Analysis of Ly-6C^{high} CD11b^{+} monocytes generated *in vitro* in inflammatory animal models

Análisis de monocitos Ly-6Chigh CD11b+ generados *in vitro* en modelos animales de inflamación

Erika Barboza Prado Lopes

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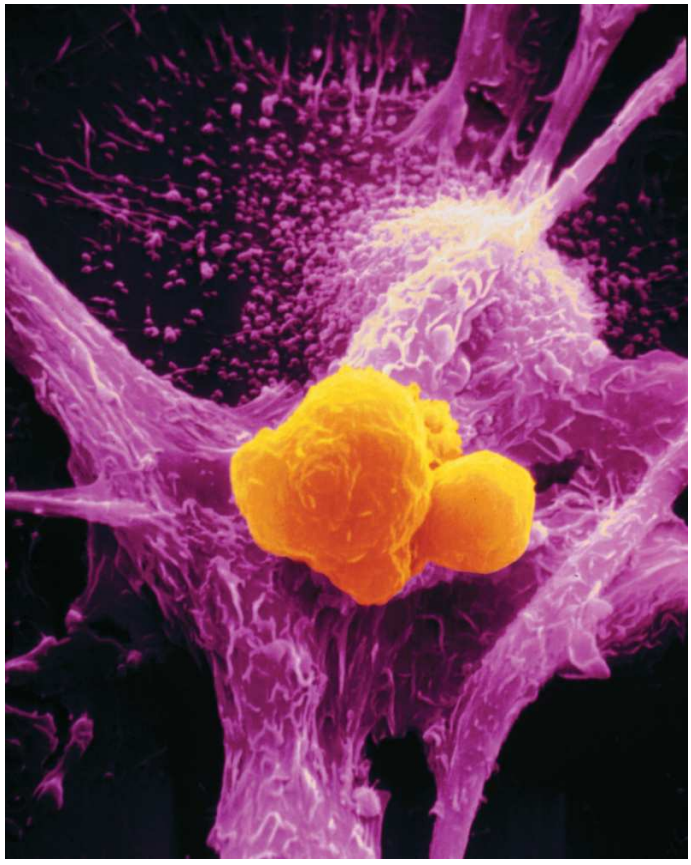

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I dedicate this thesis to my family, for my mother, Marilza, my dad Everardo, for my sister, Emilia and my husband Nicolas, who have never given up on me.

Há momentos na vida em que sentimos tanto a falta de alguém…
que o que mais queremos é tirar esta pessoa de nossos sonhos e abraça-la…
Sonhe com aquilo que você quiser…
Seja o que você quer ser…
Porque você possui apenas uma vida
E nela só temos uma chance de fazer aquilo que queremos.
Tenha felicidade bastante para faze-la doce,
dificuldades para faze-la forte,
tristeza para faze-la humana.
E esperança suficiente para faze-la feliz.
As pessoas mais felizes não tem as melhores coisas,
elas sabem fazer o melhor das oportunidades que aparecem em seus caminhos.
A felicidade aparece para aqueles que choram…
Para aqueles que buscam e tentam sempre…
E para aqueles que reconhecem a importância das pessoas que passam por suas vidas.
O futuro mais brilhante é baseado num passado intensamente vivido.
Você só terá sucesso na vida quando perdoar os erros e as decepções do passado.
A vida é curta, mas as emoções que podemos deixar…
…duram uma eternidade…

Clarice Lispector

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ABBREVIATIONS

ACD Allergic contact dermatitis

aFGF Acidic fibroblast growth factor

ALS Amyotrophic lateral sclerosis

APC Antigen presenting cell

bFGF Basic fibroblast growth factor

CCL Chemokine (C-C motif) ligand

CCR2 Chemokine- receptor-2

CD Contact dermatitis

CD11b Cluster of differentiation molecule 11b

CD11c Cluster of differentiation molecule 11c

CD14 Cluster of differentiation 14

CD62L L-selectin

CD68 Intracellular molecule macrosialin

CFU-GEMM Granulocyte, erythrocyte, monocyte, macrophage-colony forming unit

COPD Chronic obstructive, pulmonary disease

CX3CR1 CX3C chemokine receptor 1

DiR 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide

DMSO Dimethylsulfoxide

DNFB 1-Fluoro-2,4-dinitrofluorobenzene

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

F4/80 EGF-like module-containing mucin-like hormone receptor-like 1 in mice

FACS Flow cytometry assay

FBS Fetal bovine serum

FcgR IgG Fc-domains

Fizz1 Found in inflammatory zone 1

GM-CFU Granulocytic line, granulocyte, macrophage-colony forming unit

GM-CSF Granulocyte-macrophage colony-stimulating factor

Gr-1 granulocyte differentiation antigen 1

HEVs High endothelial venules

HSC Hematopoietic stem cell

ICD Irritant contact dermatitis

ICAM Intracellular adhesion molecules

IFN Interferon

IL Interleukin

IRAF Interferon regulatory factor

KLF Krüppel-like factor

LNs Lymph nodes

LPS Lipopolisaccharide

Ly-6C Lymphocyte antigen 6 complex, locus C

Ly-6G Lymphocyte antigen 6 complex, locus G

M Macrophage

M-CSF Macrophage colony-stimulating factor

M-CFUs Macrophage-colony forming units

MDPs Macrophage/dendritic cell progenitors

MHC major histocompatibility complex

MMPs Matrix metalloproteinase family

Mpcs Myogenic precursor cells

MPS Mononuclear phagocyte system

MRC Mannose receptor

MRF Myogenic regulatory factor

NF-kB Nuclear factor-kB

NK Natural Killer cells

NO nitric oxide

NOS2 Nitric oxide synthase 2

NX Notexin

PAMPs pathogen-associated molecular patterns

PDGF Platelet-derived growth factor

PGE Prostaglandin E

PPAR Peroxisome proliferator-activated receptor

PSGL-1 P-selectin glycoprotein ligand 1

RMs Regulatory macrophages

RSD Reflex Sympathetic Dystrophy

STAT Signal transducer and activator of transcription

TA Tibialis anterioris

TAM Tumor-associated macrophage

TGF-β Transforming growth factor beta

Th T helper lymphocyte

TLR toll like receptor

TNF-α Tumor necrosis factor-alpha

VCAM Vascular adhesion molecules

VEGF Vascular endothelial growth factor

YM1 Chitinase 3-like 3

INTRODUCTION

I. INTRODUCTION

1. General introduction

Inflammation is the first responses of the immune system against infection (Rocha and Silva M.1994; Benaroyo L. 1994). The clinical signs of an inflammatory process are the following: redness, swelling, heat, and pain, all these symptoms are caused by the augment of the blood flow into affected tissue. This inflammatory process is conducted by signaling molecules (eicosanoids) and cytokines, which are released by injured or infected cells. Cytokines and chemokines are responsible for communication between white blood cells, chemotaxis and have anti-viral effects, such as shutting down protein synthesis in the host cell (Le Y., et al. 2004). Growth factors and cytotoxic factors may also be released (Kawai T., el al. 2006). These cytokines and other chemicals recruit immune cells to the site of infection and promote healing of any damaged tissue following the removal of pathogens (Martin P., et al. 2005).

To function properly the immune system, must detect a wide variety of agents, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissue. The innate immune system is the first line of defense that recognizes pathogens, initiates an immune response and latter one activates the adaptive immune system. An interaction between the two systems is needed to eradicate the diseases. However, the immune system needs to be well regulated since a disorder in an immune response can result in autoimmune diseases, tissue destruction, inflammatory diseases and cancer (Coussens LM. and Werb Z. 2010; O'Byrne K.J. and Dalgleish A.G. 2010).

Within the context of innate immunity, in the last years, the mononuclear phagocyte system which is defined as a family of cells comprising bone marrow progenitors, blood monocytes and tissue macrophages has acquired great importance in the study of different pathologies and particularly the monocytes/macrophage cells. In this regard, recent studies demonstrate that circulating monocytes are a heterogeneous population of innate cells composed by various phenotype subtypes and distinct functions (Duan M., et al. 2012; Biswas SK., et al. 2010; Mantovani A., et al. 2002; Mosser DM., et al. 2008) which are involved in normal inflammatory processes and autoimmune diseases. Monocytes differentiate to macrophages into tissue, once differentiated macrophage phenotype depends on the tissue microenvironment and the host condition to be defined (Lech M., et al. 2012). Macrophages in a steady state or during an inflammatory process can be a crucial part of the host defense fighting pathogens, although, during an infection and wound healing, they can be harmful, causing or exacerbating inflammatory diseases such as osteomyelitis, atherosclerosis and multiple sclerosis, which indicate that these cells have a high phonotypical plasticity according to the environment conditions (Biswas SK. and Mantovani A. 2010). Therefore, it is important to have detailed knowledge of these circulating monocytes in order to develop drugs and vaccines against bacterial infections and to find therapeutic targets diseases.

2. Mononuclear phagocyte system

The phagocytic mononuclear system is composed by monocytes, macrophages and dendritic cells. These cells have similarities in the morphology, function, origin, and kinetics. By these criteria reticular cells, endothelial cells, and fibroblasts (fibrocytes) are excluded of this set. They present three main functions in the immune system: phagocytosis, antigen presentation, and cytokine production (immunomodulation). Nevertheless, monocytes and macrophages are critical

effectors and regulators of inflammation and the innate immune response, the immediate arm of the immune system. (Geissmann F., et al. 2010). (See point 3.2).

2.1 Origin and differentiation

In the past, several studies attempts to classify phagocytic mononuclear cells and to define the cell system, they considered to form-among these being the macrophage system of Metchnikoff (Brown EJ. 1995), the reticulo-endothelial system of Aschoff, and the reticulo-endothelial system proposed by Volterra and reintroduced by Thomas (Lay WH., et al. 1969). None of the previous studies were able to achieve what a group of worker proposed in 1969, a new classification of all highly phagocytic mononuclear cells and their precursors termed by mononuclear phagocyte system (MPS) (van Furth R., et al. 1972). The proposed system in 1969 is formed by hematopoietic stem cells (HSC) in the bone marrow, monocytes in the peripheral blood, and by macrophages in the tissues. However further studies added changes in this classification, reestablishing the theory.

Today's theory tells us that the development of these mononuclear phagocyte cells takes place in the bone marrow in HSC. The process of hematopoiesis is controlled by a group of growth factors and cytokines (IL-1, IL-3 and IL-6) produced by local environment induce the stem cell undergoes an unequal cell division or heteromitosis leading to a new stem cell and a pluripotent myeloid cell, CFU-GEMM (granulocyte, erythrocyte, monocyte, macrophage-colony forming unit). In the presence of IL-1 and IL-3, the generic precursor undergoes a differentiation process called determination and happens to be a common progenitor monocytic and granulocytic line, granulocyte, macrophage-colony forming unit (GM-CFU) (Figure. 1). At this point, the interleukin IL-3 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) inducing the proliferation of GM-CFUs.

However, the macrophage colony-stimulating factor (M-CSF) not only induces the proliferation of these cells, but also their differentiation into monocytic precursors called macrophage-colony forming units (M-CFUs). The terminal differentiation of M-CFUs, a process known as maturation, and subsequent generation of monocytes, also requires the presence of M-CSF (Celada A., and Nathan C. 1994; Valledor AF., et al., 1998).

Monocyte is smaller than its immediate parent, the promonocyte, but has a phagocytic capacity and a more developed lysosomal system. Normally, the monocyte lose their proliferative capacity, although under certain conditions, can become divided. Thus the basal generation of monocytes in the bone marrow is a process regulated by the balance between growth factors (M-CSF, GM-CSF and IL-3), produced by the cells themselves during their differentiation and bone marrow cells stroma as well as a series of inhibitors, such as prostaglandin E (PGE) 2, interferon b and lactoferrin (Celada A., and Nathan C. 1994; Valledor AF., et al., 1998). The monocyte is able to leave the bone marrow into the bloodstream and go from there to the tissues, through the interaction between adhesion molecules at the surface of monocytes and vascular endothelial cells. Furthermore, this process can be regulated by the body's needs (inflammation, ischemia, necrosis, apoptosis, etc.) resulting in a decrease in blood flow and thus ensuring that monocytes and endothelial cells interact more easily, thus favoring extravasation of monocytes into tissue.

Circulating monocytes are young cells that already own migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains (FcγR) and iC3b complement (Dubaniewicz A, et al. 2012). During monocyte migration (homing) into tissues, they undergo differentiation to become multifunctional tissue macrophages. Monocytes are considered to be immature macrophages (Figure 1). Once in the tissue, monocyte maturation ends and

becomes a macrophage. This process leads to increased cell size, increased lysosomal system development and content of hydrolytic enzymes and an increase in the number and size of mitochondria, and their energy metabolism. The half-life of macrophages in tissues can be very long (months-years), however, many macrophages reaching the tissues will eventually disappear by cell death processes. In tissues, a small number of macrophages will differ under the influence of cytokines and, depending tissue type (e.g. skin epithelial cells) (Figure1). Tissue macrophages are able to proliferate in these tissues under basal conditions (not activated) by the presence or production of autocrine M-CSF, as well as in the presence of cytokines and other growth factors not specific such as GM-CSF and IL-3 (Celada A. and Maki RA. 1992).

These local resident macrophages will be sentinels of innate immune system. When these cells are stimulated by mediators produced in the sites of inflammation, they stop their proliferation and become activated, increasing the capacity to develop their specialized functions (See section 2.3). Thereby, the main macrophage activator agent is a cytokine, IFN-γ, secreted by activated T helper 1 lymphocyte (Th1). However, in pathologic conditions such as a bacterial infection, e.g. the lipopolysaccharide (LPS), the main constituent of wall of Gram-negative bacteria, can also activate many of the macrophage functions. By contrast, in the absence of stimulus, macrophages die by apoptosis.

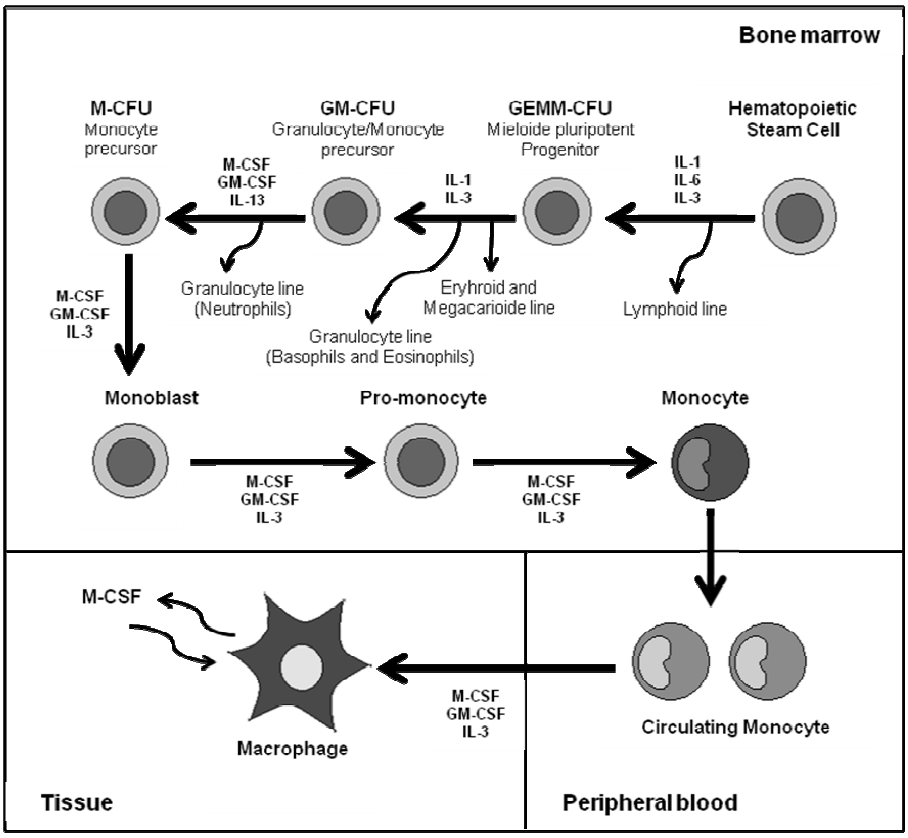

Figure 1. Macrophage differentiation process. IL, interleukin; M-CSF, colony stimulating factor from macrophages, GM-CSF, colony stimulating factor and granulocyte macrophage, CFU-GEMM, colony forming unit granulocytes, erythrocytes, megakaryocytes, and macrophages; GM-CFU, unit forming colonies of granulocytes and macrophages, M-CFU, colony forming unit macrophage. Adapted from reference (Valledor AF., et al. 1998).

