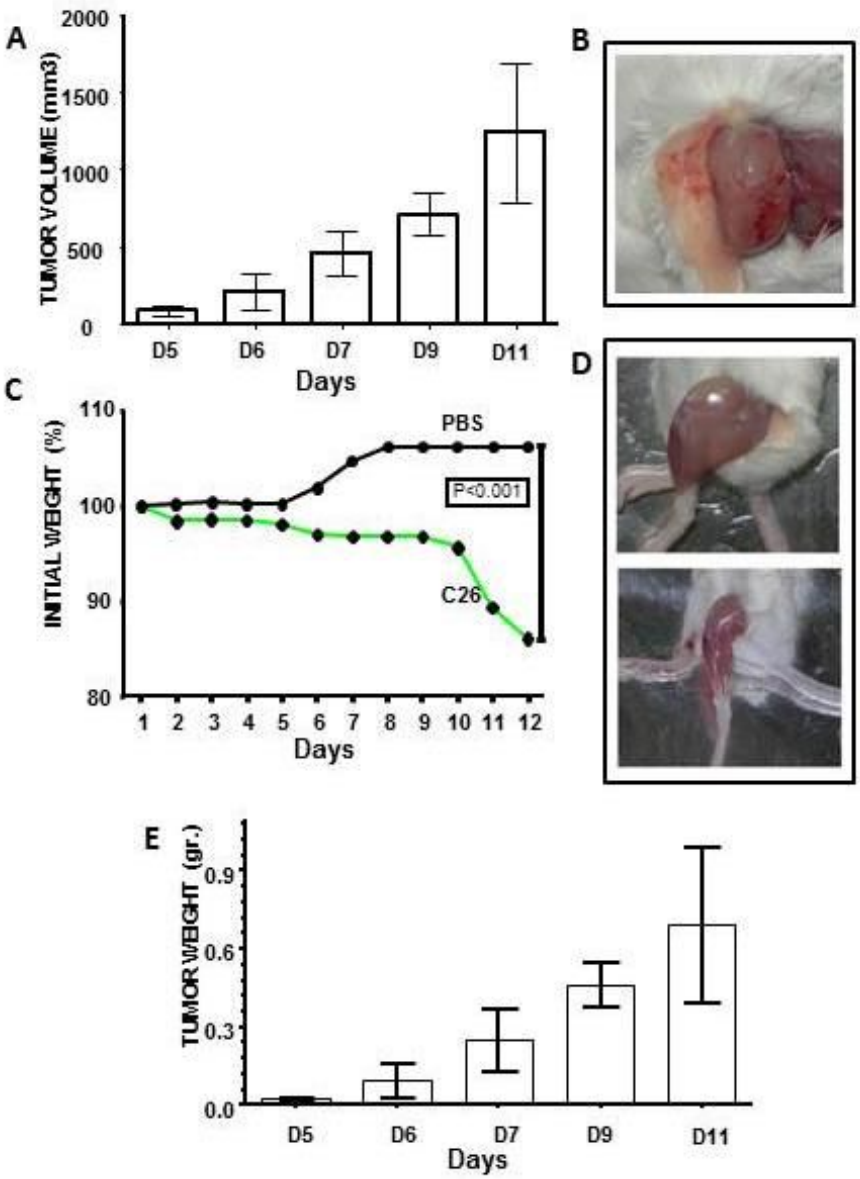

Figure 3-10. Establishment of C26 Colon Carcinoma model. A) BALB/C mice were inoculated subcutaneously with $1x10^6$ C26 cancer cells and a group of BALB/C with the same age and weight were used as control. During the tumor growth at days 5, 6, 7, 9 and 11, 4 mice were sacrificed and the tumor volume and weigh were measured. A) Tumor volume; B) Image of C26 tumor at day 9: C) Percentage of initial weight; D) Image of waste in muscle caused by severe cachexia at day 11: E) Tumor weight. Result represent the means ± SEM (n=4 in each time point of sacrifice and n=8 in control group)

Additionally, in order to check if infiltrated leukocytes to tumors increase during tumor growth, tumors obtained in each time point, were digested and single cell suspension was stained with PECY7-conjugated anti-CD45, FITC-conjugated anti-Ly6C and PE-conjugated anti-CD11b (Figure 3-12). According to tumor weight we characterized three scores in tumor growth (Figure 3-1). The results show an increase in the infiltrated $CD45^{+}Ly6C^{high}CD11b^{high}$ monocytes into tumors, that correlates with the tumor size.

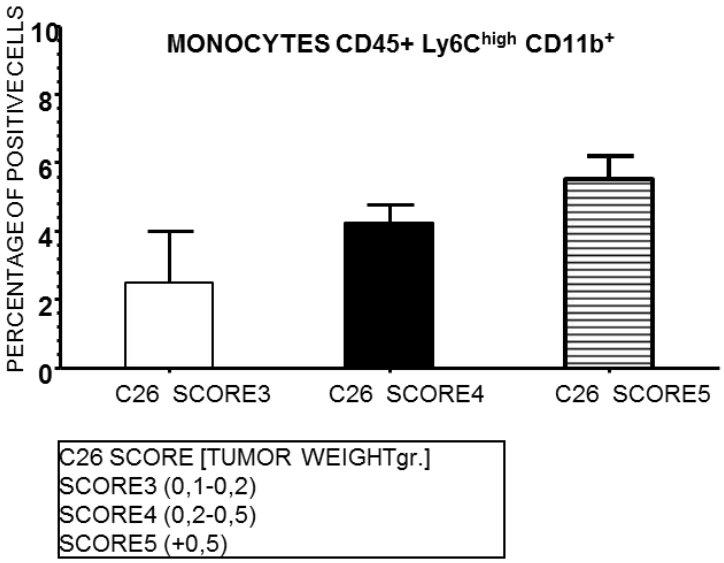

Figure 3-11 Monocytes infiltration to C26 tumor. BALB/C mice were inoculated subcutaneously with 1x106 C26 cancer cells and tumors obtained in each time point, were digested and single cell suspension was stained with PECY7-conjugated anti-CD45, FITC-conjugated anti-Ly6C and PE-conjugated anti-CD11b. According to tumor weight we characterized three scores in tumor growth. Graph shows the percentage of positive cells analyzed by FACS GALIOS. The result represent the means ± SEM (n=4).

3.3.2 Modulatory role of Ly6C^{high} monocytes in C26 colon carcinoma

To study the role of *in vitro* generated Ly6C^{high} monocytes on C26 colon carcinoma cancer growth, we outlined an experimental design. In this model after subcutaneously injection of 1x10^6 C26 cells in 8 week olds female BALB/C, mice were randomly divided in 2 groups of 8 animals: one group receiving intra-vein injection of PBS as control and the treatment group, 2x10^6 Ly6C^{high} monocytes at days 0, 3 and 6 after cancer cells injection. The aim of this experiment was to observe a possible modulatory impact of Ly6C^{high} monocytes on the progression of cancer comparing to control group.. Body weight of animals was measured daily and the percentage of survival was calculated. Body weight in the control group initiates the dramatic fall at day 8 post cancer injection, while the treatment group start to loss body weight, with two days delay at day 10 (Figure3-13 B). This indicates that the injection of Ly6C^{high} monocytes, slower the cancer progression and reduces the dramatic cachexia observed in the controls. More importantly, while all the control group mice were dead by day 15 after cancer injection, the group that Ly6C^{high} monocytes were injected remained alive and showed a significantly ($p<0.001$) higher survival relative comaring to control group (Figure3-13 A)

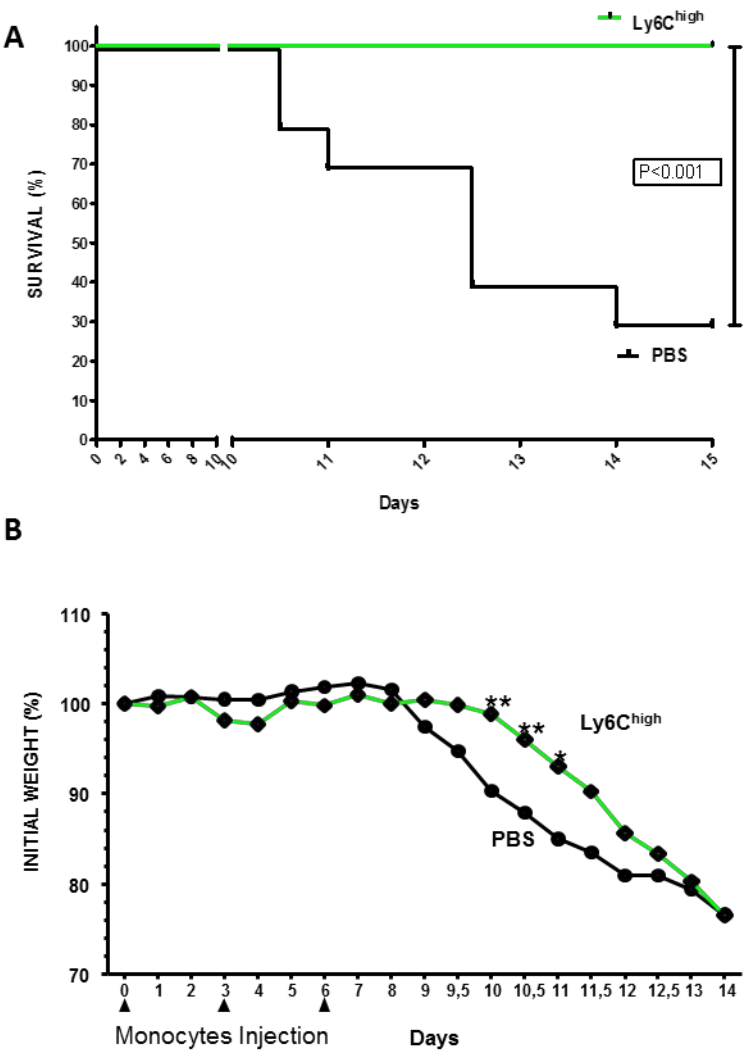

Figure 3-12. Modulatory role of Ly6C^{high} monocytes in C26 colon carcinoma. BALB/C mice were subcutaneously injected with 1x10^6 C26 cells and randomly divided in 2 groups: one group receiving IV 2x10^6 Ly6Chigh monocytes or PBS at days 0, 3 and 6 after cancer cells injection. A) Percentage of survival. B) Percentage of initial weight. (*P<0.05, ** P<0.001, *** P<0.0001) (in each group n=8).

3.3.2.1 Determination of the accurate time to inject Ly6C^{high} monocytes in C26 colon carcinoma model

In the previous experiment we observed the modulation of Ly6C^{high} monocytes on C26 colon cancer progression with three injections of these cells. We were interested to see whether one or two injections of Ly6C^{high} cells have the same impact on the survival rate. In addition, another aim of this experiment was to see which time point of cancer growth is critical to inject Ly6C^{high} monocytes and will lead to higher impact of these cells on C26 cancer growth. To perform these aims, after injection of C26 cancer cells in 8 weeks old female BALB/C, mice were randomly divided to 4 groups of 6 animals receiving either a single injection of $2x10^6$ Ly6C^{high} monocytes at day 0, 3 or 6, or double injections of $2x10^6$ Ly6C^{high} cells, at days 0 and 3, 0 and 6 or 3 and 6. As control, one group was injected with PBS.

A single injection of Ly6C^{high} monocytes in various time points of cancer growth results in higher survival rates in comparison to control group (Figure3-14 A). However, the impact of single injections is less effective when compared with twice injection of Ly6C^{high} monocytes. The group of mice with injection of Ly6C^{high} monocytes at days 3 and 6, had the highest percentage of survival among all the groups of study (Figure3-14 B).

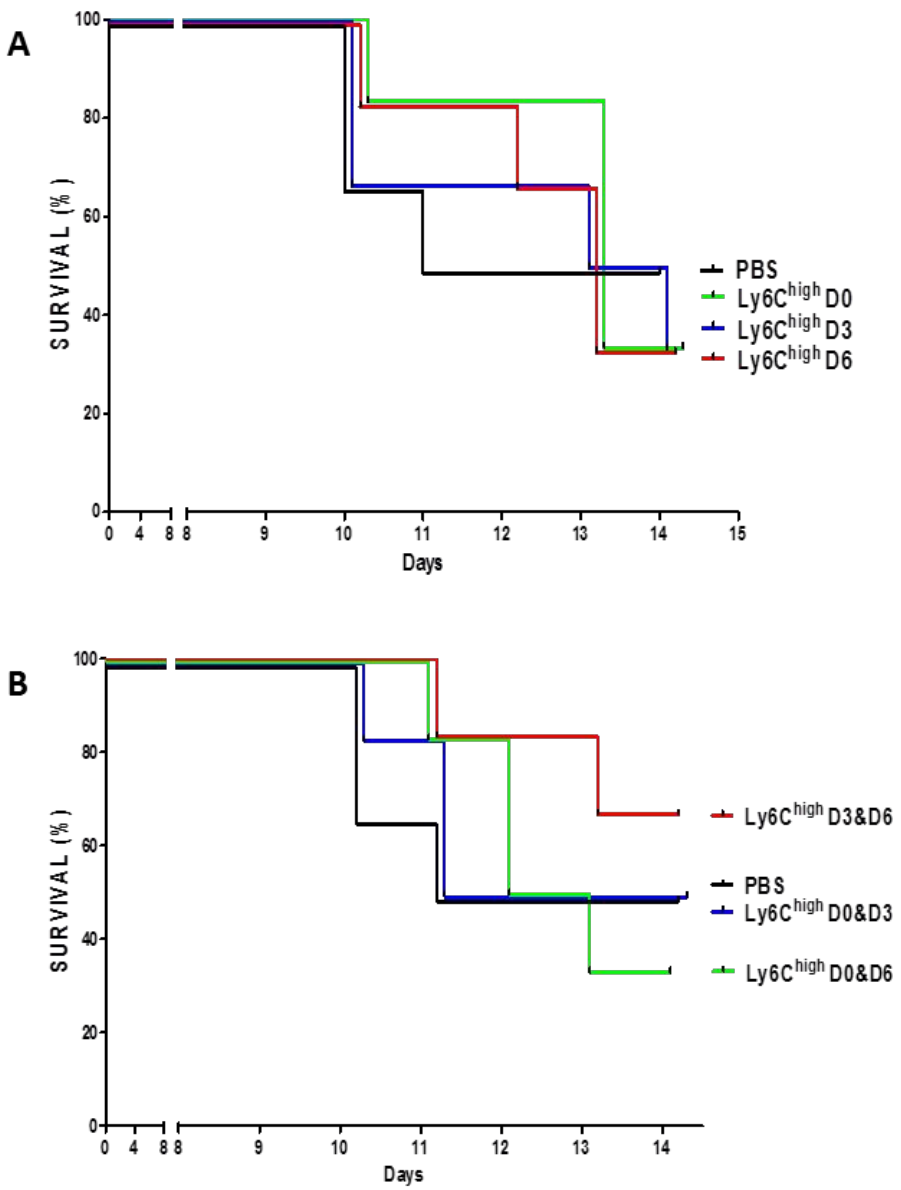

Figure 3-13. Determination of the time to inject Ly6C^{high} monocytes in the C26 colon carcinoma model. A) Percentage of survival of C26-beraing mice with single injection of Ly6C^{high} monocytes at day 0, 3 or 6 compared to mice injected with PBS. B) Percentage of survival of C26-beraing mice receiving double injections of Ly6C^{high} cells, at days 0 and 3, 0 and 6 or 3 and 6, relative to mice injected with PBS. (n=6 in each group)

3.3.3 Effect of Ly6C^{high} monocytes on the C26 tumor gene expression

To gain further insight in the potential function of Ly6C^{high} monocytes subpopulations in tumor, the expression of different surface markers was analyzed. Treatment group were injected with 2x10^6 Ly6C^{high} cells at days 3 and 6 after C26 injection and the control group with PBS. The tumors of both groups were harvested at day 10 after cancer cell injection. The whole tumor mRNA was extracted and expression of some genes was measured by Real-time PCR.

The mechanism of caquexia in C26 colon carcinoma is due to the interaction between the tumor and TAMs that release pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Bonetto et al., 2011). In our study we observed that in the group of injected Ly6C^{high} monocytes the expression of IL-6 and IL-1β is reduced comparing to the control group where PBS was injected. However, the expression of TNF-α was similar in both groups. These data suggest that the modulatory effect of Ly6C^{high} monocytes on the C26 cancer progression, might be due to recruitment and polarization of these cells towards less production of IL-6 and IL-1β (**Figure 3-14**).

In addition, the results indicates that mice injected with Ly6C^{high} monocytes exhibited lower expression of anti-inflammatory genes such as mannose receptor, arginase-1 and *cox2* (Figure3-15). This might suggest that the tumor-infiltrating monocytes down-regulate the expression of anti-inflammatory genes. The relative expression of TGF-β and MMP9 were significantly lower in the Ly6C^{high} injected tumors, which may explain the lower tumor growth rate observed.

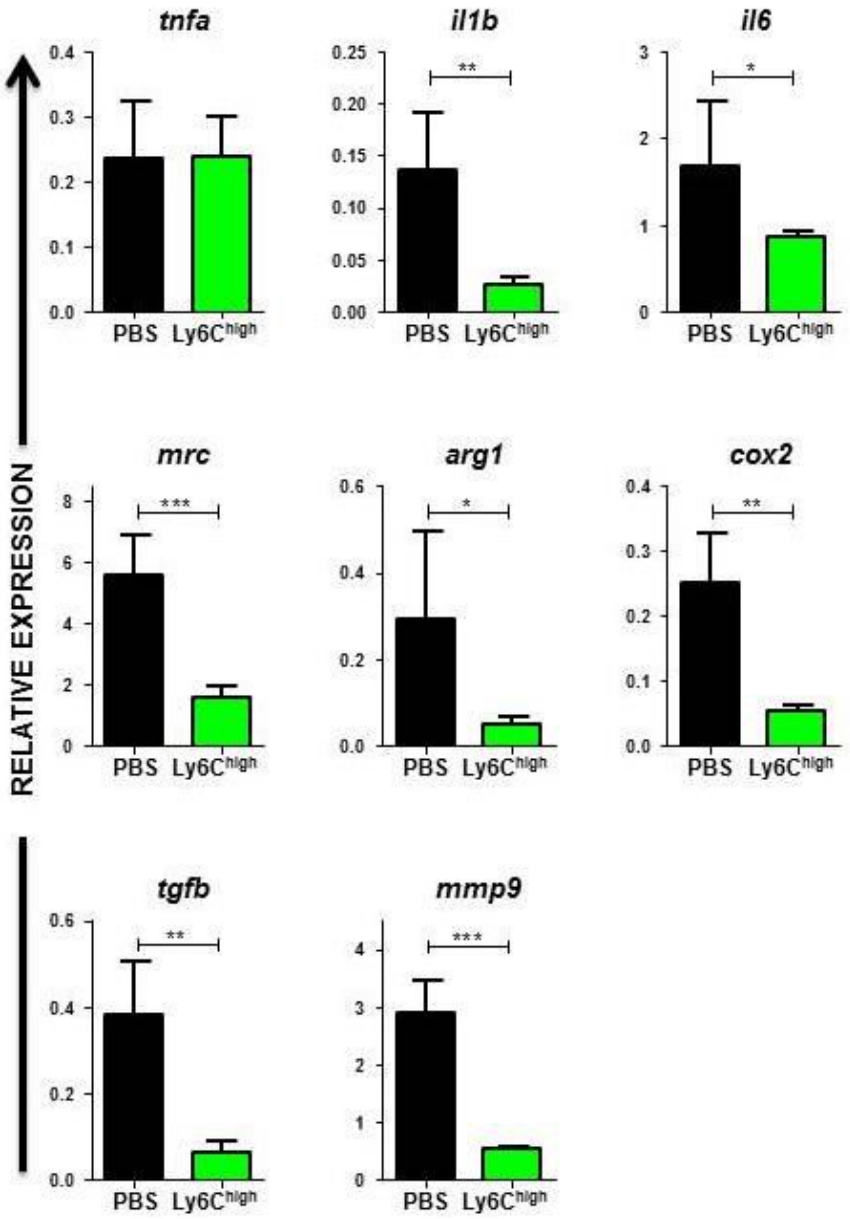

Figure 3-14. Effect of Ly6C^{high} monocytes on the C26 tumor gene expression. BALB/C. mice carrying C26 tumors receiving 2 IV injections of Ly6C^{high} monocytes at days 3 and 6 after tumor cells inoculation. As control, mice received PBS. Tumors were harvested at day 10 and mRNA was extracted and gene expressions were measured by Real-time PCR. The results represent the means ± SEM of two independent experiments with 8 mice per group. (*P<0.05, ** P<0.001, *** P<0.0001)

3.3.4 Characterization of C26 tumor leukocytes in mice receiving Ly6C^{high} monocytes

To characterize the phenotype of tumor cells, we screened the surface markers, which are associated with the monocytic/macrophage phenotype. C26 tumors were harvested after 10 days of tumor cell inoculation from both groups of PBS and Ly6C^{high} injected animals. Tumors were digested and total tumor cells were stained with PE-CY7-conjugated anti-CD45 and analyzed by flow cytometry. Ly6C^{high} injected tumors had slightly higher percentage of CD45^{+} leukocytes relative to PBS injected tumors, but this difference was not significant (Figure 3-15).