2.2 Monocytes

Monocytes constitute between 2-8% (450 monocytes/µl) of leukocytes present in human blood and around 2% (100 cells/µl) of the total peripheral blood leukocyte pool in mice (Robbins CS., et al. 2010). These cells have been described to be a nondividing cell population, able to recirculate in the bloodstream for about one day in mice and three days in humans (van Furth R., et al. 2007). In fact, this short half-

life has promoted the concept that the blood acts as a reservoir permitting myeloid precursors general continuously restocking of tissue macrophages and dendritic transitory cells (Serbina NV., et al. 2006; Geissmann F., et al. 2003; Randolph GJ., et al, 1998) (Figure 2). On the other hand, half of monocytes are stored as a reserve in the spleen in clusters in the red pulp's Cords of Billroth (Swirski FK., et al. 2009) and are subsequently released to the peripheral circulation as non dividing cells (van Furth R., et al. 1979). Circulating blood monocytes were originally classified by their irregular cell morphology and density. However, these features present a morphological variability (size, nuclear morphology and granularity) making it difficult to distinguish them by histological criteria alone, which led experts to seek tools in order to perform their characterization (Figdor CG., et al. 1982; Yasaka T., et al. 1981). Today there is a consensus that different subtypes of monocytes are found in the human and murine blood (two main subtypes), and that these cells can be distinguished by their migratory capacity, morphology and by the differential expression of the antigenic markers (Shen HH., et al. 1983; Ziegler-Heitbrock HW, et al. 1991) (See section 2.2.1). However, at the end, the enormous plasticity of these heterogeneous monocytes populations will be determined by their maturation status and by their local microenvironment in the tissue. This together will direct control of their development and function (Raes G., et al. 2012).

2.2.1 Circulating monocyte subsets

As already mentioned, two main subsets of monocytes population have been found in humans, mice and rats (Geissmann F., et al. 2003; Sunderkötter C., et al. 2004; Mack M., et al. 2001; Biburger M., et al, 2011). These subsets differ in some aspects as maturation, chemokine receptor and adhesion molecule expression, and also in the differentiation potential and migration pattern level (Table 1). Murine and human monocytes have been characterized by several markers, such as cluster of

differentiation molecule 11b (CD11b), chemokine-receptor-2 (CCR2), L-selectin (CD62L), CX3C chemokine receptor 1 (CX3CR1) and by other markers that are present in hider in mouse or in human, such as lymphocyte antigen 6 complex, locus C (Ly-6C) and cluster of differentiation 14 (CD14), respectively, which are considered phenotypical homologous proteins. The granulocyte differentiation antigen 1 (Gr-1), abbreviation, is also used as a typical monocyte marker in humans (Geissmann F., et al. 2003; Gordon S and Taylor PR. 2005) (Table 1).

However, in all markers mentioned Gr-1 and Ly-6C are the most used to distinguish different populations of monocytes, a point that deserves clarification: The antibody Gr-1 recognizes lymphocyte antigen 6 complex, locus G (Ly-6G), a granulocyte surface marker, and also Ly-6C (Fleming TJ., et al. 1993; Taylor PR., et al. 2003), which is expressed by monocytes and several other hematopoietic cell populations (Geissmann F., et al. 2003; Lagasse E., et al. 1996; Sunderkötter C., et al 2004; Serbina NV., et al. 2003). Thus, several cell types, for example, neutrophils, are also recognized by the Gr-1 mAb, so to distinguish monocytes from these other cells, the use of specific mAbs markers such as Ly-6C and Ly-6G is necessary to ensure the identity of these cells in human, since monocytes has a Ly-6C+Ly-6G- phenotype and neutrophils are Ly6C+Ly6G+ (Rose S., et al, 2012; Taylor PR., et al. 2003; Daley JM., et al. 2008).

Table 1: Phenotype of monocyte subsets in human and mice

Cell surface markers	Murine Ly-6C^{high} monocytes	Murine Ly-6C^{low} monocytes	Human CD14^{high} CD16^{low} monocytes	Human CD14^{low} CD16^{high} monocytes
F4/80	+	+	ND	ND
CD11b	+	+	+	+ /-
Ly6C	++	-	ND	ND
CD115	+	+	ND	ND
CD14	ND	ND	++	+/-
CD16	ND	ND	-	+
CD11c	-	-	+	++
MHC-II	-	-	++	+
CD62L	+	-	+	-
CD80	ND	ND	ND	ND
CCR1	ND	ND	+	-
CCR2	+	-	+	-
CCR5	+/-	+/-	-	-
CX3CR1	+	++	+	++

Adapted from reference (Geissmann F., et al. 2003; Sunderkötter C., et al. 2004; Mack M. et al. 2001). ND, not determined.

Thus, at this sense, two functional subsets among blood monocytes have been described, CD11b^{+}CCR2^{low}CX3CR1^{high}Ly-6C^{low} and CD11b^{+}CCR2^{hi}CX3CR1^{low}Ly-6C^{high} in mice and rats and CD14^{low}CD16^{high}CCR2^{low} and CD14^{high}CD16^{low}CCR2^{high} in humans. The human CD14^{low}CD16^{high}CCR2^{low} monocytes behave similar to the murine CX3CR1^{high}CCR2^{low}Ly-6C^{low} cells and are called classical or resident monocytes, while the human CD14^{high}CD16^{low}CCR2^{high} monocytes resemble murine CX3CR1^{low}CCR2^{high}Ly-6C^{high} monocytes which are called non-classical or inflammatory monocytes (Table 1) (Passlick B., et al. 1989; Geissmann F., et al. 2003; Gordon S. and Taylor PR. 2005; Auffray C, et al. 2007). Unlike what occurs in mice, in humans the CD14^{high} cells constitute 90-95% of total monocytes subpopulation (mice and rats they are decreased to 50 and 10-20%, respectively) (Tacke F. and Randolph GJ. 2006).

In murine models, distinct levels in the expression of Ly-6C, CD11b, CCR2 and some other markers classifies these cells into different subsets (Qu C., et al. 2004). Subsets that present high levels of CCR2 and CD62L but low levels of CX3CR1 are known as inflammatory monocytes or Ly-6C^{high} monocytes that represent approximately 50% of circulating white blood cells in a non-sensitized immunocompetent mouse and are rapidly recruited (approximately 8-12 hours) to sites of infection and inflammation, where they will differentiate into another cell type, inflammatory macrophages (Geissmann F., et al. 2003; Tacke F. and Randolph GJ. 2006) (Figure 2). Several studies demonstrate the role of CCR2 chemokine receptor in the migratory capacity of the inflammatory monocytes, where mice with an CCR2 deficiency present reduction of Ly-6C^{high} monocytes trafficking to sites of inflammation, what indicates the importance in the presence of these markers (Lu H, et al. 2011; Li L., et al. 2008; Willenborg S., et al 2012). However, studies also have shown that these cells can shuttle back from the blood to the bone marrow and even convert into Ly-6C^{low} bone marrow monocyte, that egress back to into the peripheral circulation, within 3 days in the absence of inflammation. (Varol C., et al. 2007; Yona S. and Jung S. 2010) (Figure 2).

In counterpart, in the second subsets of circulating monocytes (Ly-6C^{low}), high levels of CX3CR1 and low levels of CCR2 are observed, which makes these cells less prevalent in the inflammatory site (Movahedi K, et al. 2010; Anzai A, et al. 2012). These monocytes (referred as CX3CR1^{high} monocytes) are less prevalent than Ly-6C^{high} monocytes also in the blood flow, constituting only 5–10% of circulating white blood cells (Geissmann F, et al. 2003; Tacke F. and Randolph GJ. 2006). Ly-6C^{low} monocytes, known as resident monocytes, have the ability to migrate from the blood flow to the non-inflamed tissues where they will differentiate in resident cells to regulate the tissue integrity and homeostasis. Recent studies have demonstrated that these Ly-6C^{low} cells migrate to tissues such as the lungs, brain, and gut independently of inflammatory stimuli (Auffray C., 2007; Hristov M.

and Weber C, 20011; Geissmann F, et al. 2003; Gautier EL, et al 2009; Woollard KJ. and Geissmann F. 2010; Weber C, et al 2008). However, it remains unclear whether macrophage/dendritic cell progenitors (MDPs) give rinse directly to Ly-6C^{low}; if the generation of these cells requires an intermediate Ly-6C^{high} or whether both pathways exists side by side (Varol C, et al. 2007) (Figure 2).

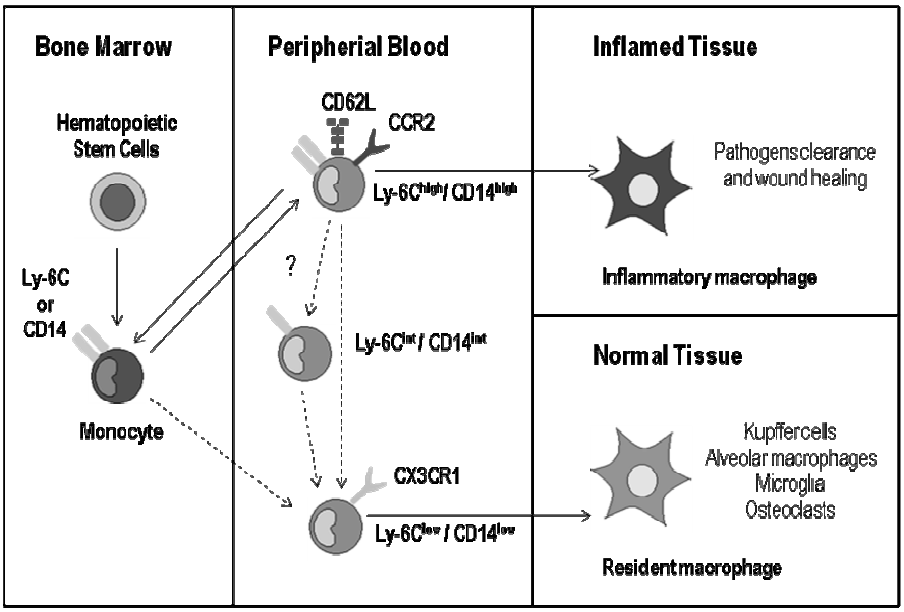

Figure 2. Ly-6C^{+} bone-marrow monocytes are released into the peripheral blood and are thought to adopt a Ly-6C^{high}/CD14^{high}, a Ly-6C^{int}/CD14^{int} and a Ly-6C^{low}/CD14^{low} phenotype. Ly-6C^{high}/CD14^{high} monocytes enter into the inflammatory tissue and change phenotype, becoming an inflammatory macrophage. In counterpart, Ly-6C^{low}/CD14^{low} cells migrate to normal tissue where they differentiate into a resident macrophage. Ly-6C^{int}/CD14^{int} monocytes function still unknown. Adapted (Gordon S. and Taylor PR. 2005).

In humans, is important to point out that distinct expression of CD14 and CD16 and of some chemokine receptors such as CCR2 in blood monocytes enabled the categorization of these cells into two major subsets: CD14^{high} CD16^{low} monocytes, which present high levels of CD14 and CCR2, and low levels of CD16, accounting for 90% of human blood monocytes, migrating to the inflammatory loci

where it will differentiate into macrophages and in a less frequency $CD14^{low}CD16^{high}$ subset that displays low levels of CD14 and CCR2 and high levels of CD16 and CCR5 and migrate in response to macrophage inflammatory protein-1α/RANTES (Passlick B., et al. 1989). Even though these cells have mostly distinct marker from the murine models, they present the same functions (Zimmermann HW., et al. 2010).

Although, two main subsets of monocytes the Ly-$6C^{high}$ and Ly-$6C^{low}$ were described, a third monocyte subset has been found. This subtype also called Ly-$6C^{int}$ is characterized by the expression of an intermediate level of Ly-6C but a high level of CCR2 markers in mice (Qu C., et al. 2004). In humans these cells are classified as $CD14^{+}CD16^{+}$, expressing also CCR2 (Ziegler-Heitbrock HW., et al. 1993). These cells are also known as transitional monocytes and their functionality has not been well studied in mice. However in humans some analysis of this population has been done. (Ancuta P., et al. 2003; Grage-Griebenow E., 2001) Interestingly, studies have been shown that this cells tend to express the whole range of CCRs that otherwise distinguish the two major subsets, in addition to unique expression of other CCRs (Ancuta P., et al. 2003). The presence of this wide range of CCR expression may make them the most flexible population of monocytes for recruitment to different chemokine signals, although low amount of these cells are found in the blood. The specific function of this intermediate phenotype it has not been described yet, but some authors suggested that they can be either resident or inflammatory monocytes depending on the inflammatory stimuli that they are submitted (Ancuta P., et al. 2003; Qu C., et al. 2004) (Figure 2).

2.3 Macrophages

Macrophages are large and heterogeneous tissue resident myeloid cells that present pseudopodia and phagocytic granules. These cells are distributed in almost every tissue of the body and have a stellate and foamy appearance. They develop distinct functions and display different patterns of surface molecules depending on what tissue they reside in and where in the tissue they are localized (Gordon S. and Taylor PR. 2005; Gordon S. 2003). Macrophages are usually the first cells to recognize an invading pathogen, a dying cell or a foreign substance. After recognition, the target is internalized by phagocytosis and destroyed in intracellular vesicles called phagosomes via several mechanisms e.g. by major pH drop or production of lytic enzymes or free radicals. They also release cytokines and chemokines which recruit neutrophils, other leukocytes and complement proteins to the site of infection and activate them to initiate the process called inflammation (Mosser DM., et al. 2008; Stuart LM., et al. 2005).

Macrophages are known also to be long-living cells, in the mice tissue where their half life range from a week to months and in human from months to years. However, recent studies have been shown that their viability is between 6 and 16 days depending on the type of tissue they are found (Papadimitriou JM., et al. 1989; Van Furth R. 1988).

2.3.2 Macrophage subtypes

Macrophages share many surface markers with neutrophils and dendritic cells, which make difficult to distinguish these cells from the others, in this sense the use of multiple markers together with cell morphology and function are needed to support phenotypic data. Examples of macrophages markers in murine models

are F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1 in mice) and intracellular molecule macrosialin (CD68). However, some of these markers are also found in dendritic cells and monocytes (Hum DA. 2006; Holness CL., et al. 1993). Furthermore, CD11b and cluster of differentiation molecule 11c (CD11c) also known as integrin alphas X and M, respectively, are also identified by subpopulations of macrophages. Nevertheless, these molecules are not exclusively expressed by macrophages and are also found on neutrophils, monocytes, natural killer (NK) cells and dendritic cells (Shortman K. and Liu YJ. 2002). In addition, due to the several controversies about the different macrophage subtypes, macrophage subtypes are better divided depending of the function (inflammation versus regeneration), the activation stimuli (cytokines), even by the secreted products (pro- vs anti-inflammatory cytokines) or the activation of several pathways (Table 2).

Briefly, several macrophage subtypes have been described, to help to differentiate macrophage population. Some of these subtypes include: M0 macrophage. Similar to the Th0 lymphocyte, the M0 macrophages are induced by CSF1, in a steady state condition as sentinels cells presenting a scavenging, homeostasis and trophic role (Table 2). On the other hand there are classically activated macrophages, also known as M1 macrophages. These cells can be are activated by different external stimuli such as LPS from Gram bacteria pathogens or inflammatory cytokines produced in the damaged tissue (GM-CSF, IFN-γ, and TNF-α), inducing an type I immunity, mediating host defense against, bacteria, viruses, protozoa, type IV hypersensitivity and tumor resistance. M1 macrophages although are characterized by the production of high levels of NO, TNF-α, IL-6 (Orme J. and Mohan C. 2012).

Furthermore, the M2 or alternative macrophages are described to be involved in tissue remodeling. These cells are divided in different subpopulations: M2a macrophage can be activated by IL-4, IL-10, IL-13 and PPARγ-agonists, this

activation induce a type II immunity that will fight parasites, allergies and profibrotic. Another M2 subtype, the M2b macrophage are activated by immunocomplexes (FCR agonists), IL-1R agonists, LPS and TLR agonists, these macrophages are involved for the immune regulation and the Th2 activation. At the end, the M2c macrophages are signaled by glucorticoid, IL-10 and TGF-β and present the capacity of immune suppression, tissue repair and matrix remodeling, regulating wound healing. However, these M2 subtypes are the most difficult to define since the same agents (e.g. IL-10) can induce different subtypes. Although there are some differences among the M2, each of these populations has mainly immunosuppressive activity and participates in the tissue repairing producing several markers such as chitinase 3-like 3 (YM1). At the end, these M2 macrophages present high express scavenging, galactose and mannose receptor (MRC) in the membrane surface, and an enhanced arginase pathway, which can permit to distinguish them from M1 or M0 macrophages (see table 2) (Arnold L., et al. 2007; Mantovani A., et al. 2004).

In the other hand another subtype of macrophage is also known, the regulatory macrophages, RMs, that are activated by CpG, LPS, transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF), and secrete high levels of IL-10 to IL-4R-mediate immune suppression. Last but not least, the tumor-associated macrophages, TAM, macrophages that differentiate in a tumor environment with M-CSF/CSF1 or in response to stimuli of the nuclear factor-κB (NF-κB), suppress tumor immunity (Biswas SK. and Mantovani A. 2010).