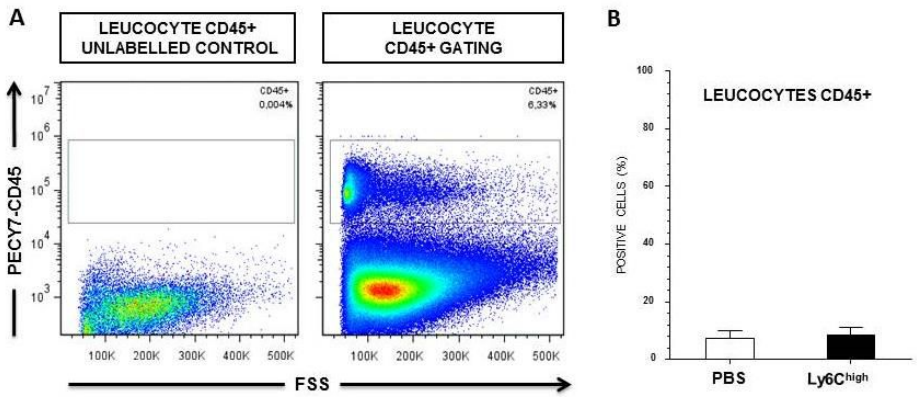

Figure 3-15. Characterization of C26 tumor leukocytes injected with Ly6C^{high} monocytes. A) Gating the C26 tumor cells stained with PE-CY7-conjugated anti-CD45. B) Percentage of CD45+ leukocytes in the tumors extracted from PBS and Ly6C^{high} injected in C26-bearing mice.

Furthermore, we characterized the subsets of leukocytes infiltrating the C26-tumor. Single cell suspensions of cell tumors were stained with several surface markers as described in Materials and methods. Around 8 % of the whole tumor cells were $CD45^+$ leukocytes in both groups (Figure 3-15). Further charachterization of this 8% $CD45^+$ leukocytes, showed the largest proportion belongs to macrophages ($CD45^+F4/80^+CD68^+$), which were 50% of leukocytes in the control tumors and almost 45% in $Ly6C^{high}$ injected tumors. Monocytes ($CD45^+Ly6C^{high}CD11b^+$) represented 10% of leukocytes in the control tumors and 15% in the $Ly6C^{high}$ treated group. T cells ($CD45^+CD3^+$) and B cells ($CD45^+CD45R^+$) percentages were similar in both groups of study and were respectively 20% and 50% of leukocytes of C26 tumors.

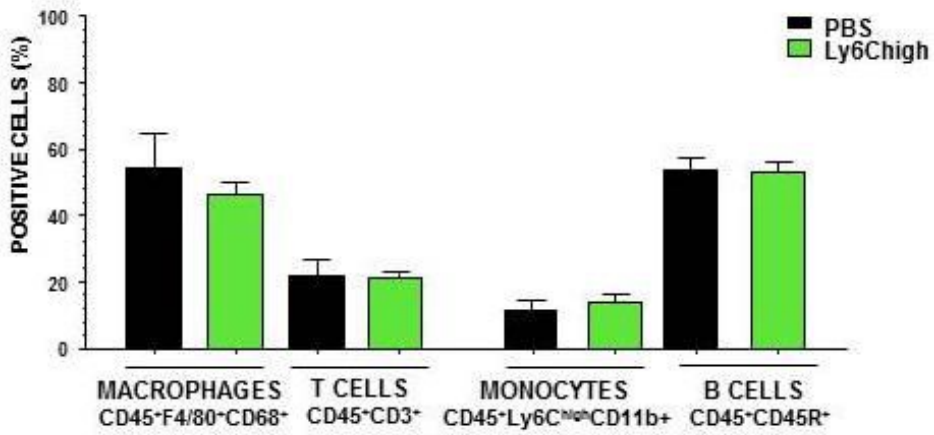

Figure 3-16. Characterization of leukocytes in C26 tumor. A) Cells from tumors of PBS and $Ly6C^{high}$ injected C26-bearing mice were extracted and stained with the following surface markers: macrophages (CD45+F4/80+CD68+), monocytes (CD45+Ly6ChighCD11b+), T cells (CD45+CD3+) and B cells (CD45+CD45R+).

3.3.5 Role of Ly6C^{high} monocytes pre-treated with IL-4 or INF-γ in C26 Colon Carcinoma

The treatment of monocytes with IFN-γ activate them towards differentiation to M1-like macrophages, whereas treatment with IL-4 induces the differentiation to M2-like macrophages (Davis et al., 2013). To study the effect of these treatments we designed a new experiment. Our goal was to see the difference in the impact of Ly6C^{high} monocytes on progression of cancer, while stimulated to polarize to M1 or M2-like phenotype. After subcutaneous injection of 1X10^{6} C26 cells in eight weeks old BALB/C female mice 4 groups of 8 animals were randomly separated. These groups received I.V injection of BPS, Ly6C^{high}, Ly6C^{high} treated for 30 minutes with IFN-γ (20μg/ml) or Ly6C^{high} treated for 30 minutes with IL-4 (10ng/ml), at days 3 and 6 after injecting cancer cells. Daily body weight and percentage of survival were measured. The results demonstrate that the groups of Ly6C^{high} and Ly6C^{high}-IL-4 treated monocytes had the highest rate of survival among all the groups and the difference between these two groups and the control group was statistically significant ($p<0.05$). In contrast, the group of mice injected with Ly6C^{high}-IFNγ treated had lower survival rate relative to those two groups (Figure3-18). These result indicate that pre-activation of Ly6C^{high} monocytes toward pro-inflammatory phenotype will decrease their impact on the progression of C26 colon cancer, whereas by activation of these cells to more anti-inflammatory polarization, they become more effective in delaying cachexia.

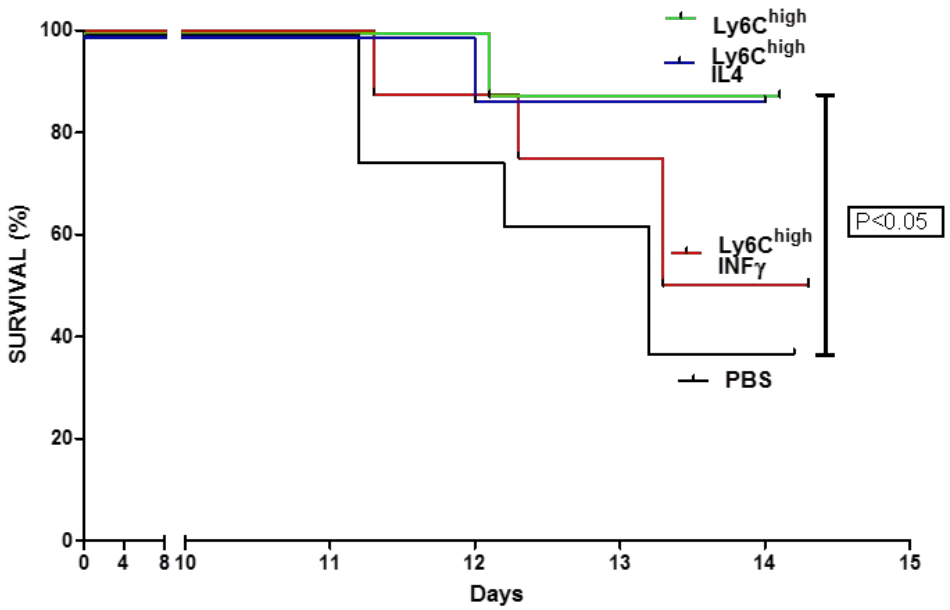

Figure 3-17. Effect of Ly6C^{high} monocytes treatment with IFN-γ or IL-4 on C26 tumor-bearing mice survival. Mice bearing C26 carcinoma were injected with Ly6C^{high} monocytes, treated with PBS, IFN-γ (20ng/ml) or IL-4 (10ng/ml), at days 3 and 6 after C26 cells subcutaneous injection (n=8 in each group).

3.3.5.1 Role of Ly6C^{high} monocytes treated with IFN-γ or IL-4 on C26 tumor growth

For further investigations on the impact of activated Ly6C^{high} monocytes with IFN-γ or IL-4 the same cancer model as described in chapter 3.3.5 was used. From each group 4 mice were euthanized at day 9 and 4 mice at day 10. The percentage of initial weight was calculated, tumors were extracted and their weights measured and compared among all groups.

As expected, regarding to our previous experiment, the tumors of the group injected with Ly6C^{high} cells treated with IL-4 were smaller comparing to the tumors of injected Ly6C^{high} cells treated with IFN-γ but still the group of Ly6C^{high} monocytes injected had the smallest and PBS injected the biggest tumors of all groups. Additionally percentage of initial weight loss shows that the rate of cachexia is approximately similar in the groups of mice injected with Ly6C^{high} and IL-4 pre-treated Ly6C^{high}, while the percentage of body weight loss is higher than these two groups in the IFN-γ pre-treated Ly6C^{high} group and the control group had the highest body weight due to cachexia (Figure3-19).

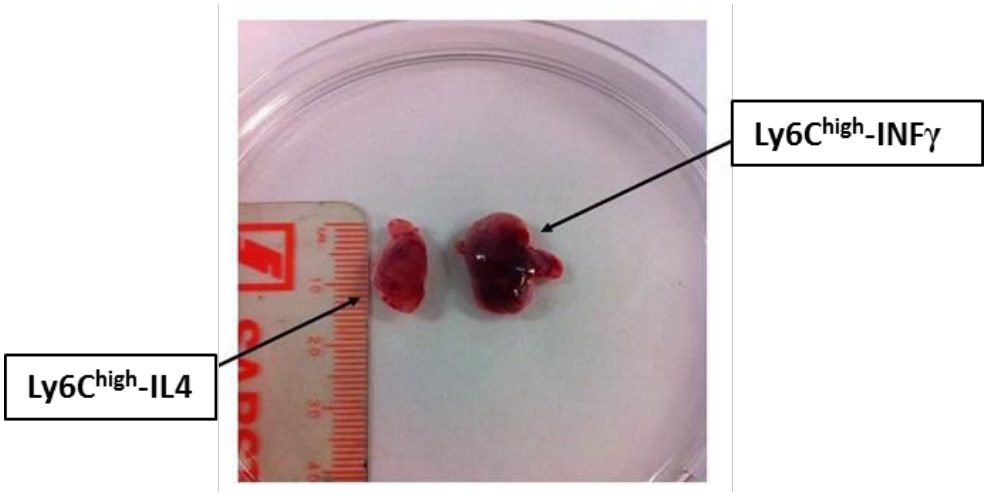

Figure 3-18. Photographic illustration from the impact of pre-activated Ly6C^{high} monocytes with IFN-γ or IL-4 on C26 tumor size. Photographic illustration of tumors excised from mice with double injections of 2x10^6 Ly6C^{high} pre-treated with IFN-γ (20μg/ml) or Ly6C^{high} pre-treated with IL-4 (10ng/ml), at days 3 and 6 after C26 cells subcutaneous injection, on the day of authanasia (day10).