In general the most known M1 and M2 macrophage have been characterized buy the expression of some genes. tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), nitric oxide synthase 2 (NOS2), and interleukin-6 (IL-6), were used to indicate M1 phenotype, in the counterpart arginase I, MRC, IL-10 and TGF-β gene expression were used as markers for M2 phenotype (Sica A. and

Bronte V. 2007; Pello OM, et al. 2012). (Figure 5). Some signaling pathways, also can be classified into M1/M2, a good example of these pathways are signal transducer and activator of transcription (STAT) 1 and STAT6. STAT1 is described to be an IFNγ signaling pathway that leads to M1 macrophage phenotype and STAT6 a signaling pathways induced by IL-4 and IL-13 is recognized to induce a M2 macrophage phenotype (Figure 3). In this study some of these markers were used to characterize M1 and M2 phenotype.

Table 2: Macrophage subsets

	Inducers	***Suggested Role***	***Phenotypical Markers***
M0	CSF1	Scavenging; Trophic role; Homeostasis	Inactivated cells
M1 (Alternative Classicaly)	GM-CSF, IFNγ, LPS, TNF-α	Type I immunity bacteria, viruses, protozoa Type IV hypersensitivity Tumor resistance	Secretion of pro-inflammatory cytokines : TNF-α, IL-1β, IL-6 and the presence of NO
M2a (Alternative Activated)	IL-4, IL-10, IL-13 and PPARγ-agonists	Type II immunity; Parasites; Allergies Profibrotic	Presence of mannose receptor (MRC) and secretion of anti-inflammatory cytokines: IL-4, IL-10, TGF-β, IL-13
M2c (Desactivated)	Glucorticoid, IL-10 and TGF-β	Immune suppression Tissue repair; Matrix remodeling	
RMs	CpG, LPS, TGF-β and VEGF	IL-4R- mediated immune supression	
TAM	M-CSF/CSF1 stimuli of the nuclear factor-κB (NF-κB)	Suppress tumor immunity	

Orme J. and Mohan C. 2012 (Adapted).

2.3.1 Monocytes/Macrophage functions

Antigen-presenting cells such as monocytes/macrophages play a major role as sentinels for the first line alerts, mediate the shape of the adaptive immune response, and display important functions during the steady state condition (Hoebe, K., et al. 2004). In a steady state condition, circulating monocytes act as a transient reservoir of macrophage waiting to be directed to the tissue to differentiate and develop their function. Monocytes also appear to be involved in the surveillance of the vascular surface, in the defence against the peripheral blood phagocyting invading agents and in the production of some cellular mediators (Liu K., et al. 2007). Indeed, besides the ability to be a cellular precursor, several studies have suggested that the only definitive monocyte function is their ability to migrate from the bone marrow to peripheral blood and into the tissues where they differentiate into macrophages or dendritic cells, cells that present an stationary or a limited potential to migrate. So, for this reason monocytes become a very important part for the acute needs of the tissue (Auffray C., et al. 2007). However, several studies have demonstrated that during the steady state resident tissue macrophages can be maintained by local proliferation (Sawyer RT., et al. 1982; Landsman L., et al. 2007; Landsman L. and Jung S.2007).

However, as previously described, different subtypes of monocytes operate in different ways (see section 2.2.1). However, under inflammatory conditions Ly-6C^{high} monocytes migrate from the blood flow to the site of inflammation, differentiating into effectors cells such as macrophage (Woollard KJ. and Geissmann F. 2010; Gautier EL., et al. 2009; Zhang D., et al. 2012; Kim YG., et al. 2011). Studies have demonstrated that in the lung, monocytes replenish two subsets of pulmonary dendritic cells during steady-state condition and convert to either dendritic cells or macrophages during inflammation (Yrlid U., et al. 2006; Landsman L., et al. 2007; Landsman L. and Jung S. 2007; Varol C., et al. 2007; Jakubzick C., et al. 2008; Lenzo JC., et al. 2012). In addition, monocytes can be

recruited to tumor sites and can inhibit tumor-specific immune defense mechanisms (Chioda M., et al. 2011; Medina-Echeverz J., et al. 2011). In the steady state Ly-6C^{low} monocytes migrate from the peripheral circulation into the tissue where they differentiate into tissue resident-dendritic cells or tissue-resident macrophage. These cells once differentiated will present various functions in the maintenance of tissue homeostatic conditions, such as clearance of senescent cells, tissue remodeling, repair, as well as the genesis and the resolution of the inflammatory response. These cells also can be influenced several chemokines and cytokines produced by the surrounding environment or by themselves to be activated (Gilroy DW., et al. 2004; Gordon S. 2007).

Monocyte once differentiated into resident or inflammatory macrophage, present their own particular functions, according to the environment that they are in. Resident tissue macrophage are defined in distinct cells according to their location, they can be histiocytes in connective tissue, Kupffer cells in the liver, alveolar macrophages in the lung, Langerhans's cell in the skin, and microglia in the central nervous system, metallophilic and marginal zone macrophages in the spleen, as well as osteoclasts and pleural and peritoneal macrophages in the serous fluids (Takahashi K., 1994). These cells present ability to scavenge dying material means that macrophage also play a vital role during organogenesis in embryonic development, where they are highly concentrated at sites of high cell death, such as developing limb buds (Hopkinson-Woolley J., et al. 1994). However macrophage also performs tissue-specific functions. For example, macrophage in the liver aid the removal of toxins from the circulation, while macrophage in the alveolar space are specialized at engulfing and eliminating inhaled environmental antigens, and osteoclasts are essential for bone remodeling. Although scavenging cellular debris may be their primary role under normal conditions, macrophage also secrete mediators that contribute to maintenance of homeostasis, including enzymes, cytokines, chemokines, arachidonic acid derivatives and glycoproteins such as fibronectin (Takemura R. and Werb Z. 1984). Nevertheless, all these functions are

independent of the immune system and are very important for the tissue maintenance.

On the other hand, macrophage act as sentinels when there is any sign of infection, inflammation or trauma, participating in an innate and adaptative immune response, where they recognize, phagocyte and clear invading pathogens or apoptotic cells through the expression of pattern recognition receptors. Macrophages can be classically activated in result from the recognition of pathogen-associated molecular patterns (PAMPs) on the pathogens surface. Macrophages also express a range of toll-like receptor (TLR) and other pattern recognition receptor which are triggered during phagocytosis resulting in the production of pro-inflammatory cytokines, TNF-α, IL-1, IL-6, IL-12 and IL-23 and chemokines which will participate in immune modulation, antigen presentation and the regulation of T cell activation and differentiation. These cells also can be alternative activated participating in resolution of inflammation and in the promotion of healing through induction of matrix synthesis, fibroblast proliferation, angiogenesis and the clearance of cellular debris (Stout RD. and Suttles J. 2004).

Microbial antigens lead to the classical activated macrophages induced by pro-inflammatory microbial molecules such as LPS in a Th1 cytokine environment (IFN-γ produced by T lymphocytes), production of TNF-α and release of inflammatory and/or microbicidal products in tissues. These cells can be identified by their ability to produce nitric oxide (NO) combined with an increased expression of major histocompatibility complex (MHC) class II and CD86 enhancing their antigen-presenting capacity (APC, antigen presenting cell) to T lymphocytes. Activated macrophages play an essential role in protection against intracellular pathogens. They also exert anti-proliferative activities secreting NO and pro-inflammatory cytokines such as TNF- α, IL-1, IL-6. However, the persistence of inflammatory processes often results in tissue damage and the immune system

faces a permanent challenge for developing anti-inflammatory mechanisms (Gordon S. 2003).

The development of these activated macrophages is inhibited by Th2 cytokines; as a result, they switch to alternative activation program (Noël W., et al. 2004). M2 macrophages are activated in the presence of IL-4 and IL-13 and deactivated in the presence of IL-10. M2 Macrophages, in contrast to M1 macrophages, fail to generate NO from L-arginine and do not efficiently limit the growth of intracellular pathogens. Moreover, they exhibit enhanced endocytic and phagocytic ability and increased expression of MHC class II molecules for antigen presentation. As such, anti-inflammatory macrophages protect organs and surrounding tissues against detrimental immune responses promoting tissue remodeling (Figure 3).

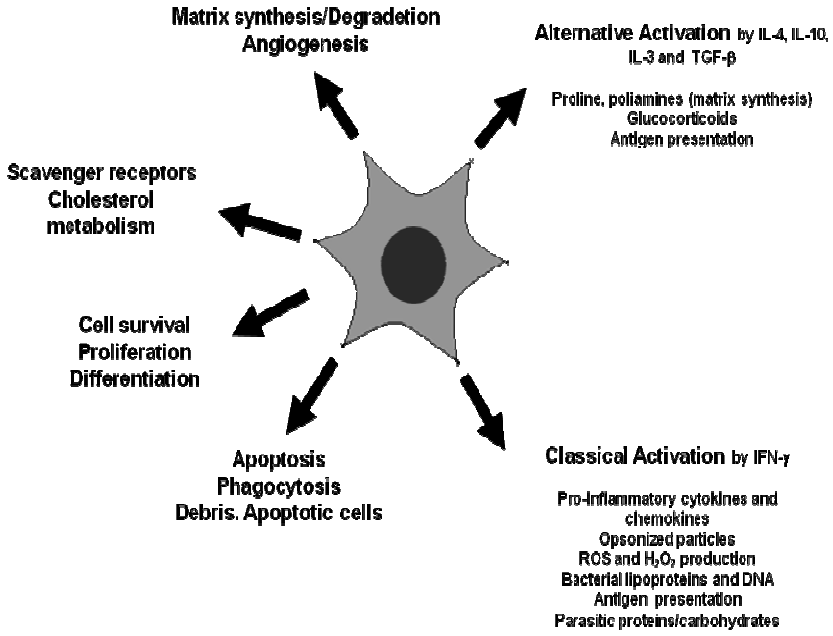

Figure 3. Biological functions of macrophages. According on the stimuli, macrophages proliferate, activate or die through apoptosis.

3. Inflammatory response

When the body suffers aggression, by pathogens, damaged cells, injury, microbes, and trauma by other physical agents like irritants suffers the purpose of the immune system is to control or eradicate these noxious agents. It forms part of the complex biological response of vascular tissues to harmful stimuli (Ferrero-Miliani L., et al. 2007). The initial reaction of the immune system to an infection depends on the site of the invasion and on the nature of the invader. Many insults can "trigger" an inflammatory response and dispatch cells and chemicals to the inflammatory site to repair the damage. Inflammation is a key part of the body's defense system, an indispensable protective response by the body's system of self-defense. In this sense, this process is also involved in the pathogenesis of chronic diseases of autoimmune origin such as atherosclerosis, rheumatoid arthritis and cancer (Gilliver SC., et al. 2011).

3.1. Inflammation

The word inflammation comes from the Latin *inflammare* (to set on fire). Cornelius Celsus, a Roman, wrote: the signs of an inflammation are four: redness and swelling with heat and pain (Celsus. 1935). Rudolf Virchow later added: *loss of function*. Two centuries after Celsus, Galen considered inflammation as a beneficial response to injury; on the other hand, many centuries later Virchow viewed inflammation as inherently pathological. In nowadays inflammation concepts are much more complex, which is classified as a protective attempt by the orgamism to remove the injuries stimuli and to initiate the healing process. However, this process is a stereotyped response, and therefore is considered as a mechanism of innate immunity as compared to adaptative immunity, with is specific for each pathogen (Abbas AB., et al. 2009). The inflammatory response is dependent on the type of aggressive agent that induces, if the offending intruder is dust or food, an

allergic response type will occur, if the invading agent is bacterial infection a classical inflammatory response type will be induced, if the aggressive agent are gastrointestinal nematodes, an alternative inflammatory response will happen. All these inflammatory responses will be distinct as they lead to different types of response, eg Th1 or Th2.

3.1.1. Inflammation acute vs chronic

Furthermore inflammation can be classified as either *acute* or *chronic*. *Acute inflammation* is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes), neutrophils (primarily), basophils (inflammatory response), eosinophils (respose to helminth worms and parasites), and mononuclear cells (monocytes, macrophages) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue; this response is immediate and last for few days and can result in to resolution of the inflammatory process, to an abscess formation or to a chronic inflammatory process (Figure 4). Prolonged inflammation, known as *chronic inflammation*, leads to a progressive shift in the type of cells present like mononuclear cells (monocytes, macrophages, lymphocytes, and plasma cells), fibroblasts at the inflammatory loci and is characterized by simultaneous destruction and healing of the inflamed tissue; this response are delayed and can take months or years to end, the outcomes of this process can give rise to the tissue destruction, fibrosis and necrosis (Table 3) (Singer AJ, et al. 1999), that can develop into different pathologies such as allergy, inflammation and autoimmune diseases.

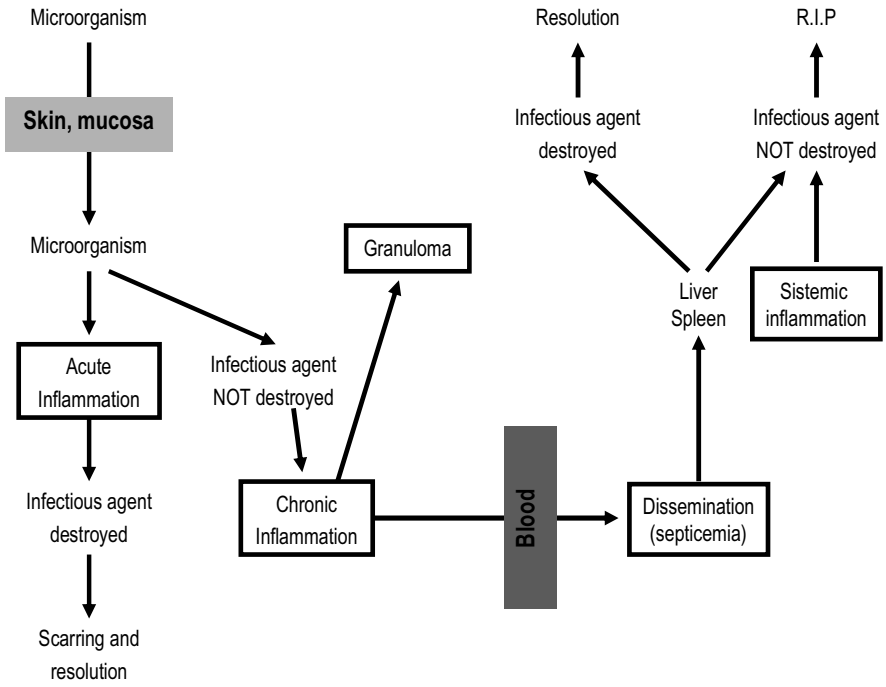

Figure 4. Inflammation evolution.

Briefly, in these acute inflammation described, the main components produced are the change in flow and vascular caliber, increased vascular permeability and inflammatory exudates and finally the passage of leukocytes from the vascular space to the extravascular (Table 3). The origin of these disorders is due to the release of chemical mediators such as TNF-α, IL-8, IL-6, IL-1, prostaglandin E2 and NO by neutrophils (primarily), basophiles (inflammatory response), eosinophils (response to helminthes worms and parasites), and mononuclear cells (monocytes, macrophages). These chemical mediators also participate in the activation of leukocytes, inducing phagocytosis and bacteria lysis. The activation of coagulation system, fibrinolysis and complement enhance the inflammatory process. Increased expression of adhesion molecules on endothelial cells, such as intracellular adhesion molecules (ICAM) class I, vascular adhesion molecules (VCAM) class I or

E-selectin facilitates diapedesis (migration of immune cells to inflammatory loci). In the first 6 to 24 hours in local inflammation neutrophils are found, these are replaced by monocytes and macrophages arriving at loci within 24 hours. These cell types are responsible to phagocytose microorganisms, altered cell and tissue debris (Table 3). At this stage the acquired immune response is triggered by antigens, resulting from phagocytosis by macrophages, dendritic cells and B cells when the innate immune response is not capable to eliminate the pathogen or eradicate the inflammation. These antigens are displayed by antigen presenting cells trough their MHC class I or MHC class II to CD8+ cytotoxic T cells or CD4+ helper T cells respectively. Naïve T helper cells can be hider activated by MHC class II antigens in macrophages or dendritic cells or by the presentation of the antigen by B cells, in each case the T cells are known by different names Th1 cell and Th2 cells respectively. Th1 cells once activated produce IFN-γ a cytokine capable to activate macrophages which will phagocyte the pathogen once again. If the cell mediated response occurred IFN-γ in addition to activate macrophages will inhibit the antibody response by the plasma cells. Furthermore, Th2 cells can cooperate with and can activate B cells by the release of cytokines, IL-4, IL-10 and IL-13. These cytokines will induce B cells to proliferate and to give rise to memory cells and plasma cells that will produce antibodies against these specific pathogens. When this antibody response take place IL-10 produced by Th2 cells will prevent Th1 cells form produce IFN-γ, suppressing the cell mediated response once the tissue is clear o invaders, leading to inflammation resolution. Although, macrophages may also display parasite peptides in the context of MHC class I molecules to cytotoxic T cells that activate proliferation and secretion of IFN-γ that will destroy the infected macrophage. In result to all previously mentioned, acute inflammation can progress to full resolution, by substituting for the healing tissue, abscess formation, or to the progression of tissue response to a chronic inflammation (Ingersoll MA., et al. 2011).

Table 3: Division in the Immune system		
	Acute	***Chronic***
Causative agent	Bacterial Pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions
Cells involved	Neutrophils (primarily), basophils (inflammatory response), and eosinophils (respose to helminth worms and parasites), mononuclear cells (monocytes, macrophages)	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months, or years
Outcomes	Resolution, abscess formation, chronic inflammation	Tissue destruction, fibrosis, necrosis

Chronic inflammation is characterized by the cellular infiltrate, is occupied mainly macrophages, lymphocytes and plasma cells, overriding the formation of fibrous tissue. This process of chronic inflammation can occur for various reasons such as the progression of acute inflammations, recurrent episode of inflammation by acute and frequent intracellular infections. Most of the cases leads to chronic pathologies such as artrithuis reumatoid, encephalomyelitis, Amyotrophic lateral sclerosis (ALS), Alzheimer's disease and other dementias, arthritis, asthma, cancer, chronic obstructive, pulmonary disease (COPD), cystic Fibrosis, diabetes, eating disorders, end stage renal disease, heart disease, obesity, oral health, osteoporosis, Reflex Sympathetic Dystrophy (RSD) Syndrome, among others (Hodge SJ., *et al.* 2003).

Lymphocytes play an important role in this type of inflammation which is present in continues manner in the locus, using substances produced by

macrophages to their migration. In turn, secrete IFN-γ, activator of macrophages, thus establishing the basis for the persistence of the inflammatory reaction. Macrophages also produce biologically active substances such as cytokine, TNF-α, IL-1β, among others substances, that can be destructive when produced in an uncontrolled manner. The accumulation of macrophages persists in chronic inflammation for the continued recruitment of monocytes from blood flow, to the inflammatory loci, that differentiate in macrophages after migration, and proliferate in the inflamed area (Table 3). On the other hand, plasma cells produce antibodies, against the antigen that persist in the inflamed area or against altered tissue components. All of this produces a vicious cycle that leads to chronic inflammation in which all immunological cells are involved (Matsuzaki H., et al. 2012).

3.2. Steps of inflammation response

Inflammatory resolution (wound healing) involves a cascade of sequential biological processes (Enoch and Price 2004). Cellular and biochemical activities including phagocytosis, cell migration, cell proliferation, collagen synthesis and cytokine production are well coordinated in normal wound healing (Singer AJ. and Clark RA. 1999; Martin P. 1997). The process takes place in 3 phases or steps: after injury or infection or antigen contact, there is cell recruitment (3.3.1), monocytes and leukocyte extravasation from the blood flow to the inflammatory loci (3.3.2) and inflammation resolution (3.3.3).

3.2.1 Cell recruitment

Cell recruitment is can be divided into different steps: mobilization from the bone marrow to the blood flow, rolling, adhesion to endothelial cells, and influx into

the tissue. The first contact is between the endothelium and cell surface receptor such as CD62L and P-selectin glycoprotein ligand 1 (PSGL-1) with PNAd and P/E-selectins, respectively. Integrins, mediator of the attachment between a cell and the tissue, can also form contacts during this stage. During rolling step, leukocytes-chemokine receptors recognize chemokines presented by glucosaminoglycans on the endothelium, leading to the activation of integrins on the leukocytes, enabling engagement with counter receptors present in the interior surface of blood vessels and lymphatic vessels (Ley K., et al. 2007). The result of this is a firm arrest of the leukocyte to the vessel wall. The next step is the strengthening adhesion, spreading and crawling before the cells transmigrate through a paracellular or transcellular pathway. All of this process is initiated and performed by chemokines.

Chemokines are a family of small cytokines, or proteins secreted by cells also known as small basic proteins that are named after the position and number of cysteine residues, and are called C, CC, CXC or CX3C chemokines. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells, been the ones responsible to control the migration of cells into and out of the tissue. Glucosaminoglycans or sulfated proteoglycans present on the cell surface or in the extracellular matrix bind to chemokines, to concentrate them in the tissue and prevent them from being drained out by the blood when they are presented on endothelial cells (Thelen M. 2001). Furthermore, chemokines bind also to different types of glucosaminoglycans with different affinities, therefore adding an extra control level (Rot A. and von Andrian U. H. 2004). Some chemokines are considered pro-inflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection, while others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development.

During inflammation or infection, pro-inflammatory cytokines and TLR ligands are the ones responsible to induce the production of chemokines that attract

phagocytes as well as lymphocytes to the inflammatory loci (Sallusto F., et al. 2000). In addition, cytokines and TLR ligand guides the migration of the individual cell in the tissue by changing the repertoire of distinct chemokine receptors on the cell surface. For example, CCR2 is up regulated on monocytes in response to CCL2, LPS or TNF-α and also CXCR2 is down regulated on human neutrophils in response to CXCL2, LPS or TNF-α (Khandaker MH., et al. 1999; Luster AD. 2002).

Endothelial cells from the high endothelial venules (HEVs) produce chemokines, which are also produced by neighboring cells inside the lymph node and transported to the luminal surface of the HEVs (Palframan, R. T., et al. 2001). These chemokines produced in tissues are transported to the draining lymph node presented on HEVs. A good example of this is the production of the CCL2, which is produced in inflamed skin and transported to lymph node where it induces adhesion of inflammatory monocytes on the HEVs (Janatpour, M. J., et al. 2001). Therefore, the association of chemokines, selectins and integrins direct the migration of the cell to distinct destines such as lymph node and/or tissue.

During homeostasis, Ly-6C^{high} monocytes and neutrophils migrate slowly to tissues, but during inflammation they are recruited very fast. Monocytes under non-inflammatory conditions do not adhere to HEVs, but under inflammatory stimuli these cells roll and adhere to HEVs. Monocyte rolling is dependent on P-selectin interacting with PSGL-1, whereas adhesion is mediated by VCAM-1, and by ICAM-1, expressed on the endothelial cells (Leon B., et al. 2008; Tedder TF., et al 1995). Monocytes recruitment during infection seems to be dependent on CD62L, whereas PSGL-1 can be replaced by other adhesion molecules. In an inflammation or an infection process the influx of Ly6-C^{high} monocytes from the bone marrow, to the blood and the inflammatory loci, increase, process dependent on CCR2. In the absence of CCR2, or the ligands CCL2 and CCL7, monocytes are not able to leave the bone marrow during inflammation and also during steady state conditions (Mack M., et al. 2001; Geissmann F., et al. 2003). After monocytes are released

into the blood, the recruitment to inflamed tissues is directed by CCR2 and its ligands during various inflammatory conditions, a deficiency in this receptor or its ligands is linked to the induction of several inflammatory diseases such as atherosclerosis and predisposal infection with *M. tuberculosis* or *Listeria* (Suresh MV., et al. 2012). In addition, the ligands to CCR2, particularly CCL2, are induced in many infected or inflamed organs (Tacke F., et al. 2007; Tsou CL., et al. 2007). This argues for a crucial role for CCR2 during monocyte recruitment to tissues in several inflammatory settings.

The absence of some chemokine receptors and ligands were described as been important to the susceptibility to bacterial infection. However, these results in the decreased exit of monocytes from the bone marrow and their accumulation in blood, leading to low amounts of monocytes at the site of inflammation, since there are very few Ly6-C^{high} monocytes in the blood. Ly-6C^{high} monocyte are known to expresses high levels of CCR2 and a low level of the fractalkine receptor CX3CR1, on the contrary Ly-6C^{low} monocyte subset expresses low level of CCR2 and a high level of CX3CR1, these cells depends on CX3CR1 to crawl along the blood vessels and, in response to infection or inflammatory signals, these receptors are the ones responsible for their transmigrate at the site of infection or inflammation (Tacke F., et al. 2007). In the other hand, Ly-6C^{high} monocytes use CX3CR1 together with CCR2 and CCR5 to migrate into inflamed tissue, while Ly-6C^{low} monocyte migration depends on other receptors such as CCR5 (Mack M., et al. 2001; Tacke F., et al. 2007).

CCR2 a receptor specific for Ly-6C^{high} monocyte and a CCR5 a receptor present in all monocyte subtypes are implicated in the migration of monocytes/macrophages to inflamed tissues such as atherosclerotic lesions. Studies have shown that an animal with a CCR5 deficiency, monocytes/macrophages recruitment to the brain is reduced during West Nile virus infection, while normal recruitment and clearance of bacteria are detected during

Listeria infection (Glass WG., et al. 2005). CCR2 expression decreases when human monocytes differentiate into macrophages in vitro while CCR5 expression increases (Zernecke, A., et al. 2006; Kaufmann, A., et al. 2001). Moreover, CCL3 a ligand of CCR5 play a role in recruitment of monocyte/macrophage into wounds (Xue ML., et al. 2007). However, the role of these receptors for monocyte migration is not clear. It is possible that the chemokine receptors and their ligands have similar and overlapping functions and are expressed in a different manner in response to inflammation and infection.

3.2.2. Role of mononuclear phagocyte system in inflammation

In an acute or chronic inflammation state monocytes are recruited from the blood into the tissues where differentiate into macrophages as already mentioned before (see section 2.2). In response to acute injury or infection, Ly-6C^{high} monocytes levels increase in the circulation, due to their high production and release from the bone marrow and the spleen, the presence of large amounts of these cells in the blood result in a rise emigration to the infected tissues, and in a further differentiation into macrophage (Drevets DA., et al. 2004; Serbina NV., et al. 2006; Nahrendorf M., et al. 2007; Tsou CL., et al. 2007). In 2008, Dunay IR., el at, shown that Ly6C^{high} monocytes migrated from the blood stream to the infected tissue where they produced TNF-α and NOS2, and upregulated F4/80, but not CD11c, yielding macrophages at the inflammatory loci. These macrophages also known as classically activated M1 macrophages or pro-inflammatory macrophages arise from the activation of the IRF5 and STAT1 signaling pathways by IFNs and TLR (Sica A., et al. 2007).

Pro-inflammatory macrophages play a key role in acute inflammatory processes, participating in the antigen processing and in the high antigen-

presenting activity associated with increased MHC class II expression, and in the phagocytic removal of necrotic material. Once in the inflamed loci, M1 macrophages upregulate interferon regulatory factor (IRAF) 5 producing high levels of pro-inflammatory cytokines, such as TNF-α, IL-12, IL-1β and IL-23, which are involved in to the Th1 and controversial Th17 cell responses (Krausgruber T., et al. 2011; Martinez FO., et al. 2009). In addition, they can induce NOS2 expression, which is required to efficiently metabolize L-arginine to generate the large amounts of NO involved in killing intracellular pathogens. Furthermore, IFN-γ derived from T cell, provide a positive feeds back that expands the population of M1 cells while also increase their microbicidal and tumoricidal activity (Gordon S., et al. 2010; Mantovani A., et al. 2002). M1 cells are generally believed to serve a protective role in tumorigenesis by antagonizing the suppressive activities of tumor-associated macrophages, alternatively activated macrophages and regulatory macrophages, which promote tumor growth, invasion and metastasis by suppressing adaptive antitumor immune responses. On the other hand, uncontrolled M1 inflammation secretes large amounts of TNF-α and IL-1β and participates in the differentiation of Th17 cells, thereby promoting further inflammation causing gastroenteritis, urinary tract infections, neonatal meningitis, and sepsis (Cavaillon JM. and Adib-Conquy M. 2006; Krausgruber T, et al. 2011). However, these exacerbated response by M1 also drivers to tissue damage, a chronic inflammation and autoimmune diseases, including rheumatoid arthritis, atherosclerosis, pulmonary fibrosis and Crohn's disease (Murray PJ. and Wynn TA. 2011). The maintenance of M1 macrophages are thought to derive entirely from circulating blood monocytes, but recently it has been shown that alternatively activated macrophage that arise in the Th2 setting may also be maintained through self-renewal, a process that is highly dependent on IL-4 (Ogawa M. 1994).

Finally, the microenvironment can also direct Ly-6C^{high} monocytes, resident and M1 macrophages to convert to an anti-inflammatory phenotype once the

inflammatory response decrease. Ly-6C^{high} monocytes recruitment during tissue injury was demonstrated to phagocyte cell debris proliferate and differentiate into alternatively activated macrophages or M2 cells, which produced anti-inflammatory cytokines and promoted tissue repair (Figure 5). Although in some cases Ly-6C^{low} also can differentiate into anti-inflammatory macrophage in the tissue (Galkina E., et al. 2007). Hence, the microenvironment and cause of inflammation determines the fate and function of newly recruited monocytes.

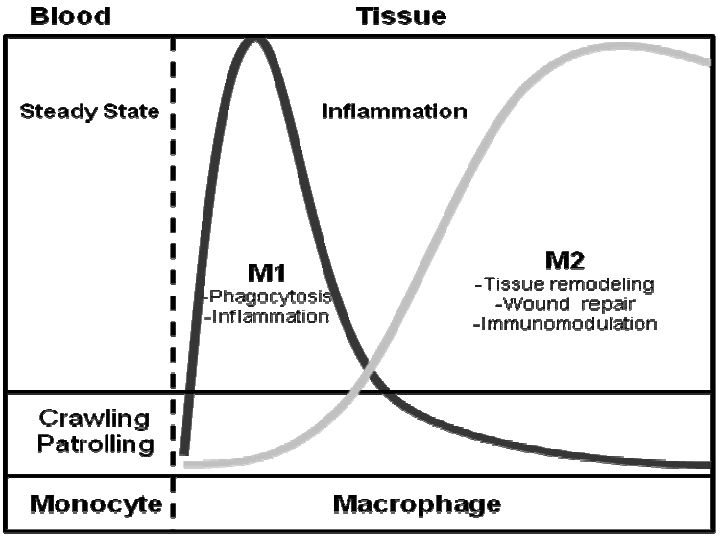

Figure 5. Monocyte/macrophage phenotypes switch during a steady state and inflammatory response.

When immune response is reduced by signals including IL-10, glucocorticoid hormones, molecules released from apoptotic cells, and immune complexes or by the activation by IL4, IL-13 and IL-33 cytokines from Th2 lymphocytes, M1 macrophages can themselves polarize into an alternative state, in the inflammatory foci (Arnold, L. et al 2007; Biswas, S. K. and Mantovani, A. 2010) (Figure 5). These cells also known as M2 macrophages, in contrast to pro-inflammatory and

antimicrobial M1 macrophage responses, present an anti-inflammatory activity producing IL-4, IL-10, IL-13, TGF-β as well as the pro-angiogenic cytokine such as vascular endothelial growth factor, cytokines that suppress antitumor immunity and also induce wound healing, tissue remodeling and angiogenesis (Wynn, T. A. 2004; Xiao, W., et al. 2008; Sindrilaru, A. et al. 2011). This phenotype switch is controlled by the activation of transcription factors IRF4, STAT6 and peroxisome proliferator-activated receptor (PPAR) γ, among others (Figure 6). These alternatively activated macrophages are appreciated during allergy, parasitic infection, and in the healing phase after tissue damage and are characterized by the presence of the MRC on their surface, by their proline secretion and by their phagocytic activity.

The anti-inflammatory macrophages population is more complex than the pro-inflammatory macrophages, being divided in distinct subtypes, with different specializations (see section 2.3). All these M2 macrophages subtypes together induce expression of anti-inflammatory genes such as MRC, arginase-I, found in inflammatory zone 1 (Fizz1), Ym1, PPARγ among others when the IL-4 type I and II receptors activate the STAT6 pathway. In the other hand when IL-10 activates the STAT3 pathway other genes are expressed such as IL-10, TGF-β1 and MRC and also produce growth factors that stimulate epithelial cells and fibroblasts, including TGF-β1 and platelet-derived growth factor (PDGF) (Barron L. and Wynn TA. 2011) (Figure 6). Macrophage-derived TGF-β1 contributes to tissue regeneration and wound repair (Sunderkotter, C.,et al. 1994; Roberts AB., et al. 1986; Shimokado, K. et al. 1985). Additionally, M2-secreted IL-4, IL-13, and IL-10 may support the generation of anti-inflammatory Th2 cells and immunosuppressive Treg, favoring alternative inflammation. An uncontrolled activation of the M2 cells may induce many chronic inflammatory such as atherosclerosis, fibrotic diseases and allergy responses (Chinetti-Gbaguidi G. and Staels B. 2011).