3.3.5.2 Impact of pre-treated Ly6C^{high} monocytes with IFN-γ or IL-4 and Ly6C^{high} monocytes on C26 tumor growth, when injected in more advanced tumor stages

For further investigations on the impact of pre-activated Ly6C^{high} monocytes with IFN-γ and IL-4 the same cancer model as described in chapter 3.3.5 was repeated and this time injections were performed at day 6 and 9. All mice were sacrificed on day 10 and tumor growth and survival was monitored among all groups.

Results demonstrate that this delay in the injection of Ly6C^{high} and pre-treated Ly6C^{high} monocytes when the tumor is more grown, will decrease their effect on the progression if the C26 cancer, because as shown in Figure 3-20 tumors of all the three groups injected with monocytes at days 6 and 9 grow faster comparing to those of injected at days 3 and 6 post-cancer induction and relatively cachexia is more sever in this condition.

Therefore, we can conclude from this experiment that Ly6C^{high} and pre-treated Ly6C^{high} monocytes have higher impact on tumor growth when they are injected in the early stages of C26 colon cancer.

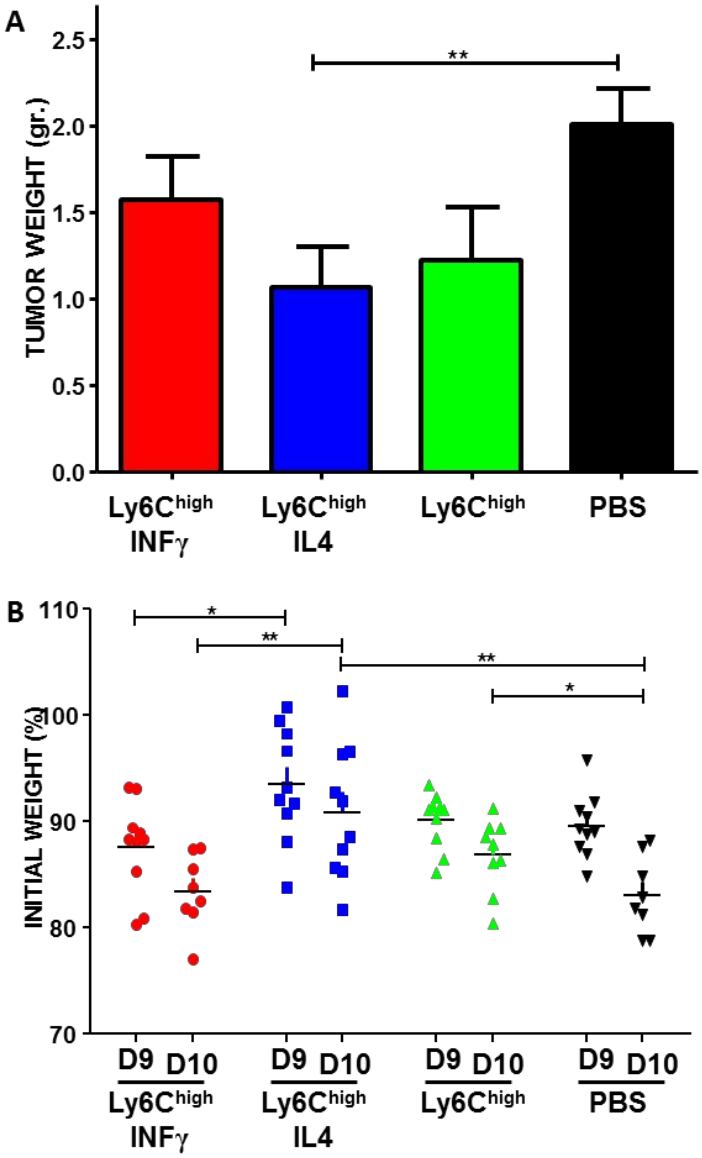

Figure 3-19. Impact of pre-treated Ly6C^{high} monocytes with IFN-γ or IL-4 and Ly6C^{high} monocytes on C26 tumor growth, when injected in more advanced tumor stages. A) C26 tumor weight of mice with double injections of $2x10^6$ Ly6C^{high}, Ly6C^{high} pre-treated with IFN-γ (20μg/ml) or Ly6C^{high} pre-treated with IL-4 (10ng/ml), at days 6 and 9 after C26 cells subcutaneous injection, relative to PBS injected mice. (n=4 in each time-point). B) Percentage of initial weight compared between mentioned groups.

4 DISCUSSION

Discussion

Established therapies currently used to treat cancer include surgery, local radiotherapy and chemotherapy. However, even when the primary tumor was initially removed, remaining micrometastases and cancer stem cells frequently lead to tumor relapse and therapeutic failure. Therefore, it became clear that it is not sufficient to develop strategies only to eliminate cancer cells but also approaches are needed that stimulate anti-tumor immune responses. For example dendritic cells pulsed with tumor antigens are broadly applied in cancer patients (Mosser and Edwards, 2008). Adoptive cell therapy using autologous tumor-infiltrating T cells was an effective treatment for patients with metastatic melanoma (Duffield et al., 2005)

Researchers over the last decades observed that tumor-infiltrating cells play a dual role in cancer. They do not only suppress tumor growth by destroying cancer cells or inhibiting their outgrowth, they also promote tumor progression by inducing immune suppression. Understanding how these cells affect tumor development and progression is one of the most challenging questions in tumor immunology. Immune cells are involved in tumor immune-surveillance and elimination. Certain immune cells were shown to exhibit major role on cancer progression. These cells include macrophages and monocytes. They accumulate in blood and spleen, but are also found in the tumor tissue, where they are recruited by tumor-derived factors (Mehlen and Puisieux, 2006; Onitilo et al., 2009).

Further knowledge about the exact potential of macrophages and monocytes populations within the tumor microenvironment, including their phenotypical and functional properties, might improve therapeutic strategies to overcome immune suppression within the tumor tissue. The present study was undertaken to understand the interaction of tumor cells, with immune

cells like monocytes/macrophages to establish new insights into such crosstalk.

Macrophages are a major population of immune cells within tumors and based on their contrasting biochemical, killer and healer, phenotypic properties, M1 and M2 TAMs have opposing effects on the progression of cancer. M1 TAMs by expressing high levels of pro-inflammatory cytokines are capable of killing pathogens and leading to anti-tumor immune responses. Which by contrast, M2 TAMs, by expressing arginase and other anti-inflammatory cytokine enhance tumor cell growth by promoting tumor angiogenesis and tissue remodeling (Bernacki et al ., 1995; Minois et al ., 2011). In fact, shifting the M1/M2 balance towards M1 phenotype is the strategy of multiple studies recently. A major proportion of TAMs is originated from the infiltrating monocytes to tumors under, which this makes monocytes of great importance to study as a major player in cancer related immunology (Franklin et al., 2014).

TAMs can be indicators of either a positive or negative prognosis in cancer. Given their phenotypic plasticity, it has long been recognized that activated macrophages can distinguish tumor cells from their normal cellular counterparts and are capable of reducing tumor cell growth and achieving tumor cytotoxicity without the aid of specific antibodies. On the other hand, sufficient evidence has also accumulated to conclude that macrophages, under certain circumstances, can stimulate cancer growth. High macrophage infiltration in stomach cancer results in a favorable prognosis (Satoshi Ohno, 2003). However, there are conflicting evidences for the role of macrophages in prostate, lung and brain tumors (Allavena et al., 2008). In bladder, esophageal and breast cancer, high numbers of macrophages correlate with a poor prognosis (Mantovani et al., 2007). Although the mechanism underlying this dual nature of macrophages remains unclear, recent studies have shown that the pathways by which macrophages metabolize arginine may influence their tumoricidal function.

Among the three subsets of monocytes described in mice, which are Ly6C^{high}, Ly6C^{low} and Ly6C^{intermediate}, Ly6C^{high} monocytes have been indicated in several studies to recruit selectively to the site of inflammation in many models of inflammatory diseases, such as bowel disease (Zigmond et al., 2012), artherosclerosis (Combadière et al., 2008), rheumatoid arthritis (Brühl et al., 2007) and central nervous system infection (King et al., 2009). In these models Ly6C^{high} monocytes have been reported to recruit to the site of inflammation and according to environmental state differentiate to macrophages with distinct phenotype. Therefore, knowing the ability of Ly6C^{high} monocytes subset to recruit to inflamed tissue, we were interested to investigate on this specific population of monocytes.

Having special features, Ly6C^{high} monocytes have acquired great importance among various groups to study in the last few years. However, since Ly6C^{high} cells are just 2-5% in the mice blood and due to difficulties for their acquisition (Hammond et al., 2014), current studies on these cells are limited to transgenic models (i.e. CCR2$^{-/-}$; GFP-CX3CR1 models) (Wohleb et al., 2013; Shi et al., 2010), clodronate models or expensive techniques to acquire maximum number Ly6C^{high} monocytes from mice blood (Helk et al., 2013). Acquisition of large number of Ly6C^{high} monocytes from blood, increase the costs and delay experiments results. Meanwhile, alteration in fate and function of these populations during this assessment remain unclear. All of these limitations impair the study of these cells. Therefore, the generation of Ly6C^{high} monocytes *in vitro* from bone marrow, gives us a powerful and unique tool to investigate the role of Ly6C^{high} monocytes in inflammation. In this study we focused on their role in cancer.

The results obtained in this thesis are based on a new method of generating Ly6C^{high} monocytes from bone marrow of mice with a cocktail of growth factors (protocol under patent). This novel protocol rise the efficiency

of Ly6C^{high} monocytes production to 100 times more than the current techniques used to obtain these cells. Using this protocol, we are able to produce a heterogeneous cell population composed mostly of the Ly6C^{high}CD11b^{high} phenotype. This population has been described in the blood of mice by several studies (Sunderkötter et al., 2004). Afterward, since we were interested to work only with the pure Ly6C^{high} monocytes, this sub-population was sorted. The population enriched by sorting technique has the same phenotype described from sorted cells of mice in peripheral blood, which means they can have the same function as the Ly6C^{high} monocytes circulating in the blood.

The role of *In vitro* generated Ly6C^{high} monocytes have been studied in our group in different models of inflammation. In this thesis, we were interested to study their role in cancer progression, which for this aim at first we chose the xenograft MDA-MB-231 breast cancer in nude BALB/C as a widely used mice model in cancer research. In this model injection of MDA-MB-231 breast cancer cells in the mammary fat pad of mice leads to appearance of a xenograft tumor.

Because *In vitro* generated Ly6C^{high} monocytes express CCR2, a molecule well known to be involved in inflammatory monocyte recruitment (Imhof and Aurrand-Lions, 2004), it was proposed that these cells are able to rapidly recruit to sites of inflammation. To confirm this migratory capacity, was the first important task of this study. For this aim, *in vitro* generated Ly6C^{high} monocytes were injected in the blood flow of breast tumor-bearing mice. *In vivo* analysis of IVIS images and also microscopic images of tumor tissue revealed that, Ly6C^{high} monocytes are capable to migrate in the blood and home to breast tumor.

The next step is to see whether the migrated Ly6C^{high} monocytes have any impact on the process of tumor growth. The first issue to accomplish was to determine the proper time to inject Ly6C^{high} monocytes during tumor

progression. It is thought that monocytes in the peripheral circulation are recruited to the tumor site by the release of chemotactic cytokines (Bauer et al., 2014; Daurkin et al., 2011).

Several types of cells within the tumor produce chemotactic factors for mononuclear phagocytes to induce chemo-tactic migration of monocytes. Release of chemokines like monocyte chemoattractant protein-1 (MCP-1/CCL2) is one of the main regulators of monocyte migration and infiltrations to tumors (Qian et al., 2011; Sanford et al., 2013). Based on these data, we assumed that, when the progression of cancer and accordingly the tumor volume is advanced enough, injected Ly6C^{high} CCR2^{+} monocytes are able to move toward high local concentration of chemokines. In addition, since *in vivo* tracking of injected Ly6C^{high} monocytes in the blood stream showed us that the accumulation of these cells in the body decrease after few days, we decided to inject Ly6C^{high} monocytes several times during breast tumor progression.

The results obtained from this study, shows that multiple injection of Ly6C^{high} monocytes, modulate breast tumor progression comparing to control group. The “inflammatory” Ly6C^{high} monocytes are believed to recruit to tumors and become inflammatory TAMs with an M1 phenotype. Our data of gene expression results, illustrates an alteration when we injected Ly6C^{high} monocytes in the balance between M1 and M2 macrophages of the breast tumor. We observed significant up-regulation of TNF-α in the Ly6C^{high} injected tumors, while expressing less anti-inflammatory cytokines such as arginase-1 and MRC. Several studies have shown that the activation of a pro-inflammatory program by stimulating M1-polarized macrophage respond to tumor growth regulation (Eming et al., 2007). In general, the restoration of an M1 phenotype in TAMs may provide a therapeutic benefit by promoting antitumor activities (Ong et al ., 2007; Guiducci et al ., 2005). In our study, we believe that the injection of Ly6C^{high} monocytes has led to their recruitment to breast tumor into a more pro-inflammatory phenotype. Thus, the shift from

anti-inflammatory to pro-inflammatory TAMs, might be the reason why we observed a more moderate tumor progression in our treatment group of breast tumors compared to untreated tumors. Interestingly, in the Ly6C^{high} injected tumors it was a down-regulation of TGF-β and MMP9. Considering the beneficial role of these two genes for tumor growth and angiogenesis, this down-regulation is another evidence for modulatory abilities of infiltrated the Ly6C^{high} monocytes on tumor growth.

STAT6 is involved in IL-4 signaling pathway, which is responsible for the switch of pro to anti-inflammatory macrophages (Hebenstreit et al., 2006; Kis et al., 2011). STAT6 deficient mice are unable to differentiate Th2 cells and have an impaired proliferative response (Chapoval et al., 2010; Ueta et al., 2008; Kaplan et al., 1999). Due to importance of TAMs phenotype in cancer progression, STAT6 knockout mice provide a great tool to investigate cancer development. In mouse model of STAT6$^{-/-}$ with impaired IL-4 signaling, it has been shown that there is more M1 macrophage activation and an absence of M2 macrophages and arginase 1 , and this promotes the rejection of syngeneic 4T1 mammary carcinomas as well as limits metastasis (Sinha et al., 2005). As a part of our study we used STAT6 knockout mice for *in vitro* generation of Ly6C^{high} monocytes. Data shows that cancer progression is even more affected when we inject Ly6C^{high} monocytes extracted from STAT6$^{-/-}$mice, which might be an influence of pro-inflammatory polarization of STAT6$^{-/-}$ Ly6C^{high} monocytes.

To sum up, shifting the balance between the M1 and M2 TAMs more to M1 phenotype is our main hypothesis to explain the impact of recruited Ly6C^{high} monocytes in the breast cancer microenvironment. Our investigation on the role of infiltrated Ly6C^{high} monocytes in breast cancer is in agreement with previous findings that the role of immune response in cancer progression seems to be possibly dual and requires more research to be well understood.

Immune-deficient mice engrafted with human cells like MDA-MB-231 have provided an exciting alternative for the study of human immune-biology. However, there are a number of limitations in such humanized mouse models, such as: a)Background strain features: we believe, for a more complete study of *in vitro* generated Ly6C^{high} monocytes role in cancer, a single strain of mice should be used for generation of Ly6C^{high} monocytes from bone marrow and induction of cancer; b) Function of various endogenous immune system components. Nude mice lack thymus; therefore they are unable to generate mature T lymphocytes and mount most types of immune system and c) human tumors in mice are a heterologous system where several factors involving the recognition between species need to be into account to understand the results.

Due to these reasons, we believe that using the same strain of mice for both generation of Ly6C^{high} monocytes and also as a cancer animal model, will rise to more reliable results in our study. So, as the next animal model of cancer, we chose C26 colon carcinoma. This tumor was induced in BALB/C mice by a chemical mutagenesis. The subcutaneous inoculation of C26 colon carcinoma cells in BALB/C mice cause severe cachexia and rapid growth of the tumor which leads to mortality.

Inflammation is a very important factor for the development of colon cancer, but the molecular mechanisms by which inflammation promotes colon cancer, are still uncovered. It has been established that pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 are major players in inducing and promoting the development of cancer cachexia. IL-6 plays a substantial role in inducing cachexia and muscle wasting in mice bearing C26 cancer (Bonetto et al., 2011; Carson and Baltgalvis, 2010).

In the present study, at first the cachectic C26 cancer model was established. Subcutaneous inoculations of C26 results in a rapid growing tumor which induce sever cachexia. Body weight loss as a result of cachexia

started at day 8 after tumor inoculation and mice were found to loss almost 20% of initial body weight during until day 11 and start to dye. Characterization of tumor lymphocytes, showed an increase in infiltrating CD45Ly6C^{high}CD11b^{high} cells, relative to tumor growth. These results lead us to the conclusion that since the progression of cancer in this model is a very fast, to see the impact of Ly6C^{high} monocytes on tumor growth, administration of these cells to animal model requires being at early stages of cancer growth.

Based on the above results and after confirmation of the migratory capacity of *in vitro* generated Ly6C^{high} monocytes in breast cancer model, we designed C26 colon carcinoma model to investigate the role of Ly6C^{high} monocytes in colon cancer. We believe that the modulatory outcome of our immune-based study depends upon the number of injected cells or the size of the tumor at the time of injection. Therefore the first step of the study was to determine the proper time-points to inject Ly6C^{high} monocytes. At first, we designed an experiment with three injections of Ly6C^{high} monocytes at days 0, 3 and 6 after cancer cell injection. Comparison of tumor growth and survival rate between the Ly6C^{high} and PBS injected groups, revealed that C26 tumor growth and its induced cachexia is more moderate when we inject Ly6C^{high} monocytes, and as a result higher survival rate is observed. The next question for us was, how many times of injection of Ly6C^{high} monocytes are required to see their modulatory impact in C26 cancer model and which of the time-points and stages of cancer progression are more critical to inject monocytes. To address these questions we performed an experiment with administration of single or double injection of Ly6C^{high} monocytes in various days after cancer cells injection. Survival rate analyzing in this experiment showed the most promising days to observe beneficial response on cancer progression, due to injection of Ly6C^{high} cells is the double injection at days 3 and 6. Overall, the data obtained from these experiments shows that the effect of *in vitro* generated Ly6C^{high} monocytes on the progression of C26 colon carcinoma, is completely related to the stage of cancer at which these cells are injected in mice.

The genetic profile of the tumor microenvironment and its potential correlation with anti-tumor immune responses has become an area of increased study in recent years. A pro-inflammatory gene expression profile within the tumor microenvironment was associated with survival following administration of a protein-based vaccine in patients with metastatic melanoma (Harris and Drake, 2013). Anti and pro-inflammatory gene expression profile of C26 tumors from the treatment and control groups, shows down-regulation in Ly6C^{high} injected tumors in both M1 and M2 associated genes. This down-regulation is especially interesting about IL-1β and IL-6, since as explained before elevated expression of these genes is the major mechanism for wasting in C26 cachexia. These inflammatory cytokines widely known as cachectic factors are produced by host immune cells in response to C26 tumor, or by C26 tumor cells themselves (Fearon et al., 2012). Additionally, TNF-α, IL-1β and IL-6 were shown to be produced and secreted by muscle fibers in cachectic mice. Administration of Ly6C^{high} monocytes to C26 tumors, induced down-regulation of IL-1β and IL-6, and since in cachectic body all muscles are inflamed having chemo-attractant signals for monocyte recruitment, injected Ly6C^{high} monocytes could be infiltrated to muscles and altering the macrophage polarization at these sites too. This can be a reason to explain the delay in occurring cachexia when mice are injected with Ly6C^{high} monocytes. Additionally, the same result as in breast cancer was observed in gene expression profile of TGF-β and MMP9 when the Ly6C^{high} monocytes were injected, meaning that they were both down-regulated in this case.

Displaying substantial heterogeneous phenotype is a well known character of Ly6C^{high} monocytes from mice peripheral blood, which reflects the specialization of individual macrophages population within their microenvironments (Auffray et al., 2009; Francke et al., 2011). Our group has studies the ability of Ly6C^{high} monocytes to switch phenotype *in vitro*, and for this purpose these cells were activated *in vitro* under pro and anti-

inflammatory conditions with INF-γ or IL-4, cytokines known to be responsible for the activation of macrophages in a pro-inflammatory (classical activation or M1) or anti-inflammatory (alternative activation or M2) manner, respectively. It has been proven in previous studies conducted in our group that generated Ly6C^{high} gain the ability to express pro-inflammatory markers such as *nos2* and *tnfα* when stimulated by INF-γ and to express anti-inflammatory markers such as mannose receptor and arginase-1 when stimulated with IL-4.