As mentioned before, several mechanisms including signaling pathways, transcriptional factors, epigenetic mechanisms, and posttranscriptional regulators

are known to be involved in the switch of the M1 to M2 phenotype. It has been suggested that in phagocytosis, STAT6 coordinates and synergize with PPARγ and Krüppel-like factor (KLF) 4, to induce M2 activation and M1 inhibition via sequestration of coactivators required for NF-κB activation, such as KLF2 that inhibit M1 activation by inhibiting NF-κB/HIF-1α activities (Cao Z., et al. 2010; Liao X., et al. 2011; Mahabeleshwar GH., et al. 2011; Szanto A., et al. 2010). However, NF-κB also activates a genetic program essential for resolution of inflammation and for M2 polarization (Lawrence T. and Gilroy DW. 2007). Figure 5 summarize the differents pathways that lead to M1 and M2 phenotypes (Figure 6).

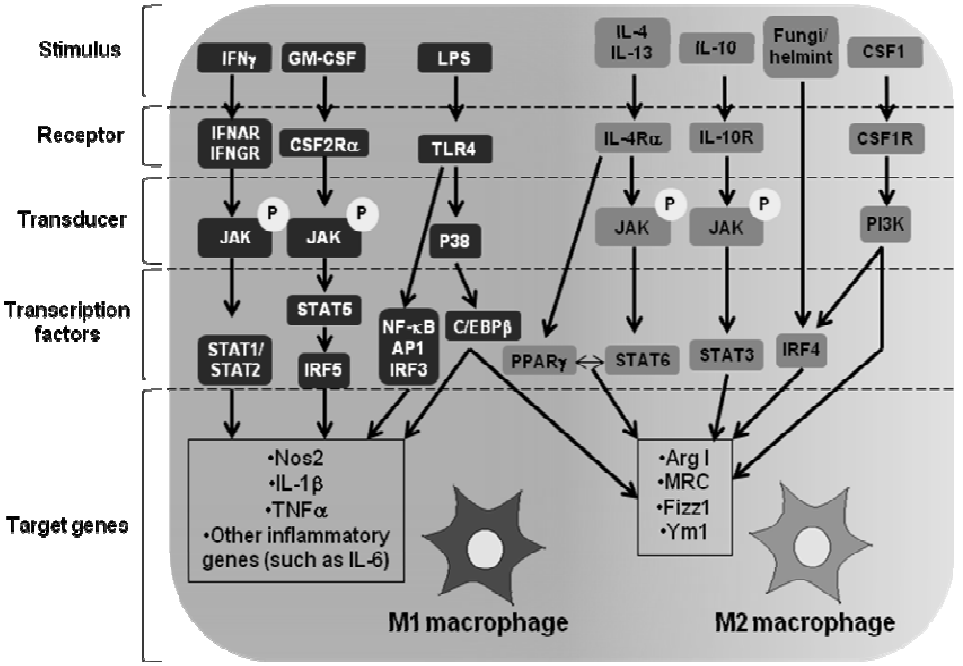

Figure 6: Signalling pathways implicated in M1 and M2 macrophage polarization are indicated. A stimulus leads the activation of sequence-specific transcription factors that mediate the changes in phenotype. The main genes that are characteristic of either the M1 or the M2 polarized state are also shown. Adapted (Lawrence T. and Natoli G. 2011).

3.2.3 Inflammation resolution

The survival of an organism depends on the ability to repair damage from trauma, from toxins exposure or from infections. For this propose, cellular and biochemical activities including phagocytosis, inflammatory cell migration and proliferation, collagen synthesis and cytokine production are well coordinated in normal tissue healing. The resolution of tissue injury in addition to inflammatory cells also requires the presence of fibroblasts, in order to produce new temporary extracellular matrix (ECM) components, such as collagen types I and III, fibronectin, elastin, proteoglycans, and laminin, which stabilize the tissue, and act as a scaffold for the new tissue (Lluri G., et al. 2006). The ECM formation and degradation is mediated by the expression of proteases and their specific inhibitors during tissue repair, it degradation also leads to the generation of protein fragments that mediate activities required to facilitate normal tissue repair (Chen X. and Li Y. 2009). Finally, in addition to ECM remodeling, angiogenesis facilitates the development of a new vascular network at the site of injury, while newly formed tissue undergo growth and maturation. Those activities occur in 4 major overlapping steps, inflammation, re-epithelialization, granulation tissue formation and tissue remodeling (Singer AJ. and Clark RA. 1999; Martin P. 1997).

Tissue injury can cause leakage of blood from damaged blood vessels. The formation of a blood clot reestablishes hemostasis and provides a provisional matrix through which cells can migrate during the repair process. The clot acts as a reservoir of cytokines and growth factors. Vasoactive mediators and chemotactic factors generated at the site of the wound attract neutrophils to cleanse the bacteria and foreign particles. In response to specific chemoattractants such as TGF-β produced by platelets, monocytes also migrate to the wound site and later become activated macrophages stimulated by the binding to the extracellular matrix (Singer AJ. and Clark RA. 1999; Dipietro LA., et al. 2001). The binding of macrophage to extracellular matrix induces the production of many important cytokines that are

necessary for tissue repair such as TGF-β, IL-1, TGF-α, PDGF. For example, TGF-β-1 and β-2 are related to induce cell migration while PDGF is related to induce fibroblast proliferation (Singer AJ. and Clark RA. 1999). Moreover, macrophages are also important in the phagocytosis of any remaining damaged cell debris (Brown, 1995). After 2-3 days, the number of neutrophils begins to decline but macrophages continue to accumulate at the wound site (Figure 7).

Approximately 3 or 4 days after injury, dermal fibroblasts begin to migrate into the provisional matrix where they proliferate and contribute to new extracellular matrix production (Clark JW. 1993). As previously mentioned, the degradation of the provisional extracellular matrix is required to permit fibroblasts and endothelial cells to move into the wound space. In order to do so, an active proteolytic system of enzymes including plasminogen activator and various members of the matrix metalloproteinase family (MMPs) such as, MMP2 and MMP 9 (Arumugam S., et al 1999) play an important role. After injury, the fibroblasts stimulated by TGF-β1 will be responsible for the synthesis of a rich collagen matrix, which will replace the provisional matrix and give the wound tensile strength. During the formation of the granulation tissue, angiogenesis occurs to form new blood vessels/ capillaries to support the formation of granulation tissue. Macrophages provide a good source of cytokines such as acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), TGF-β and VEGF which can stimulate wound angiogenesis (Wiseman DM., et al 1988; Bates DO. and Jones RO. 2003; Tonessen, et al 2000). When most of the provisional extracellular matrix is replaced by collagen in the granulation tissue, the formation of new blood vessels stop and those new blood vessels will be degenerated (Tonessen, et al 2000).

During tissue regeneration, macrophages and fibroblasts are essential cells in the normal wound healing process, where a balance between pro- and anti-inflammatory cytokines exist, although when an dysfunction in cytokine production

or extracellular matrix regulation by macrophages and fibroblasts occurs an impairment of wound healing happens (Figure 7). Macrophages and fibroblasts both demonstrated defective migration or proliferation in wounds in some disease and can decrease the release of chemokines such as TNF-α and IL-1β and by VEGF, a potent angiogenic factor by both fibroblasts and macrophages (Zykova SN., et al 2000; Wetzler C., et al 2000). On the other hand during a chronic inflammatory response, the release of chemokines and growth factor can be excessive, which leads to aberrant cell proliferation and differentiation resulting in abscess, fibrosis and scar formation (Figure 7).

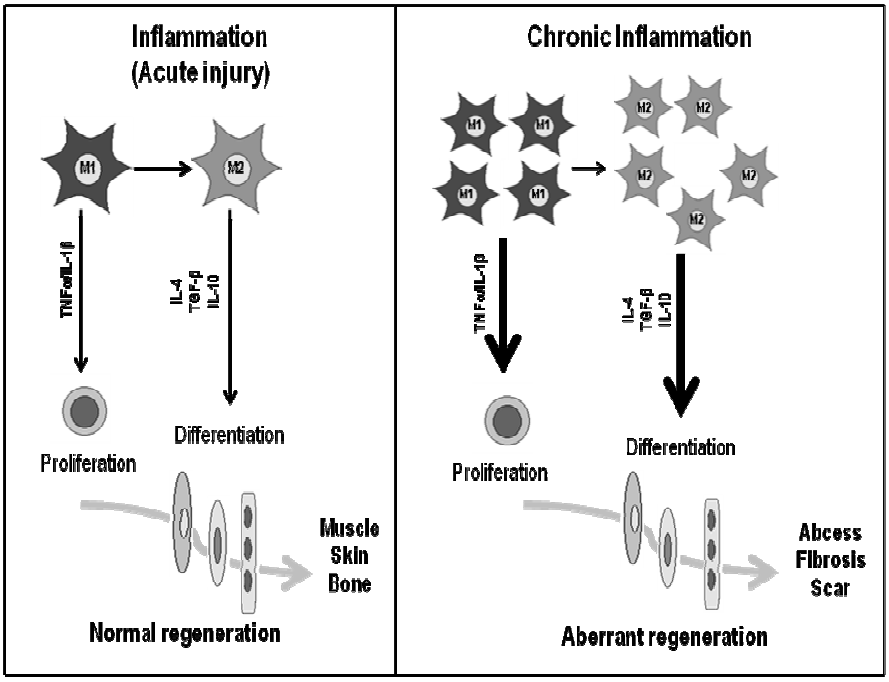

Figure 7. Inflammation resolution. Normal and aberrant regeneration process. Adapted (Mann CJ., et al. 2011).

4. Animal models of inflammation

Animal models are necessary to study the pathogenic mechanisms of disease that occur in humans and are also important to understand the complex interactions occurring in the body during an inflammatory response, in a way that would be impossible to examine with cell cultures. They can be used to study common and selective pathways that cause inflammation. Although none of the experimental models can completely mimic human disease, they always share many common features with them. Therefore animal models are of great importance in understanding the mechanisms of autoimmune diseases and consequently in designing therapeutic tools to combat these illnesses like rheumatoid arthritis, atherosclerosis, pulmonary fibrosis and Crohn's disease (Denney L., et al 2012; Yi T. and Song SU. 2012).

In the literature there are several animal models that permit to study the migration of monocytes; one of this is the 1-fluoro-2,4-dinitrofluorobenzene (DNFB) skin model of inflammation (see section 4.1.). However, the role of macrophages in an *in vivo* inflammation is difficult to determine due to the presence of other immune cells in this loci that play a crucial role during the inflammatory response making difficult to discriminate the specific involvement of macrophages in these models. Recently, it has been described by Ludovic and co-workers that in a model of muscle inflammation produced by Notexin (NX) (see section 4.2.), snake venom, the macrophages are the main mediators of the inflammatory response.

4.1. DNFB skin model

DNFB is an irritant agent that acts as a hapten and induces skin irritation in mice ear, resulting in ear swelling, itching, hypertrophy of lymphoid structures,

recruitment of inflammatory cells (such as monocyte/macrophages) and infiltration and activation of keratinocytes cells in the skin (Rijnierse, A., et al. 2006). The result of this irritation is caused by cytokines and infiltrating neutrophils and monocyte-derived macrophages (Sakai, S. et al. 2010; Ahmed, A. et al. 1983). This model resembles to the human contact dermatitis provoked by different agents like chemical irritants and physical irritants such as solvents (alcohol, xylene, turpentine, esters, acetone, ketones, and others); latex; kerosene; ethylene oxide; alkalies and low humidity from air conditioning; plants directly irritate the skin, respectively (Morris-Jones R, et al. 2002).

Irritant Contact dermatitis (ICD) is an inflammatory disease induced by skin exposure to low molecular weight chemicals, that present a proinflammatory and antigenic properties (Rowland et al., 2001; Saint-Mezard P., et al., 2004). It presents as acute, subacute or chronic eczema (Saint-Mezard P., et al. 2004; Fyhrquist-Vanni N, et al. 2007; Slodownik D, et al. 2008; Bonneville M, et al. 2004). (Figure 8).

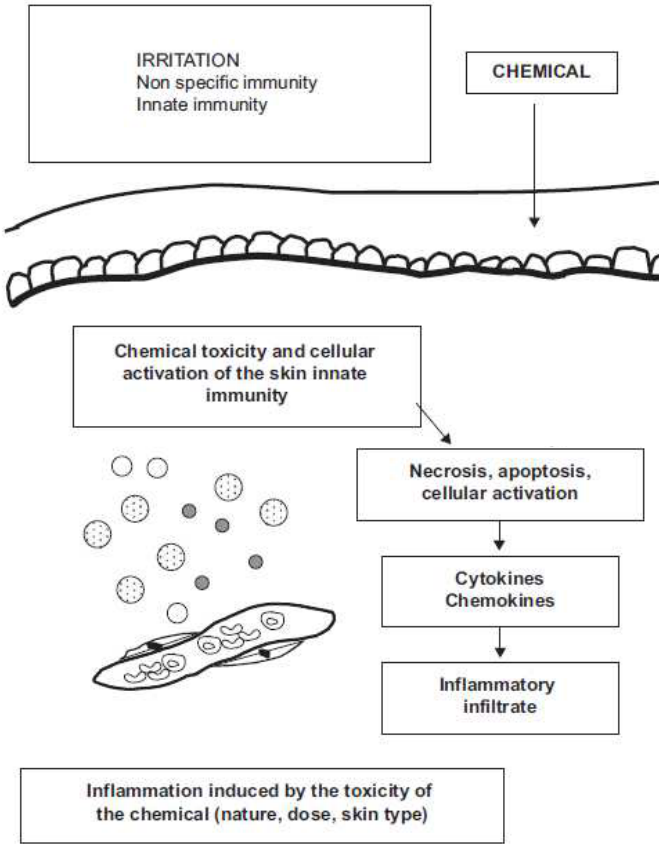

Figure 8. Immune mechanisms in ICD. ICD is induced by skin contact with chemicals. The chemical is pro-inflammatory by its direct "toxicity" on the skin cells in ICD. The later stages giving rise to an eczema lesion may, be very similar and involve cytokines, chemokines, phenomena of apoptosis and cellular necrosis and the recruitment of a polymorphic inflammatory infiltrate (Nosbaum A., et al. 2009).

ICD is the most common contact dermatitis and is known to be a non-antigen-specific skin inflammation brought about by activation of the innate immune system by the pro-inflammatory properties by haptens contact (Basketter DA., et al. 1999). During the skin irritation chemical penetrate through the different layers of the skin, notably the epidermis and the dermis, is responsible for the release of a large number of cytokines and chemokines by different cell types such as keratinocytes responsible for the induction of inflammation (Bonneville M., et al. 2004; de Jongh

CM., et al. 2007). Other cell types like mast cells, macrophages/dendritic cells, endothelial cells and NK cells are activated by the chemicals and contribute to the induction of inflammation and development of apoptosis/necrosis lesions of the epidermal cells with a compensatory proliferation of keratinocyte (Petersen TK. 2006; Kammeyer A., et al. 2008). The profile of cytokine expression during ICD varies over time and also depends on the nature, environment and dose of the chemical (de Jongh CM,et al. 2007). However, the inflammation is mainly linked to IL-1β, TNF-α, chemokine (C-C motif) ligand (CCL) 20 and derivatives of arachidonic acid, which are essential for the recruitment of leukocytes to the altered skin site, activation of macrophage and dendritic cells precursors and in the migration of skin DC to draining lymph nodes (LNs) (Pastore S., et al., 2004; Saint-Mezard P., et al., 2004).

Clinical studies demonstrate that the concentration and the nature of hapten (ranging from strong to weak sensitizers) are important and necessary to induce a cellular migration during the skin inflammation process (Kimber I., et al., 2003; Smith HR., et al., 2002). The hapten concentration necessary to induce ICD in a no sensitized patient or animal is higher than that necessary to induce ICD in pre-sensitized individual. Wherever the sensitized patients, ICD occurs 12 to 96 hours after contact with high concentrations of hapten. However, evidence that the quality and magnitude of the ICD reaction depends on the nature and strength of the inflammatory signals delivered by haptens during the course of ICD reactions is still lacking (Nosbaum A., et al. 2009). In this sense, the study of the skin disease through the DNFB skin model in addition to their economic advantages, presents an ideal capacity to understand migration of monocytes and their function during skin inflammation.

4.2. Notexin muscle model

Notexin a myotoxic phospholipase, from *Notechis scutatus, snake* venom, called Notexin. In this model an injection with Notexin in the tibialis anterioris (TA) on a mice paws, induce myoinjury by the previously degeneration of the sarcolemma and late fiber muscle necrosis, this inflammation model reassembles the muscle inflammation pathologies such as muscular strain, autoimmune reactions such as Duchenne's muscular dystrophy (Tidball JG., et al. 2005) and the skeletal muscle is the site of immune reactions during inflammatory processes like myopathies, muscular dystrophy, graft-versus-host disease, intramuscular vaccination, and therapeutic cell or gene transfer (Greenberg SA, et al. 2004; Hohlfeld R, et al 2007).