Using C26 cancer model we were interested to study the impact of activation of Ly6C^{high} monocytes toward pro or anti-inflammatory phenotype on the progression of cancer. We conducted the C26 cancer model injecting Ly6C^{high} monocytes treated with IL-4 or INF-γ before injection. Among the groups of treatment, Ly6C^{high} monocytes activated with IL-4 were the most beneficial on cancer progression, since they had the highest survival rate and the least tumor volume rise among all groups. It has been shown in our group that treated Ly6C^{high} monocytes with IL-4 facilitate muscle and ear repair in Notexin and DNFB animal models, by contrast treated Ly6C^{high} monocytes with INF-γ delayed muscle repair in the Notexin experimental model (unpublished results). These data supports our findings in C26 models related to activated Ly6C^{high} monocytes toward pro or anti-inflammatory phenotype. Since the whole cachectic muscles are inflamed and injured in C26-bearing mice and activated Ly6C^{high} monocytes injected in this cancer model apart from recruiting to tumor site have played substantial role by affecting cachectic muscle repair.

5 Conclusion

In conclusion, we defined a new regulatory role of recruiting Ly6C^{high} monocytes in cancer, which might be clinically relevant in developing novel immunotherapeutic strategies. Although, underlying mechanism by which Ly6C^{high} monocytes influences the tumor progression have yet to be established and it requires further studies to characterize the phenotype of these cells after recruitment in cancer. So far, since the inflammatory genes involved in tumor progression were differently regulated in tumors infiltrated with Ly6C^{high} monocytes, our hypothesis is that the recruitment of Ly6C^{high} monocytes, alter the balance of pro-inflammatory/anti-inflammatory pool of macrophages in the cancer and this is the main reason why modulation impacts occur in this study. While pro-inflammatory macrophages will be able to induce wound hilling and revascularization, the anti-inflammatory macrophages will block the tumor growth through the production of fibrosis.

6 REFERENCES

References

Allavena, P., Piemonti, L., Longoni, D., Bernasconi, S., Stoppacciaro, A., Ruco, L., and Mantovani, A. (1998). IL-10 prevents the differentiation of monocytes to dendritic cells but promotes their maturation to macrophages. Eur. J. Immunol. *28*, 359–369.

Allavena, P., Sica, A., Solinas, G., Porta, C., and Mantovani, A. (2008). The inflammatory micro-environment in tumor progression: The role of tumor-associated macrophages. Crit. Rev. Oncol. Hematol. *66*, 1–9.

Allred, D.C., Clark, G.M., Elledge, R., Fuqua, S.A.W., Brown, R.W., Chamness, G.C., Osborne, C.K., and McGuire, W.L. (1993). Association of p53 Protein Expression With Tumor Cell Proliferation Rate and Clinical Outcome in Node-Negative Breast Cancer. J. Natl. Cancer Inst. *85*, 200–206.

Ancuta, P., Rao, R., Moses, A., Mehle, A., Shaw, S.K., Luscinskas, F.W., and Gabuzda, D. (2003). Fractalkine Preferentially Mediates Arrest and Migration of CD16+ Monocytes. J. Exp. Med. *197*, 1701–1707.

Ardavín, C., Amigorena, S., and e Sousa, C.R. (2004). Dendritic Cells: Immunobiology and Cancer Immunotherapy. Immunity *20*, 17–23.

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., Rooijen, N. van, Plonquet, A., Gherardi, R.K., and Chazaud, B. (2007). Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J. Exp. Med. *204*, 1057–1069.

Auffray, C., Fogg, D., Garfa, M., Elain, G., Join-Lambert, O., Kayal, S., Sarnacki, S., Cumano, A., Lauvau, G., and Geissmann, F. (2007). Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior. Science *317*, 666–670.

Auffray, C., Sieweke, M.H., and Geissmann, F. (2009). Blood

Monocytes: Development, Heterogeneity, and Relationship with Dendritic Cells. Annu. Rev. Immunol. *27*, 669–692.

Balkwill, F. (2004). Cancer and the chemokine network. Nat. Rev. Cancer *4*, 540–550.

Balkwill, F., Charles, K.A., and Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell *7*, 211–217.

Belge, K.-U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberger, B., Espevik, T., and Ziegler-Heitbrock, L. (2002). The Proinflammatory CD14+CD16+DR++ Monocytes Are a Major Source of TNF. J. Immunol. *168*, 3536–3542.

Berthold, F. (1981). Isolation of human monocytes by ficoll density gradient centrifugation. Blut *43*, 367–371.

Biswas, S.K., Gangi, L., Paul, S., Schioppa, T., Saccani, A., Sironi, M., Bottazzi, B., Doni, A., Vincenzo, B., Pasqualini, F., et al. (2006). A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). Blood *107*, 2112–2122.

Bonetto, A., Aydogdu, T., Kunzevitzky, N., Guttridge, D.C., Khuri, S., Koniaris, L.G., and Zimmers, T.A. (2011). STAT3 Activation in Skeletal Muscle Links Muscle Wasting and the Acute Phase Response in Cancer Cachexia. PLoS ONE *6*, e22538.

Boring, L., Gosling, J., Chensue, S.W., Kunkel, S.L., Farese, R.V., Broxmeyer, H.E., and Charo, I.F. (1997). Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J. Clin. Invest. *100*, 2552–2561.

Brühl, H., Cihak, J., Plachý, J., Kunz-Schughart, L., Niedermeier, M., Denzel, A., Rodriguez Gomez, M., Talke, Y., Luckow, B., Stangassinger, M., et al. (2007). Targeting of Gr-1+,CCR2+ monocytes in collagen-induced arthritis. Arthritis Rheum. *56*, 2975–2985.

Burke, B., Sumner, S., Maitland, N., and Lewis, C.E. (2002). Macrophages in gene therapy: cellular delivery vehicles and in vivo targets. J. Leukoc. Biol. *72*, 417–428.

Carson, J.A., and Baltgalvis, K.A. (2010). Interleukin-6 as a Key Regulator of Muscle Mass during Cachexia. Exerc. Sport Sci. Rev. *38*, 168–176.

Celada, A., and Nathan, C. (1994). Macrophage activation revisited. Immunol. Today *15*, 100–102.

Chapoval, S., Dasgupta, P., Dorsey, N.J., and Keegan, A.D. (2010). Regulation of the T helper cell type 2 (Th2)/T regulatory cell

(Treg) balance by IL-4 and STAT6. J. Leukoc. Biol. *87*, 1011–1018.

Colombo, M.P., and Mantovani, A. (2005). Targeting Myelomonocytic Cells to Revert Inflammation-Dependent Cancer Promotion. Cancer Res. *65*, 9113–9116.

Combadière, C., Potteaux, S., Rodero, M., Simon, T., Pezard, A., Esposito, B., Merval, R., Proudfoot, A., Tedgui, A., and Mallat, Z. (2008). Combined Inhibition of CCL2, CX3CR1, and CCR5 Abrogates Ly6Chi and Ly6Clo Monocytosis and Almost Abolishes Atherosclerosis in Hypercholesterolemic Mice. Circulation *117*, 1649–1657.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860–867.

Donnelly, D.J., Longbrake, E.E., Shawler, T.M., Kigerl, K.A., Lai, W., Tovar, C.A., Ransohoff, R.M., and Popovich, P.G. (2011). Deficient CX3CR1 Signaling Promotes Recovery after Mouse Spinal Cord Injury by Limiting the Recruitment and Activation of Ly6Clo/iNOS+ Macrophages. J. Neurosci. Off. J. Soc. Neurosci. *31*, 9910–9922.

Duffield, J.S., Forbes, S.J., Constandinou, C.M., Clay, S., Partolina, M., Vuthoori, S., Wu, S., Lang, R., and Iredale, J.P. (2005). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J. Clin. Invest. *115*, 56–65.

Eming, S.A., Krieg, T., and Davidson, J.M. (2007). Inflammation in Wound Repair: Molecular and Cellular Mechanisms. J. Invest. Dermatol. *127*, 514–525.

F M Marincola, E.M.J. (2000). Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. Adv. Immunol. *74*, 181–273.

Francke, A., Herold, J., Weinert, S., Strasser, R.H., and Braun-Dullaeus, R.C. (2011). Generation of Mature Murine Monocytes from Heterogeneous Bone Marrow and Description of Their Properties. J. Histochem. Cytochem. *59*, 813–825.

Gabrilovich, D., Ishida, T., Oyama, T., Ran, S., Kravtsov, V., Nadaf, S., and Carbone, D.P. (1998). Vascular Endothelial Growth Factor Inhibits the Development of Dendritic Cells and Dramatically Affects the Differentiation of Multiple Hematopoietic Lineages In Vivo. Blood *92*, 4150–4166.

Gajewski, T.F., Meng, Y., Blank, C., Brown, I., Kacha, A., Kline, J., and Harlin, H. (2006). Immune resistance orchestrated by the tumor microenvironment. Immunol. Rev. *213*, 131–145.

Gajewski, T.F., Schreiber, H., and Fu, Y.-X. (2013). Innate and adaptive immune cells in the tumor microenvironment. Nat. Immunol. *14*, 1014–1022.

Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties. Immunity *19*, 71–82.

Geissmann, F., Auffray, C., Palframan, R., Wirrig, C., Ciocca, A., Campisi, L., Narni-Mancinelli, E., and Lauvau, G. (2008). Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol. Cell Biol. *86*, 398–408.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., and Ley, K. (2010). Development of monocytes, macrophages and dendritic cells. Science *327*, 656–661.

Getts, D.R., Terry, R.L., Getts, M.T., Müller, M., Rana, S., Shrestha, B., Radford, J., Rooijen, N.V., Campbell, I.L., and King, N.J.C. (2008). Ly6c+ "inflammatory monocytes" are microglial precursors recruited in a pathogenic manner in West Nile virus encephalitis. J. Exp. Med. *205*, 2319–2337.

Van Ginderachter, J.A., Movahedi, K., Hassanzadeh Ghassabeh, G., Meerschaut, S., Beschin, A., Raes, G., and De Baetselier, P. (2006). Classical and alternative activation of mononuclear phagocytes: Picking the best of both worlds for tumor promotion. Immunobiology *211*, 487–501.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.-M., Stanley, E.R., Randolph, G.J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. Nat. Immunol. *7*, 265–273.

Gordon, S., and Taylor, P.R. (2005). Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. *5*, 953–964.

Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, Inflammation, and Cancer. Cell *140*, 883–899.

Groh, V., Wu, J., Yee, C., and Spies, T. (2002). Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature *419*, 734–738.

Guo, C., Buranych, A., Sarkar, D., Fisher, P.B., and Wang, X.-Y. (2013). The role of tumor-associated macrophages in tumor vascularization. Vasc. Cell *5*, 20.

Hagemann, T., Lawrence, T., McNeish, I., Charles, K.A., Kulbe, H., Thompson, R.G., Robinson, S.C., and Balkwill, F.R. (2008). "Re-

educating" tumor-associated macrophages by targeting NF-?B. J. Exp. Med. *205*, 1261–1268.

Hammond, M.D., Taylor, R.A., Mullen, M.T., Ai, Y., Aguila, H.L., Mack, M., Kasner, S.E., McCullough, L.D., and Sansing, L.H. (2014). CCR2+ Ly6C(hi) inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage. J. Neurosci. Off. J. Soc. Neurosci. *34*, 3901–3909.

Harris, T.J., and Drake, C.G. (2013). Primer on tumor immunology and cancer immunotherapy. J. Immunother. Cancer *1*, 12.

Hebenstreit, D., Wirnsberger, G., Horejs-Hoeck, J., and Duschl, A. (2006). Signaling mechanisms, interaction partners, and target genes of STAT6. Cytokine Growth Factor Rev. *17*, 173–188.

Heil, M., Ziegelhoeffer, T., Pipp, F., Kostin, S., Martin, S., Clauss, M., and Schaper, W. (2002). Blood monocyte concentration is critical for enhancement of collateral artery growth. Am. J. Physiol. - Heart Circ. Physiol. *283*, H2411–H2419.

Helk, E., Bernin, H., Ernst, T., Ittrich, H., Jacobs, T., Heeren, J., Tacke, F., Tannich, E., and Lotter, H. (2013). TNFα-Mediated Liver Destruction by Kupffer Cells and Ly6Chi Monocytes during Entamoeba histolytica Infection. PLoS Pathog *9*, e1003096.

Hettinger, J., Richards, D.M., Hansson, J., Barra, M.M., Joschko, A.-C., Krijgsveld, J., and Feuerer, M. (2013). Origin of monocytes and macrophages in a committed progenitor. Nat. Immunol. *14*, 821–830.

Houthuys, E., Movahedi, K., De Baetselier, P., Van Ginderachter, J.A., and Brouckaert, P. (2010). A method for the isolation and purification of mouse peripheral blood monocytes. J. Immunol. Methods *359*, 1–10.

Hu, X., Chen, J., Wang, L., and Ivashkiv, L.B. (2007). Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. J. Leukoc. Biol. *82*, 237–243.

Imhof, B.A., and Aurrand-Lions, M. (2004). Adhesion mechanisms regulating the migration of monocytes. Nat. Rev. Immunol. *4*, 432–444.

Jacquelin, S., Licata, F., Dorgham, K., Hermand, P., Poupel, L., Guyon, E., Deterre, P., Hume, D.A., Combadière, C., and Boissonnas, A. (2013). CX3CR1 reduces Ly6Chigh-monocyte motility within and release from the bone marrow after chemotherapy in mice. Blood *122*, 674–683.

Joyce, J.A., and Pollard, J.W. (2009). Microenvironmental regulation of metastasis. Nat. Rev. Cancer *9*, 239–252.

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., and Littman, D.R. (2000). Analysis of Fractalkine Receptor CX3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion. Mol. Cell. Biol. *20*, 4106–4114.

Kalupahana, R.S., Mastroeni, P., Maskell, D., and Blacklaws, B.A. (2005). Activation of murine dendritic cells and macrophages induced by Salmonella enterica serovar Typhimurium. Immunology *115*, 462–472.

Kaplan, M.H., Wurster, A.L., Smiley, S.T., and Grusby3, M.J. (1999). Stat6-Dependent and -Independent Pathways for IL-4 Production. J. Immunol. *163*, 6536–6540.

Karlmark, K.R., Weiskirchen, R., Zimmermann, H.W., Gassler, N., Ginhoux, F., Weber, C., Merad, M., Luedde, T., Trautwein, C., and Tacke, F. (2009). Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology *50*, 261–274.

Kim, J., Nam, K.-H., Kim, S.-O., Choi, J.-H., Kim, H.-C., Yang, S.-D., Kang, J.-H., Ryu, Y.-H., Oh, G.T., and Yoo, S.-E. (2004). KR-31378 ameliorates atherosclerosis by blocking monocyte recruitment in hypercholestrolemic mice. FASEB J.

King, I.L., Dickendesher, T.L., and Segal, B.M. (2009). Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. Blood *113*, 3190–3197.

Kis, L.L., Gerasimčik, N., Salamon, D., Persson, E.K., Nagy, N., Klein, G., Severinson, E., and Klein, E. (2011). STAT6 signaling pathway activated by the cytokines IL-4 and IL-13 induces expression of the Epstein-Barr virus–encoded protein LMP-1 in absence of EBNA-2: implications for the type II EBV latent gene expression in Hodgkin lymphoma. Blood *117*, 165–174.

Klimp, A.H., Hollema, H., Kempinga, C., Zee, A.G.J. van der, Vries, E.G.E. de, and Daemen, T. (2001). Expression of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase in Human Ovarian Tumors and Tumor-associated Macrophages. Cancer Res. *61*, 7305–7309.

Larco, J.E.D., Wuertz, B.R.K., and Furcht, L.T. (2004). The Potential Role of Neutrophils in Promoting the Metastatic Phenotype of Tumors Releasing Interleukin-8. Clin. Cancer Res. *10*, 4895–4900.

León, B., López-Bravo, M., and Ardavín, C. (2007). Monocyte-

Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania. Immunity *26*, 519–531.

Lewis, C.E., and Pollard, J.W. (2006). Distinct Role of Macrophages in Different Tumor Microenvironments. Cancer Res. *66*, 605–612.

Lewis, C., and Murdoch, C. (2005). Macrophage Responses to Hypoxia: Implications for Tumor Progression and Anti-Cancer Therapies. Am. J. Pathol. *167*, 627–635.

Lin, W.-W., and Karin, M. (2007). A cytokine-mediated link between innate immunity, inflammation, and cancer. J. Clin. Invest. *117*, 1175–1183.

Lin, E.Y., Nguyen, A.V., Russell, R.G., and Pollard, J.W. (2001). Colony-Stimulating Factor 1 Promotes Progression of Mammary Tumors to Malignancy. J. Exp. Med. *193*, 727–740.

Lin, S.L., Castaño, A.P., Nowlin, B.T., Lupher, M.L., and Duffield, J.S. (2009). Bone marrow Ly6Chigh monocytes are selectively recruited to injured kidney and differentiate into functionally distinct populations. J. Immunol. Baltim. Md 1950 *183*, 6733–6743.

Lowe, D.B., Shearer, M.H., Aldrich, J.F., Winn, R.E., Jumper, C.A., and Kennedy, R.C. (2010). Role of the Innate Immune Response and Tumor Immunity Associated with Simian Virus 40 Large Tumor Antigen. J. Virol. *84*, 10121–10130.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. *23*, 549–555.

Mantovani, A., Sica, A., Allavena, P., Garlanda, C., and Locati, M. (2009). Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. Hum. Immunol. *70*, 325–330.

Mehlen, P., and Puisieux, A. (2006). Metastasis: a question of life or death. Nat. Rev. Cancer *6*, 449–458.

Merad, M., Manz, M.G., Karsunky, H., Wagers, A., Peters, W., Charo, I., Weissman, I.L., Cyster, J.G., and Engleman, E.G. (2002). Langerhans cells renew in the skin throughout life under steady-state conditions. Nat. Immunol. *3*, 1135–1141.

Merad, M., Hoffmann, P., Ranheim, E., Slaymaker, S., Manz, M.G., Lira, S.A., Charo, I., Cook, D.N., Weissman, I.L., Strober, S., et

al. (2004). Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. Nat. Med. *10*, 510–517.

Mosser, D.M., and Edwards, J.P. (2008). Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. *8*, 958–969.

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stangé, G., Bossche, J.V. den, Mack, M., Pipeleers, D., Veld, P.I., Baetselier, P.D., et al. (2010a). Different Tumor Microenvironments Contain Functionally Distinct Subsets of Macrophages Derived from Ly6C(high) Monocytes. Cancer Res. *70*, 5728–5739.

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stangé, G., Bossche, J.V. den, Mack, M., Pipeleers, D., Veld, P.I., Baetselier, P.D., et al. (2010b). Different Tumor Microenvironments Contain Functionally Distinct Subsets of Macrophages Derived from Ly6C(high) Monocytes. Cancer Res. *70*, 5728–5739.

Muller, W.A. (2001). New Mechanisms and Pathways for Monocyte Recruitment. J. Exp. Med. *194*, f47–f52.

Nesbit, M., Schaider, H., Miller, T.H., and Herlyn, M. (2001). Low-Level Monocyte Chemoattractant Protein-1 Stimulation of Monocytes Leads to Tumor Formation in Nontumorigenic Melanoma Cells. J. Immunol. *166*, 6483–6490.

Newton, K., and Dixit, V.M. (2012). Signaling in Innate Immunity and Inflammation. Cold Spring Harb. Perspect. Biol. *4*, a006049.

Ojalvo, L.S., King, W., Cox, D., and Pollard, J.W. (2009). High-Density Gene Expression Analysis of Tumor-Associated Macrophages from Mouse Mammary Tumors. Am. J. Pathol. *174*, 1048–1064.

Onitilo, A.A., Engel, J.M., Greenlee, R.T., and Mukesh, B.N. (2009). Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. Clin. Med. Res. *7*, 4–13.

Paik, S., Shak, S., Tang, G., Kim, C., Baker, J., Cronin, M., Baehner, F.L., Walker, M.G., Watson, D., Park, T., et al. (2004). A Multigene Assay to Predict Recurrence of Tamoxifen-Treated, Node-Negative Breast Cancer. N. Engl. J. Med. *351*, 2817–2826.

Pollard, J.W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. Nat. Rev. Cancer *4*, 71–78.

Polly, C.P. Impact Journals presents.

Priceman, S.J., Sung, J.L., Shaposhnik, Z., Burton, J.B., Torres-Collado, A.X., Moughon, D.L., Johnson, M., Lusis, A.J., Cohen, D.A.,

Iruela-Arispe, M.L., et al. (2010). Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. Blood *115*, 1461–1471.

Pucci, F., Venneri, M.A., Biziato, D., Nonis, A., Moi, D., Sica, A., Serio, C.D., Naldini, L., and Palma, M.D. (2009). A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. Blood *114*, 901–914.

Qu, C., Edwards, E.W., Tacke, F., Angeli, V., Llodrá, J., Sanchez-Schmitz, G., Garin, A., Haque, N.S., Peters, W., Rooijen, N. van, et al. (2004). Role of CCR8 and Other Chemokine Pathways in the Migration of Monocyte-derived Dendritic Cells to Lymph Nodes. J. Exp. Med. *200*, 1231–1241.