After injury, muscles present a great capacity to regenerate, mainly due to satellite cells, which represent a population of adult myoblasts that remain normally quiescent (Mauro A**.** 1961). After muscle damage and the initial step of necrosis and phagocytosis of injured myofibers, satellite cells are activated and then proliferate, fuse, and differentiate to form new myofibers or to repair damaged ones (Allbrook D. 1981). Myofiber damage and repair may be linked to autoimmune reactions by several mechanisms. Moreover, myofiber damage induces an inflammatory reaction that, could favor autoimmune reactions (Tidball JG, et al. 2005). Consistently, monocytes, macrophages and dendritic cells, cells that link innate and adaptive immunity, are abundant in the different types of myositis, in which they can initiate or perpetuate specific muscle immune reactions (Page G., et al. 2004; Greenberg SA., et al. 2005; Greenberg SA., et al. 2007).

Interactions between myeloid cells and skeletal muscle cells can influence muscle cell proliferation, differentiation and injury through mechanisms that are only beginning to be understood. *In vitro* and *in vivo* findings offer strong evidence that Ly-6C^{high} monocyte infiltration in to injured skeletal muscle exhibit an inflammatory

profile that induce phagocytosis in which macrophages can increase muscle membrane lysis, and presumably thereby increase muscle injury (Wehling M., *et al.* 2001; Nguyen HX. and Tidball JG. 2003*a*, 2003*b;* Ludovic A., et al. 2007). Membrane lysis by macrophages *in vitro* occurs through a NO-dependent and superoxide-independent process (Nguyen HX. and Tidball JG. 2003*a*). In addition, NO-mediated lysis of muscle membranes by macrophages *in vitro* is exacerbated by the presence of neutrophils (Nguyen HX. and Tidball JG. 2003*a*), which suggests that signalling between myeloid cell populations can affect their cytotoxicity. These cytotoxic interactions between myeloid cells and muscle are also apparently modulated by muscle-derived factors, at least *in vitro*, where muscle-derived factors increase NO release by macrophages (Nguyen HX. and Tidball JG. 2003*a*). This sequence of cellular events is accurately regulated, especially by myogenic regulatory factors (MRFs) that act as transcription activators and regulate the transcription of muscle-specific genes (Kablar B., et al. 2003; Zhao P. and Hoffman EP. 2004). Some markers that determinate the MRF are MyoD, an early MRF mainly involved in satellite cell activation and proliferation, another marker is myogenin a late-acting MRF, expressed during differentiation (Hannon K., et al 1992; Nabeshima Y., et al. 1993; Cooper RN., et al. 1999).

Furthermore, macrophages can also rapidly convert in to an antiinflammatory cell during a muscle injury environment in which they are able to promote muscle growth and repair through the myogenesis and fiber growth stimulation (Ludovic A., et al 2007). *In vitro* findings show that conditioned media from peritoneal macrophages or macrophage cell lines can increase proliferation of myoblasts in culture and elevate the proportion of myoblasts that express MyoD (Cantini M. and Carraro U. 1995; Cantini M., *et al.* 2002), which indicates a role for macrophage-derived factors in muscle growth and differentiation. *In vivo* observations may also support a positive role for macrophages in muscle growth and repair. Muscle repair by transplanted whole-muscle grafts is diminished if the graft recipients are

irradiated before transplantation (Lescaudron *et al.* 1999), which reflects a role for proliferative cells, such as macrophages, in muscle regeneration.

Some works have described that partial depletion of monocytes/macrophages impairs muscle regeneration, whereas reconstitution of bone marrow restores regeneration (Grounds MD. 1987; Shireman PK., et al. 2006; Summan M., et al. 2006; Warren GL., et al, 2004; Contreras-Shannon V., et al, 2007; Ludovic A., et al 2007). (Figure 9).

As previously described, monocyte-derived macrophages enhance myogenic cell growth (Chazaud B., et al 2003). Macrophages release mitogenic growth factors for myogenic precursor cells (mpcs) and establish cell–cell interactions that protect mpcs from apoptosis (Sonnet C., et al. 2006; Chazaud B. et al. 2003). However, little is known about both monocyte/macrophage subsets and phenotypes at work during muscle regeneration.

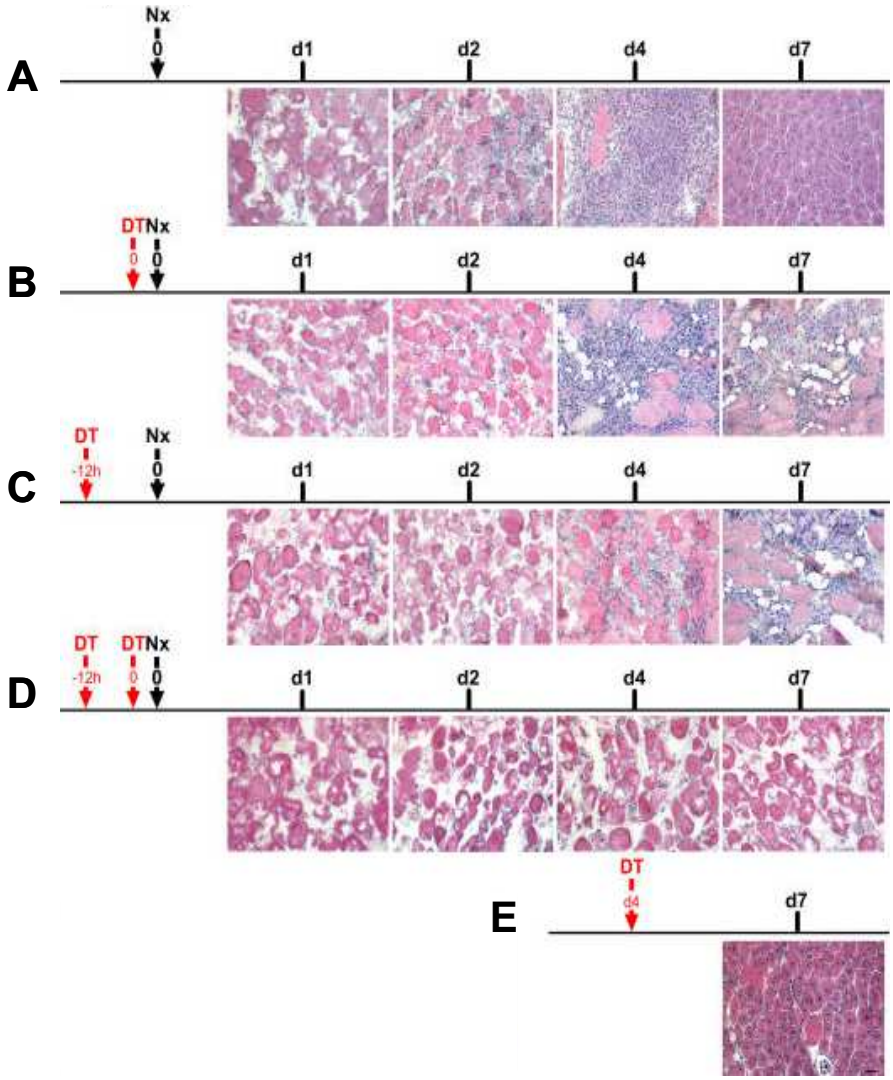

Figure 9. Monocyte/macrophage are critical for muscle regeneration. (A–E) Animals injected with Notexin (Nx) into the tibialis anterioris (TA) at day 0 and with diphtheria toxin (DT) intravenous (i.v.) at various times, indicate that the depletion of monocytes/macrophages (with DT) at earlier stages of the inflammation process impairs muscle regeneration, which do not occur when the depletion is later on in inflammation (Ludovic A., et al. 2007).

In mice, the response to inflammatory stimuli takes seconds/minutes after the injury occurs. In immunocompetent animal, resident mast cells degranulate and release preformed TNF-α and other inflammation mediators. Minutes/hours post injury infiltrated neutrophils accumulate in the inflammatory loci and also release TNF-α. After 8 hours, monocytes begin to appear in the inflamed tissue, along with further mast cell and neutrophil infiltration. Between 24 hours and 48 hours thereafter, macrophages become the major cell subset and remain so for days, until disappear gradually (Esser KA., et al. 1995). Initially limited to phagocytosis of necrotic fibers, the pro-regenerative role of macrophages has been shown to involve a direct stromal support function (Johnson SE. et al. 1993; Inoki K. et al. 2003). The mechanisms by which monocyte/macrophages are recruited to the site of myodamage are largely undetermined. For this since, the study of the muscle injury through the Notexin muscle model provides an ideal tool to understand the migration of monocytes, which is essential to tissue repair and their function during muscle inflammation.

OBJECTIVES

II. OBJECTIVES

Due to the low number of the Ly-6C^{high} monocytes in the peripheral blood and the low amounts obtained of these cells by several techniques (e.g. coldronate), the general objective of this thesis consisted in the generation of a novel protocol to generate high levels of Ly-6C^{high} monocytes *in vitro* and to investigate their role during inflammatory environment. For these propose our studies were focused on the following:

1. Development of an *in vitro* model that allow the generation of large amounts of Ly-6C^{high} monocytes from bone marrow from mice.

2. Characterization of the phenotype of Ly-6C^{high} monocyte generated *in vitro*.

3. Analyze the activation function of Ly-6C^{high} monocyte generated *in vitro*.

4. Study the migration of Ly-6C^{high} monocytes in to two inflammation models:
   - Skin (ear DNFB model)
   - Muscle (Notexin muscle model)

5. Analyze the therapeutic effect of Ly-6C^{high}CD11b^{+} monocytes injection in the resolution of inflammation in two experimental models of inflammation.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

1. Obtention of conditioned medium

1.1. Obtention of *Macrophage Colony-Stimulating Factor (M-CSF)* supernatant

Conditioned medium to generate macrophages from bone marrow was obtained from the supernatant of cell culture of mouse fibroblast cell line L929 (ATCC CCL 1, NCTC clone 929), capable to produce large quantities of M-CSF. The M-CSF is a growth factor required for differentiation of myeloid precursors into macrophages. This is the only growth factor produced by these fibroblasts affecting macrophages. The addition to the medium of monoclonal antibodies against M-CSF blocks production of macrophages (Lokeshwar BL. and Lin HS. 1988). L929 cells were cultured in high glucose DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) from Sigma. Cells were cultured in 150 cm^2 flasks up to confluence, moment in which the medium were switched by an equal volume of fresh medium. After 48 hours the supernatant was removed, centrifuged to remove the cells in suspension and was kept in aliquots at -80 ºC until the moment of use. Once unfrozen, the aliquots were stored at 4 ºC to prevent degradation of M-CSF due to excessive freezing and thawing cycles. The content of M-CSF was determined by a test of proliferation (^{3}H-thymidine incorporation) in macrophages from mouse bone marrow. In our studies we use the concentration of 30% of supernatant which is equivalent to 1200 U/ml of recombinant M-CSF (eBioscience,

San Diego, CA), since this dose is able to produce a saturation of the receptors for M-CSF on the surface of macrophages (Celada A., et al. 1984).

1.2. Obtention of growth factor cocktail

Conditioned medium for monocytes was obtained from the mixture of several growth factors, formula under patent (under patent process).

1.3. Obtention of IFN-γ

The IFN- was obtained from the supernatant of hybridoma T cells transfected with a called X63 constitutive expression construct capable of producing large quantities of recombinant murine IFN-γ (Gonalons E., et al. 1998). The cells were cultured in high glucose DMEM supplemented with 5% fetal bovine serum in the presence of 1 mg/mL Geneticin (G418, Sigma Chem.Co., St Louis, MO) as selective pressure for ensuring the permanence and transmission of plasmid coding for IFN-γ. Cells were cultured in 150 cm^2 flasks up to confluence, moment in which the medium were switched by an equal volume of fresh medium lacking geneticin. After 48 hours the supernatant was removed, centrifuged to remove the cells in suspension and was kept in aliquots at -80 ºC until the moment of use. Once unfrozen, the aliquots were stored at 4 ºC to prevent degradation of IFN-γ due to excessive freezing and thawing cycles. The content of IFN-γ was determined by a functional assay in bone marrow-derived macrophages comparing with recombinant IFN-γ (Genentech Inc., San Francisco, CA) (Gonalons E., et al. 1998). In our studies we use the concentration of 300 U/mL since this dose is able to produce a saturation of the receptors for IFN-γ on the surface of macrophages (Celada A., et al. 1984).

2. Growth factors and other reagents

2.1. Recombinant growth factors

Recombinant M-CSF was provided by eBioscience (San Diego, CA). Reagents were tested for the presence of endotoxins which were absent, having a 98% of purity. This reagent was provided from a lyophilized stock solution sterilized by filtration and was reconstituted and used following the manufacturer's recommendations.

2.2. IL-4

IL-4 was provided by R & D Systems (Minneapolis, MN). This cytokine results from the processing of the bacteria E. Coli with a plasmid containing the coding region for the IL-4 minus the first two amino acid residues. IL-4 has a purity of 97% and it was tested for the presence of endotoxins which were absent. The activity of this cytokine was determined by proliferation assays on cell line HT2. IL-4 was provided from a lyophilized stock solution, which was reconstituted in sterile PBS, 0.1% BSA, aliquots of 10µg/mL was made and frozen at -80^{o}C preserving its activity for three months. In our studies we use the concentration of 10ng/mL since this dose is able to produce a saturation of the receptors for IL-4 on the surface of macrophages.

2.3. Notexin

Notexin is toxic presynaptic PLA_2 with a potent myotoxic and a presynaptic (blocks the release of acetylcholine) activity (Karlsson E., et al 1972); HPLC pure. This venom was obtained from Latoxan (Valence, France) and has a purity of 93%. This product has a toxicity of a minimal lethal dose of 25 µg/kg mouse when is injected intraveinously and is wildly used in animal model as a potent myotoxic agent. Notexin is provided from a lyophilized stock solution sterilized by filtration. It was reconstituted in sterile PBS and aliquots of 125 µg/ml were made and frozen at -20°C preserving its activity for three months. In our studies we use the concentration of 25 µg/ml, since this dose is able to induce myoinjury (Ludovic A. et al. 2007).

2.4. DiR(1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide)

DiR, is a tracking dye with weakly fluorescent in aqueous phase, but highly fluorescent and photostable when incorporated into membranes or bound to lipophilic biomolecules (Pittet MJ, et al. 2006). This dye was obtained from Invitrogen (Paisley, UK). DiR is provided from a lyophilized stock. This fluorocrome has excitation and emission maxima in the near infrared region, where many tissues are optically transparent (Lassailly F., et al. 2010). This dye have a spectral property of excitation (750 nm) and emission (780 nm) and a optical filter Omega (XF112) and Chroma (41009), that must be detected using a infrared-sensitive detector in an *in vivo* imaging machine called IVIS. It was reconstituted with dimethylsulfoxide (DMSO), and aliquots of 1 mg/mL were made and frozen at -20°C preserving its activity for six months. In our studies we use the concentration of 10µM, since this dose is able to label the whole monocyte and macrophage outer

membrane allowing the noninvasive tracking of these inflammatory cells for several days *in vivo* (Eisenblätter M., et al. 2009).

2.5. DNFB (1-Fluoro-2,4-dinitrobenzene)

DNFB is an irritant hapten with low molecular weight, which in contact to skin endowed proinflammatory and antigenic properties (Rowland et al., 2001; Saint-MezardP., et al., 2004). This chemical from Sigma-Aldrich (St.Louis, MO) is widely used in mouse model studies of contact dermatitis (Bonneville M., et al 2007; Röse L., et al. 2012). In our studies we use the concentration of 10μM, since this dose is able to induce ear inflammation in non-sensitized mouse. For the *in vivo* models, DNFB was diluted in acetone (1:10 vol/vol), freshly before application and protected by light.

2.6. Other reagents

All other reagents were obtained from Sigma-Aldrich (St.Louis, MO). Deionized water that had been further purified with a Millipore Milli-Q system (Bedford, MA) was used.

3. Animals

Female BALB/c mice (8–10 weeks old, 18–20g body weight) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). STAT6 female Balb/C mice (8–10 weeks old, 18–20g body weight) wild type

and knockout mice (Metwali A., et al. 2002) were purchased from The Jackson Laboratory (Bar Harbor, Maine). All animals were healthy, housed in a barrier system (temperature: 20–26 ◦C; relative humidity: 40–70%) with a 12 h light/dark cycle. Water and standard diet were available add libitum. All animal procedures were approved by the Animal Research Committee of the University of Barcelona (number 2523), according to government guidelines for animal care.

4. Mouse bone marrow-derived Ly-6C monocytes

4.1. Generation of bone marrow-derived Ly-6C$^+$ monocyte

Mice were killed by cervical dislocation and both femurs were dissected free of adherent tissue. The ends of the bones were cut off and the bone marrow cells were harvested from flushed marrow cavities of femurs and tibiae eluted by irrigation with DMEM, containing 2 mM L-glutamine, 1 mM Na pyruvate, from uninfected Balb/C normal mice under aseptic conditions. Cells were suspended by vigorous pipetting and washed by centrifugation. To obtain Ly-6C$^+$ monocytes, an amount of 16 million cells extracted from femurs and tibiae were cultured in plastic tissue culture dishes of 60 cm^2 (Lab-Tek 4030, Miles Laboratories, Inc., Naperville, IL) in DMEM rich in glucose (Sigma, ST Louis, MO) containing 25% of FBS (Sigma), 25% of L-cell conditioned media as a source of M-CSF and other grow factors (Protocol under patentee). Media was supplemented with 50U/ml penicillin and 50µg/ml streptomycin. Cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere. Cells were grown to reach a state of subconfluence and after 7 days a heterogeneous population was observed, consisting of attached and floating cells, which forms aggregates. As described by Geissmann F, et al, Ly-6C$^+$ monocytes

form part of the floating population, for this reason only cells in suspension were used from the total culture and were characterized.

On previously indicated experiments generation of bone marrow-derived Ly-6C^{+} monocytes form STAT6 wild type and knockout Balb/C mice was carried out in the same manner as mentioned above. Furthermore, generation of bone marrow-derived Ly-6C^{+} monocytes in addition of IL-4 (10ng/ml) to total media was performed.

4.2. Obtention of enriched Ly-6C^{high} monocyte from total bone marrow culture by cell sorting

Enriched Ly-6C^{high} cells were acquired from total bone marrow culture after 7 days of differentiation with specific grow factors by a positively isolation with a sorting technique. Suspension cells were recovered from total culture after one week differentiation. Cell suspension was centrifuged and resuspended in 1ml of sorting media containing, DMEM containing 10% of FBS (PAA laboratories) and 4% of EDTA (ethylenediaminetetraacetic acid) from Sigma. Cells were counted and the necessary volume of sorting media was added to reach de density of 20x10^{6} cell/ml suspension. Before sorting procedure, neutralization of FcR was accomplished by the use of 1:200 αCD16/CD32 (BD Pharmigen) (table of antibodies), blocking was preformed for 15 minutes at 4ºC. Subsequently, Ly-6C marker labeling was performed by FITC-conjugated anti-Ly6C (BD Pharmigen) in a 1:200 concentration for 30 minutes at 4ºC (Table 1). Isotype control was made with the corresponding Rat IgM,κ monoclonal immunoglobulin isotype control (BD Pharmigen). Ly-6C^{high} monocyte purification was accomplished using MoFlo Systems sorter (Beckman Coulter, Inc. Spain). Only the higher fraction of the Ly-6C positive population was acquired. Ly-6C^{high} monocytes purity reached >98%. To assure the purity of the

sorting technique 10^6 sorted cells were labeled with CD11b, CD11c, CCR2, CD62L, F4/80 and CX3CR1 extracellular markers and were analyzed by Gallios™ flow cytometry (Beckman Coulter, Inc.) see section 6.1. For some experiments a positively acquisition of Ly-6C^{neg} monocytes was also done.

4.3. *In vitro* activation of enriched Ly-6C^{high} monocytes with IFN-γ or IL-4

One million of enriched Ly-6C^{high} monocytes per plate (24 cm^2) were treated with IFN-γ (300 U/ml) or IL-4 (10ng/ml) in total media. After 3, 6 and 24 hours, cells were collected and centrifuged for 5 minutes at 1500 rpm. Subsequently, medium was removed and cell pellet was washed with PBS 1X and processed according to section 6.2. for total RNA extraction.

4.4. Activation of enriched Ly-6C^{high} monocytes with IL-4 before *in vivo* injection

Bone marrow cells were obtained under the same conditions as in the section 4.1. After 7 days of enriched Ly-6C^{high} cells were acquired from total bone marrow culture (see section 4.2.). Enriched Ly-6C^{high} monocytes where were treated with IL-4 (10ng/ml) for 15 minutes in a falcon (15ml) with total media at 37ºC. Subsequently, medium was removed and cell pellet was washed with PBS 1X and resuspended with 200µl of 1% of EDTA (50mM) from Sigma, diluted in sterile PBS 1X. For in vivo injection, cells where processed according to section 4.6.

4.5. Activation of enriched Ly-6C^{high} monocytes with IFN-γ before *in vivo* injection

Bone marrow cells were obtained under the same conditions as in the section 4.1. After 7 days of enriched Ly-6C^{high} cells were acquired from total bone marrow culture (see section 4.2.). Enriched Ly-6C^{high} monocytes where were treated with IFN-γ (300 U/ml) for 15 minutes in a falcon (15ml) with total media at 37ºC. Subsequently, medium was removed and cell pellet was washed with PBS 1X and resuspended with 200µl of 1% of EDTA (50mM) from Sigma, diluted in sterile PBS 1X. For in vivo injection, cells where processed according to section 4.6.

4.6. Ly-6C^{high} monocyte DiR tracking dye labeling

Enriched Ly-6C^{high} monocytes were labeled with DiR (Invitrogen) in order to proceed to in vivo imaging by IVIS. DiR 10µM stock solution was prepared in sterile PBS 1X at 37ºC previous to cell dilution. Cells were resuspended at a 10x10^6 cell/ml concentration also in sterile PBS 1X at 37ºC. DiR labeling was accomplished by 1:1 (cell suspension/DiR stock) incubation for 15 minutes at 37ºC. Cells were washed several times with sterile heated PBS 1X in order to eliminate the excess of unbound tracer and were resuspended with 200µl of 1% of EDTA (50mM) from Sigma, diluted in sterile PBS 1X. Labeled cells were kept on ice until *in vivo* injection, see section 4.6.

4.7. Ly-6C^{high} monocytes *in vivo* injection

One million of enriched Ly-6C^{high} monocytes (see section 4.2); incubated with IL-4 (see section 4.4); incubated with IFN (see section 4.5) or generated from Balb/C STAT6 WT or KO mice, were resuspended in 200µl of 1% of EDTA (50mM) from Sigma, diluted in sterile PBS 1X and with a 1ml syringe (0.45x10mm) were injected intravenous in mice tail, see section 5.1 or section 5.2.

5. Animal models

5.1. Animal model of DNFB-induced ear irritation

The original procedure of the mouse ear swelling test has been described in detail elsewhere (Garrigue JL., et al, 1994; Bour H., et al, 1995) and was adept by our group where a different concentration of the hapten was single applied to induce skin inflammation. This chemical once in contact to the skin induce a irritant contact dermatitis (ICD), when applied in high doses, which closely reflect the characteristics of chronic T-cell-dependent inflammatory dermatoses as pronounced keratinocyte proliferation, strong hypervascularization, immune cell infiltration such as inflammatory monocyte and over expression of T cell and inflammatory cytokines (Bonneville M., et al 2007; Röse L., et al. 2012). Briefly, at day zero mice right ear was received 10µl of 1% DNFB (diluted in acetone (10µM)) applied to outer surface ear, whereas the same volume of acetone was applied on the contra lateral ear. Simultaneously to ear irritation, tree thousand to one million of DiR stained enriched Ly-6C^{high} monocytes were injected intravenously in mice tail in a volume of 200µl of 1% EDTA (Sigma) in PBS 1X. To observe cell migration *in*

vivo image were performed after 12, 24 and 48 hours, see section 6.5. Further, one to fifteen days later, mice were killed by neck dislocation and ear punch was obtained for further analysis. Net ear weight over the time course was determined by the subtraction from the left ear to the right ear weight values. RNA extraction (see section 6.2.) was performed for gene expression. Histological images (see section 6.6.) and dermis, epidermis and total ear thickness analysis were also evaluated after hematoxilin&eosin staining, in the whole image (10x objectives) in each mouse by a Fiji ImageJ2 Software (San Francisco, CA).

On previously indicated experiments one million of enriched Ly-6C^{high} monocytes activated with IL-4 or generated from Balb/C STAT6 WT or KO mice, injection was carried out simultaneously to ear irritation. Further, one to fifteen days later, mice were killed by neck dislocation and ear punch was obtained for further analysis. RNA extraction (see section 6.2.) was performed for gene expression. Histological images (see section 6.6.) and dermis, epidermis and total ear thickness analysis were also evaluated after hematoxilin&eosin staining, in the whole image (4x or 10x objective) in each mouse by a Fiji ImageJ2 Software (San Francisco, CA). In addition, one million of Ly-6C^{neg} monocytes injection was carried out simultaneously to ear irritation and cell migration *in vivo* image were performed 24 hours later see section 6.5.

5.2. Animal model of notexin-induced muscle injury

In the notexin-induced muscle injury model, has been described in detail by Ludovic and co-workers which demonstrated that inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis (Arnold L., et al. 2007). For this since, previous injections of notexin, Balb/C animals were anesthetized with Isoflurane (Xie Z., et al. 2007) to reduce pain and animal stress. Subsequently, intramuscular injection of 10µl of

Notexin (25 µg/ml in PBS; Latoxan) was performed in tibialis anterioris (TA) of mice right leg, whereas the same volume of PBS 1X was applied on the contra lateral leg, simultaneously one to two million of DiR labeled enriched Ly-6C^{high} monocytes were injected intravenously in mice tail in a volume of 200µl of cell suspension of 1% EDTA (Sigma) in PBS 1X. On previously indicated experiments cell injection was carried out 24 hours after induction of inflammation with notexin. To observe cell migration in both cases, *in vivo* image was performed after 24 and 48 hours, see section 6.5. One to ten days after cell injection, mice were killed by neck dislocation and TA was obtained for histology analysis (see section 6.6.) and total RNA extraction (see section 6.3.). Muscle analysis was performed on the entire injured area: approximately seven fields (20x objective; PL Flustar; Carl Zeiss MicroImaging, Inc.) For histological analysis, muscles were prepared as previously described (Sonnet C., et al, 2006), see section 6.6. Myofiber was evaluated after hematoxilin&eosin immunolabeling on approximately seven fields (20x objective) in each mouse, 250–350 necrotic and centrally nucleated myofibers were quantified and evaluated in muscle sections with Fiji ImageJ2 Software (San Francisco, CA). In PBS-injected mice, the punctured fascicule was omitted from analysis.

On previously indicated experiments one million of enriched Ly-6C^{high} monocytes activated with IL-4 or IFN-γ, injection was carried out simultaneously to muscle inflammation. Further, 24 to 48 hours later, mice were killed by neck dislocation and TA was obtained for total RNA extraction (see section 6.3.). In addition, one million of Ly-6C^{neg} monocytes injection was carried out simultaneously to muscle inflammation and cell migration *in vivo* image was performed 24 hours later see section 6.5.

6. Experimental Analysis

6.1. Flow cytometry assay (FACS)

To analyze the expression of cell surface molecules such as Ly-6C, F/4/80, CD11b, CD11c, CCR2, CX3CR1, 1 million cells were suspended in 100 µl FACS buffer (140 mM NaCl, 2.5 mM CaCl2, 10 mM HEPES pH 7.4), and receptors Fcγ were blocked with CD16/CD32 (receptor FcγIII/II) antibody for 15 minutes at 4ºC, subsequently 1:50 of either FITC-conjugated anti-Ly6C antibody and of PE-conjugated anti-CD11b; PE-conjugated anti-CD11c; PE-conjugated anti-CCR2; PE-conjugated anti-F4/80; PE-conjugated anti-CD62L and APC-conjugated anti-CX_3CR_1 antibody for 30 minutes at 4ºC, protected against light (see table of antibodies). Cells were washed with 2ml of buffer and centrifuged for 5 minutes at 1500rpm. Supernatant was discarded and cells were resuspended with 1ml of FACS buffer with 2% paraformaldehyde before analysis by Gallios™ Flow Cytometer (Beckman Coulter, Inc. Spain). For the result analysis dead cells are excluded by their small size and low level of granularity.

Table 1: Antibodies

Antibodies	*Reference*	*Commercial house*	***Concentrations***
Purified mouse anti-rat CD16/32	553142	BD Pharmigen	0,5mg/ml
FITC-conjugated anti-Ly6C	553104	BD Pharmigen	0,5mg/ml
PE-conjugated anti-CD11b	557397	BD Pharmigen	0,2mg/ml
PE-conjugated anti-CD11c	557401	BD Pharmigen	0,2mg/ml
PE-conjugated anti-F4/80	12-4801	eBioscience	0,2mg/ml
PE-conjugated anti-CD62L	12-0621	eBioscience	0,2mg/ml
PE-conjugated anti-CCR2	FAB5538P	R&D Systems	25µg/ml
APC-conjugated anti-CX3CR1	FAB5825A	R&D Systems	25µg/ml

All antibodies previously mentioned were used for flow cytometry and for sorting analysis. For the respective antibodies APC-, FITC- or PE-conjugated monoclonal immunoglobulin isotype was purchased.

6.2. Isolation of total RNA from enriched Ly-6C^{high} monocytes

RNA was extracted by Trizol solution from the commercial house Invitrogen, Chomczynski and Sacchi method (Chomczynski P. and Sacchi N. 1987). Homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Addition of 200µL chloroform per 1 mL of TRIzol® Reagent was performed for homogenization. Tube was shacked vigorously by hand for 15 seconds and incubated for 2–3 minutes at room temperature. Samples were centrifuged at 12000 rpm for 15 minutes at 4°C. The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume. The aqueous phase

was removed and placed into a new tube with 1:1 volume of 70% ethanol (Sigma-Aldrich) diluted in water. For RNA isolation, 700 µl of total solution was added in columns from PureLink® RNA Mini Kit Ambion (Alcobendas, Madrid) where RNA extraction was performed according to the manufacturer protocol.

6.3. Isolation of total RNA from tissue

Animal tissue (ear and muscle) was obtained from both animal models (see section 5) cut in small pieces and were stored in RNAlater (QIAGEN®. Valencia. CA) at -80ºC until RNA extraction to assure RNA stability. Tissue was ruptured with T 10 basic Ultra-Turrax (IKA®-Werke GmbH & Co. KG. Staufen, Germany) in 1ml of TRI reagent (Sigma-Aldrich) previous RNA extraction. RNA extraction was performed (see section 6.2.).

6.4. Real-Time (RT-PCR)

For cDNA synthesis and quantitative RT-PCR analysis, 1µg RNA of each experimental sample was retrotranscriptated with a mix of M-MLV reverse transcriptase RNase H minus, Point Mutant, oligo$(dT)_{15}$ primer and PCR nucleotide from Promega Corporation, as described by the manufacturer and by Sebastián C., et al. 2009. For quantitative PCR analysis SYBR Green PCR Core Reagents and the ABI Prism 7900 Detection System were used from Applied Biosystems (Arlington Heights, IL). The relative quantification of gene expression was performed as described in the SYBR Green user's manual using L14 as a housekeeping gene. The threshold cycle (C_T) was defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected. The PCR arbitrary units of each gene were defined as the mRNA levels normalized

to the L14 expression level in each sample. The primers used to amplify cDNA for real-time monitoring were described in Table I.

Gene	Forward primer	Reverse primer
L14	5'TCCCAGGCTGTTAACGCGGT3'	5'GCGCTGGCTGAATGCTCTG3'
Arginase I	5'TTGCGAGACGTAGACCCTGG3'	5'CAAAGCTCAGGTGAATCGGC3'
NOS2	5'GCCACCAACAATGGCAACA3'	5'CGTACCGGATGAGCTGTGAATT3'
IL-10	5'TCCTTAATGCAGGACTTTAAGGG3'	5'GGTCTTGGAGCTTATTAAAAT3'
TNF-α	5'CCAGACCCTCACACTCAGATC3'	5'CACTTGGTGGTTTGCTACGAC3'
IL1β	5' TGGGCCTCAAAGGAAAGAAT 3'	5' CAGGCTTGTGCTCTGCTTGT 3'
Mannose receptor	5'AATGAAGATCACAAGCGCTGC3'	5'TGACACCCAGCGGAATTTCT3'
IL-6	5'CCAGAGATACAAAGAAATGATGG3'	5'ACTCCAGAAGACCAGAGGAAAT3'
Myogenin	5'GGGCATGTAAGGTGTGTAAGAG3'	5'TAGGCGCTCAATGTACTGGAT3'

6.5. *In vivo* imaging of Ly-6C^{high} monocyte migration analysis

Animals injected with labeled cells were anesthetized with Isoflurane (Xie Z., et al. 2007) and placed in near-infrared scanning machine from Xenogen - IVIS ® Spectrum (Advanced Molecular Vision, Inc. Grantham, UK) for detection of *in vivo* image. Matched controls (age- and sex-matched animals) were imaged on the same scan to provide reliable and objective representation of the background and/or autofluorescence signals. Post 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. Image displays were set up so that fluorescence did not come from the control region of interest.