Randolph, G.J., Sanchez-Schmitz, G., Liebman, R.M., and Schakel, K. (2002). The CD16+ (Fc?RIII+) Subset of Human Monocytes Preferentially Becomes Migratory Dendritic Cells in a Model Tissue Setting. J. Exp. Med. *196*, 517–527.

Rauh, M.J., Ho, V., Pereira, C., Sham, A., Sly, L.M., Lam, V., Huxham, L., Minchinton, A.I., Mui, A., and Krystal, G. (2005). SHIP Represses the Generation of Alternatively Activated Macrophages. Immunity *23*, 361–374.

Robbins, C.S., Chudnovskiy, A., Rauch, P.J., Figueiredo, J.-L., Iwamoto, Y., Gorbatov, R., Etzrodt, M., Weber, G.F., Ueno, T., Rooijen, N. van, et al. (2012). Extramedullary Hematopoiesis Generates Ly-6Chigh Monocytes That Infiltrate Atherosclerotic Lesions. Circulation *125*, 364–374.

Roberti, M.P., Barrio, M.M., Bravo, A.I., Rocca, Y.S., Arriaga, J.M., Bianchini, M., Mordoh, J., and Levy, E.M. (2011). IL-15 and IL-2 increase Cetuximab-mediated cellular cytotoxicity against triple negative breast cancer cell lines expressing EGFR. Breast Cancer Res. Treat. *130*, 465–475.

Roscic-Mrkic, B., Fischer, M., Leemann, C., Manrique, A., Gordon, C.J., Moore, J.P., Proudfoot, A.E.I., and Trkola, A. (2003). RANTES (CCL5) uses the proteoglycan CD44 as an auxiliary receptor to mediate cellular activation signals and HIV-1 enhancement. Blood *102*, 1169–1177.

Ruffell, B., DeNardo, D.G., Affara, N.I., and Coussens, L.M. (2010). Lymphocytes in cancer development: Polarization towards pro-tumor immunity. Cytokine Growth Factor Rev. *21*, 3–10.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. *179*, 1109–1118.

Sallusto, F., Mackay, C.R., and Lanzavecchia, A. (2000). The Role of Chemokine Receptors in Primary, Effector, and Memory Immune Responses. Annu. Rev. Immunol. *18*, 593–620.

Sans-Fons, M.G., Yeramian, A., Pereira-Lopes, S., Santamaría-Babi, L.F., Modolell, M., Lloberas, J., and Celada, A. (2013). Arginine Transport Is Impaired in C57Bl/6 Mouse Macrophages as a Result of a Deletion in the Promoter of Slc7a2 (CAT2), and Susceptibility to Leishmania Infection Is Reduced. J. Infect. Dis. *207*, 1684–1693.

Seeger, F.H., Tonn, T., Krzossok, N., Zeiher, A.M., and Dimmeler, S. (2007). Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. Eur. Heart J. *28*, 766–772.

Seliger, B., Maeurer, M.J., and Ferrone, S. (2000). Antigen-processing machinery breakdown and tumor growth. Immunol. Today *21*, 455–464.

Shechter, R., London, A., Varol, C., Raposo, C., Cusimano, M., Yovel, G., Rolls, A., Mack, M., Pluchino, S., Martino, G., et al. (2009). Infiltrating Blood-Derived Macrophages Are Vital Cells Playing an Anti-inflammatory Role in Recovery from Spinal Cord Injury in Mice. PLoS Med *6*, e1000113.

Shi, C., Velázquez, P., Hohl, T.M., Leiner, I., Dustin, M.L., and Pamer, E.G. (2010). Monocyte Trafficking to Hepatic Sites of Bacterial Infection Is Chemokine Independent and Directed by Focal Intercellular Adhesion Molecule-1 Expression. J. Immunol. *184*, 6266–6274.

Sica, A., Saccani, A., Bottazzi, B., Polentarutti, N., Vecchi, A., Damme, J.V., and Mantovani, A. (2000). Autocrine Production of IL-10 Mediates Defective IL-12 Production and NF-κB Activation in Tumor-Associated Macrophages. J. Immunol. *164*, 762–767.

Sica, A., Larghi, P., Mancino, A., Rubino, L., Porta, C., Totaro, M.G., Rimoldi, M., Biswas, S.K., Allavena, P., and Mantovani, A. (2008). Macrophage polarization in tumour progression. Semin. Cancer Biol. *18*, 349–355.

Sinha, P., Clements, V.K., and Ostrand-Rosenberg, S. (2005).

Reduction of Myeloid-Derived Suppressor Cells and Induction of M1 Macrophages Facilitate the Rejection of Established Metastatic Disease. J. Immunol. *174*, 636–645.

Smyth, M.J., Crowe, N.Y., Hayakawa, Y., Takeda, K., Yagita, H., and Godfrey, D.I. (2002). NKT cells — conductors of tumor immunity? Curr. Opin. Immunol. *14*, 165–171.

Smyth, M.J., Swann, J., Cretney, E., Zerafa, N., Yokoyama, W.M., and Hayakawa, Y. (2005). NKG2D function protects the host from tumor initiation. J. Exp. Med. *202*, 583–588.

Spiotto, M.T., Rowley, D.A., and Schreiber, H. (2004). Bystander elimination of antigen loss variants in established tumors. Nat. Med. *10*, 294–298.

Stefater III, J.A., Ren, S., Lang, R.A., and Duffield, J.S. (2011). Metchnikoff's policemen: macrophages in development, homeostasis and regeneration. Trends Mol. Med. *17*, 743–752.

Steidl, C., Lee, T., Shah, S.P., Farinha, P., Han, G., Nayar, T., Delaney, A., Jones, S.J., Iqbal, J., Weisenburger, D.D., et al. (2010). Tumor-Associated Macrophages and Survival in Classic Hodgkin's Lymphoma. N. Engl. J. Med. *362*, 875–885.

Stout, R.D., Jiang, C., Matta, B., Tietzel, I., Watkins, S.K., and Suttles, J. (2005). Macrophages Sequentially Change Their Functional Phenotype in Response to Changes in Microenvironmental Influences. J. Immunol. *175*, 342–349.

Sunderkötter, C., Nikolic, T., Dillon, M.J., Rooijen, N. van, Stehling, M., Drevets, D.A., and Leenen, P.J.M. (2004). Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response. J. Immunol. *172*, 4410–4417.

Tacke, F., Ginhoux, F., Jakubzick, C., van Rooijen, N., Merad, M., and Randolph, G.J. (2006). Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. J. Exp. Med. *203*, 583–597.

Taylor, P.R., and Gordon, S. (2003). Monocyte Heterogeneity and Innate Immunity. Immunity *19*, 2–4.

Thieblemont, N., Weiss, L., Sadeghi, H.M., Estcourt, C., and Haeffner-Cavaillon, N. (1995). CD14lowCD16high: A cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. Eur. J. Immunol. *25*, 3418–3424.

Tjiu, J.-W., Chen, J.-S., Shun, C.-T., Lin, S.-J., Liao, Y.-H., Chu, C.-Y., Tsai, T.-F., Chiu, H.-C., Dai, Y.-S., Inoue, H., et al. (2008). Tumor-Associated Macrophage-Induced Invasion and Angiogenesis of

Human Basal Cell Carcinoma Cells by Cyclooxygenase-2 Induction. J. Invest. Dermatol. *129*, 1016–1025.

Ueta, M., Hamuro, J., Ueda, E., Katoh, N., Yamamoto, M., Takeda, K., Akira, S., and Kinoshita, S. (2008). Stat6-Independent Tissue Inflammation Occurs Selectively on the Ocular Surface and Perioral Skin of I B -/- Mice. Invest. Ophthalmol. Vis. Sci. *49*, 3387–3394.

Vakkila, J., and Lotze, M.T. (2004). Inflammation and necrosis promote tumour growth. Nat. Rev. Immunol. *4*, 641–648.

Varol, C., Landsman, L., Fogg, D.K., Greenshtein, L., Gildor, B., Margalit, R., Kalchenko, V., Geissmann, F., and Jung, S. (2007). Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. J. Exp. Med. *204*, 171–180.

Wang, B., Li, Q., Qin, L., Zhao, S., Wang, J., and Chen, X. (2011). Transition of tumor-associated macrophages from MHC class IIhi to MHC class IIlow mediates tumor progression in mice. BMC Immunol. *12*, 43.

Wohleb, E.S., Powell, N.D., Godbout, J.P., and Sheridan, J.F. (2013). Stress-Induced Recruitment of Bone Marrow-Derived Monocytes to the Brain Promotes Anxiety-Like Behavior. J. Neurosci. *33*, 13820–13833.

Wyckoff, J.B., Wang, Y., Lin, E.Y., Li, J., Goswami, S., Stanley, E.R., Segall, J.E., Pollard, J.W., and Condeelis, J. (2007). Direct Visualization of Macrophage-Assisted Tumor Cell Intravasation in Mammary Tumors. Cancer Res. *67*, 2649–2656.

Wynn, T.A. (2013). Myeloid-cell differentiation redefined in cancer. Nat. Immunol. *14*, 197–199.

Xaus, J., Comalada, M., Cardó, M., Valledor, A.F., and Celada, A. (2001). Decorin inhibits macrophage colony-stimulating factor proliferation of macrophages and enhances cell survival through induction of p27Kip1 and p21Waf1. Blood *98*, 2124–2133.

Xu, H., Manivannan, A., Dawson, R., Crane, I.J., Mack, M., Sharp, P., and Liversidge, J. (2005). Differentiation to the CCR2+ Inflammatory Phenotype In Vivo Is a Constitutive, Time-Limited Property of Blood Monocytes and Is Independent of Local Inflammatory Mediators. J. Immunol. *175*, 6915–6923.

Yrlid, U., Jenkins, C.D., and MacPherson, G.G. (2006). Relationships between Distinct Blood Monocyte Subsets and Migrating Intestinal Lymph Dendritic Cells In Vivo under Steady-State Conditions. J. Immunol. *176*, 4155–4162.

Zhang, L., Yoshimura, T., and Graves, D.T. (1997). Antibody to Mac-1 or monocyte chemoattractant protein-1 inhibits monocyte recruitment and promotes tumor growth. J. Immunol. *158*, 4855–4861.

Zhu, B., Bando, Y., Xiao, S., Yang, K., Anderson, A.C., Kuchroo, V.K., and Khoury, S.J. (2007). CD11b+Ly-6Chi Suppressive Monocytes in Experimental Autoimmune Encephalomyelitis. J. Immunol. *179*, 5228–5237.

Ziegler-Heitbrock, L. (2007). The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J. Leukoc. Biol. *81*, 584–592.

Zigmond, E., Varol, C., Farache, J., Elmaliah, E., Satpathy, A.T., Friedlander, G., Mack, M., Shpigel, N., Boneca, I.G., Murphy, K.M., et al. (2012). Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells. Immunity *37*, 1076–1090.