6.6. Histological analysis

Representative samples from animal models, ear and anterior tibialis, were obtained from animals that underwent cell injection, were fixed in a 3% formalin solution (Leica Biosystems. Barcelona. Spain) for 24 hours and processed whole through a routine 15 hours cycle to paraffin wax embedding. Paraffin blocks were cut into 50µm thick sections using a microtome and mounted on Superfrost Plus slides (Fisher Scientific. Toronto, ON). Sections were dried overnight at 37ºC. The slides were dewaxed in Ottixs baths and staining according to routine hematoxylin and eosin (H&E) staining procedure from Leica Biosystems. Data was presented as individual target scores as well as a composite score from all the tissues. All slides analysis was read in a blinded fashion. Images were visualized with a Nikon E800 microscope (Nikon Instruments Inc. Melville, NY). Image acquisition was performed with an Olympus DP70 digital camera (Olympus Iberia, S.A.U. Barcelona. Spain) with x4 or x10 or x20 objective and Image Pro Plus 6.3 software package (Media Cybernetics, Inc. Rockville, MD).

6.7. Statistical analysis

If the contrary is not indicated in a particular figure legend, all results showed the collected data corresponding of at least 3 separate experiments, being each experiment performed using at least 5 animals per point. Data is expressed as the mean+SD or mean+SEM ($n \geq 3$). Statistical analysis used was two-tailed t test of the indicated groups and significance of each particular group comparison was represented as "*" or "#" when $p \leq 0.05$, "**" or "##" when $p \leq 0.01$ or "***"or "###" when $p \leq 0.001$. Statistically significances of group comparisons previously showed where not further indicated in subsequent panels or figures in order to simplify figure representation.

RESULTS

IV. RESULTS

PART 1: Generation of Ly-6C monocytes *in vitro* from bone marrow

Monocytes are known to be originated in the bone marrow from a common myeloid progenitor. These cells are released into the peripheral blood, where they circulate for several days and then migrate from blood to vessels and develop into macrophages in the tissues. The morphology of mature monocytes in the peripheral circulation is heterogeneous, and these cells constitute ~5-10% of peripheral-blood leukocytes in humans. Several subsets of circulating monocytes are found in blood of humans and mouse. In mouse, a subset of so-called inflammatory monocytes that express the cell surface phenotype CX3CR1^{low}Ly-6C^{high}CD62L^{pos}CCR2^{high} represent approximately 2-5% of circulating white blood cells in an uninfected mouse and have been shown to traffic to sites of inflammation playing a physiologic role in animal models of infection. A second major subset of circulating monocytes in mice expresses high levels of CX3CR1 and low levels of CCR2 (CD192) and Ly-6C. In the bloodstream, these Ly-6C^{low} monocytes are less prevalent that Ly-6C^{high} monocytes (King IL, et al. 2009).

One of the big inconveniences in the study of the role of monocytes subsets *in vitro* and *in vivo* is the low amount of monocytes obtained from the blood flow of mice and human. This inconvenience reduces the number of studies performed and recently, only few studies can be found in the literature. It has been established that the number of Ly-6C^{high} cells obtained from blood of healthy mouse is less than 2,5x10^5 cells and can be expanded more than 60-fold immediately after inflammation (King IL, et al. 2009). Due to the importance of these cells in macrophage differentiation and in inflammatory process, one of the main objectives

of this Doctoral Thesis consisted in the optimization of a protocol that allows the generation of high amounts of Ly-6C^{high} cells from bone marrow of mice in to those obtained from bloodstream. The generation of a large number of Ly-6C^{high} cells will allow us to use them as a tool to investigate the role of these monocytes in several pathologies in which the activation of macrophages are involved.

Generation and characterization of Ly-6C^{+} monocytes subtype from *in vitro* differentiation of bone marrow cells in mice.

As previously described, chemokines, cytokines and several grow factors are involved in the enrichment/augmented and mobilization of Ly-6C^{high} cells from the bone marrow into the circulation (Inaba K., et al. 1992; King IL., et al. 2009). For this reason, for this Doctoral thesis we decided to study whether pre-established protocol for differentiation of bone marrow cells to macrophages, was able to produce large amounts of Ly-6C^{+} monocytes. Thus, the first aim was to check if the growth factor involved in the bone marrow-macrophage cells differentiation could differentiate Ly-6C^{+} cells from bone marrow of mice (Celada A., 1984). In order to do that, 16 millions of bone marrow cells were incubated with different percentage of supernatants from cultured fibroblasts cell line L929 (L-cell) as a source of M-CSF as previously described (Priceman SJ, et al. 2010). A daily monitoring of cell culture was done by microscope, where was observed the change of a cellular floating population in a population of adherent cells, a significant increase of adherent cells in relation to floating cells was observed at day 5, presenting 99% of adherent cells on day 7. After this period, cells were recollected and stained with Ly-6C and CD11b (integrin α_M chain) markers to characterize monocytes/macrophage subset and CD11c (integrin a^X chain) as a characteristic marker of dendritic cells (Figure 1A).

The results presented in Figure 1A demonstrate that M-CSF induced high expression of F4/80 and CD11b with almost 98% cells expressed these markers. However, although CD11b is highly expressed by these macrophages, the Ly-6C surface expression was reduced and only 18.35% were double positive for these two markers (Figure 1A). Similar results with these markers were obtained when macrophages were incubated for 5 and 7 days (Calatina Rincón master thesis). Therefore, the results presented here demonstrated that M-CSF is specific growth factor for differentiation to macrophages lineages from bone marrow mice. Moreover, this growth factor was not able to produce high amounts of Ly-6C^{+}CD11b^{+} population which could be considered as monocytes subtype (Figure 1A).

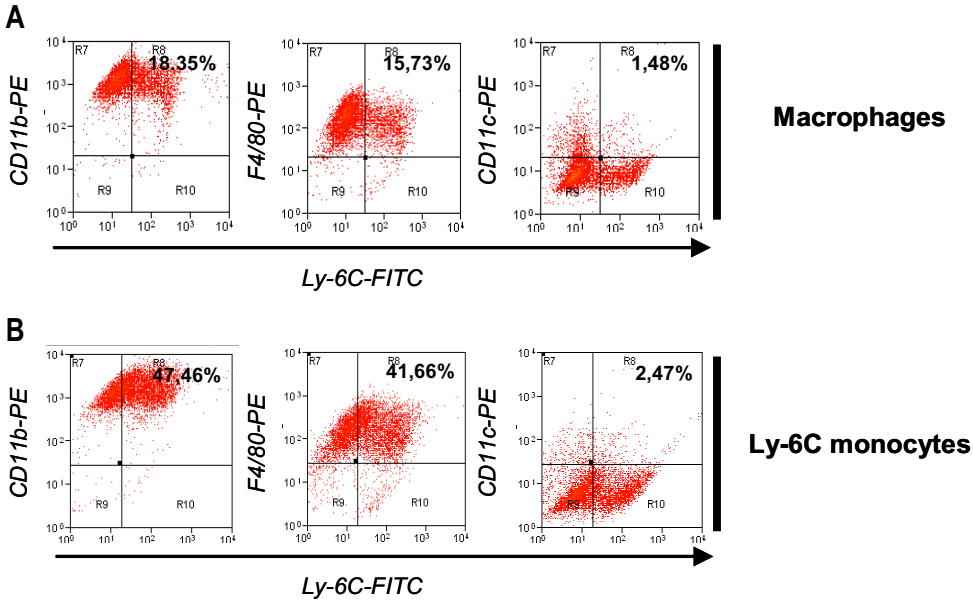

Figure 1. Generation and characterization of Ly-6C monocytes obtained from *in vitro* differentiation of bone marrow cells in mice. Bone marrow cells obtained from tibias and femurs were incubated with M-CSF (A) or growth factor cocktail (B) during 7 days. After this period the cells were recollected and stained with anti-Ly-6C and anti-CD11b; anti-CD11c; anti-F4/80 as previously described in material and methods and analysed by FACS. Then, the percentage of each cellular population was calculated. Results are representative of at least three independent experiments.

In order to improve the number of Ly-6C$^+$CD11b$^+$ monocytes subtype we decided to combine several growth factors and develop a growth factor cocktail (under patent process). In this regard, a serial of experiments using bone marrow cells and it different combination of amounts of supernatants of growth factor cocktail for 7 days were performed. In figure 1B we only represented data corresponding to the best combination of growth factors.

The results demonstrated that the presence of the growth factor cocktail in the cell culture after 7 days induced a production of monocyte/macrophage cell population, which was predominantly CD11b and F4/80, and did not induce significant values of CD11c, indicating that the cells did not exhibit markers of dendritic cells. The cells positive for CD11b and F4/80 also presented elevated expression of Ly-6C markers, where 47,46% were double positive for CD11b and Ly-6C, and 41,66% where double positive for F4/80 and Ly-6C markers, respectively. On the other hand, only 2,47% cells where double positive for CD11c and Ly-6C (Figure 1B). These results suggest that the new combination cocktail created differentiates bone marrow cell into a Ly-6C$^+$CD11b$^+$F4/80$^+$CD11c$^-$ population and that the optimal combination of these growth factors in order to obtain the higher amount of Ly-6C positive monocytes is 15%, where 47,46% of cells are positive for Ly-6C$^+$CD11b$^+$, 41,66% are Ly-6C$^+$F4/80$^+$ and only 2,47% express Ly-6C$^+$CD11c$^+$ (Figure 1B).

PART 2: Enrichment of Ly-6C^{high} monocytes generated *in vitro* from total bone marrow culture by cell sorting

Interestingly, the microscopically analysis of these Ly-6C$^+$CD11b$^+$ monocytes culture with the growth factor cocktail combination during 7 days showed different cell populations in comparation with M-CSF, that presented a

homogeneous adherent population. In the plates with the growth factor cocktail, an adhered population formed by macrophages and dendritic cells has been observed, and also a second population of floating cell forming aggregates, composed mainly by Ly-6C monocytes, indicated by the arrow (Figure 2A). The analysis of forward- and side-scatter characteristics discarded the granulocytes morphology in this cells culture (data not shown). For these reason we explored total population of Ly-6C cells in the plates, and three populations of cells were found in the total culture (Figure 2B): a population with 42,8% of Ly-6C^{high} monocytes, a Ly-6C^{int} cell population (29.1%) and a Ly-6C^{low} (26.5%) population was detected by flow cytometry (Figure 2B). These results are in agreement with other authors who demonstrate that in blood there are different states of Ly-6C monocytes subsets: an inflammatory monocytes subset (Ly-6C^{high}), a not well known monocyte (Ly-6C^{int}) and a resident monocyte (Ly-6C^{low}) subset. In order to study the monocytes involved in the inflammatory process we decided to work only with Ly-6C^{high} cells and for this reason in the following experiments we sorted the floating cells from total culture using a Moflo flow cytometre machine in orther to have the higher amount of the purest population of Ly-6C^{high} monocytes.

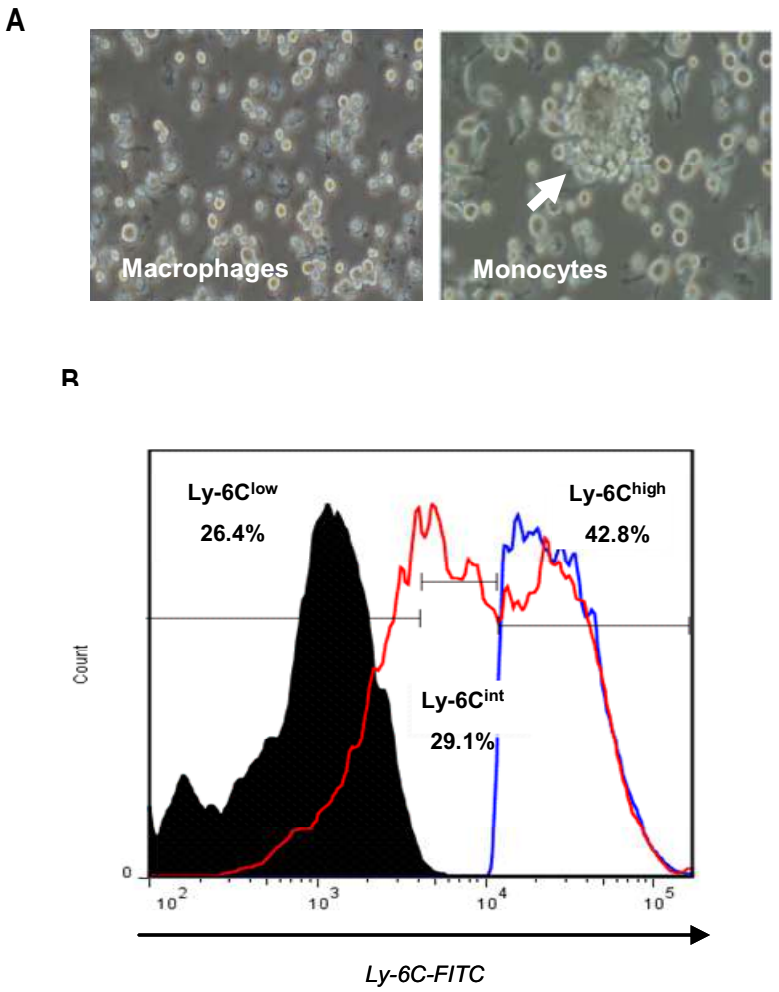

Figure 2. Bone marrow cells under grow factor cocktail conditions generate different Ly-6C monocyte population. A) Bone marrow cells cultured during 7 days with M-CSF or growth factor cocktail and were microscopically analyzed. B) Heterogeneous Ly-6C monocyte population were evaluated by Flow Cytometer Galios™. Results are representative of at least three independent experiments.

To better characterize the phenotype of enriched Ly-6C^{high} monocytes differentiated in a growth factor cocktail combination from bone marrow cells, we stained these cells with several cellular markers (Figure 3) comparing the total Ly-6C culture population (Figure 4A) with the Ly-6C^{high} enriched population (Figure

3B). In addition to CD11b, CD11c and F4/80 antibodies we added other specific markers such as CD62L (L-selectin) a cell adhesion molecule, which is involved in homing of lymphocytes (Robbins SL., et al. 1998), the CCR2 receptor which specifically mediates monocyte chemotaxis and CX3CR1 a chemokine receptor involved in the adhesion and migration of leukocytes, that plays major role in the survival of monocytes (Bursill CA., et al 2004). Our results demonstrated that in total culture the majority of cells were $CD11b^{+}CD62L^{+}CCR2^{+}F4/80^{+}$, and $CD11c^{low}CX3CR1^{low}$, which means a predominant population of Ly-$6C^{+}$ monocytes (Figure 3A). On the other hand the Ly-$6C^{high}$ enriched monocyte increased the purity of the population presenting the followed phenotype: $CD11b^{+}$ (98%) $CD62L^{+}$ (69%) $CCR2^{+}$ (70%) $F4/80^{+}$ (79%) $CX3CR1^{low}$ (5%) (Figure 3B).

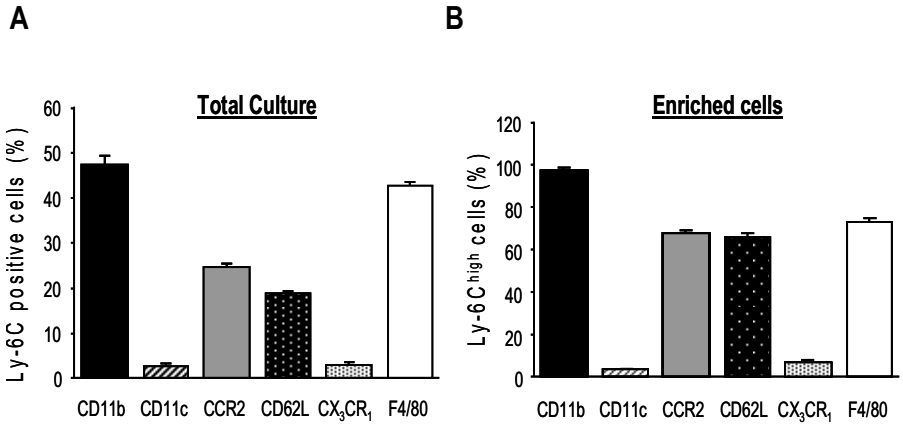

Figure 3. Phenotypical characterization of Ly-6C monocytes generated *in vitro*. Bone marrow cells were incubated with growth factor cocktail during 7 days and the cells were collected and co-stained with FITC-Ly-6C and different PE-markers; CD11b, CD11c, CCR2, F4/80 and CX3CR1. A) Percentage of double Ly-6C positive cells was represented. B) Ly-$6C^{high}$ monocytes were separated as previously described in material and methods by sorting and then stain with different markers. Results represent the means ± SEM of three independent experiments with pooled cells from 2 mice per experiment.

Thus, we have characterized an in vitro protocol for generation of enriched Ly-$6C^{high}$ monocytes from bone marrow cells. From one mice where there are

approximant 200 million of HSC we can obtain almost 30 millions of Ly-6C^{high} monocytes a 10-100 times more than obtained from the blood of mice. This will allow the study of this cellular population in an easy manner in further experiments.

PART 3: Study of heterogeneity of Ly-6C^{high} monocytes upon stimulation

It has long been known that circulating monocytes give rise to a variety of tissue resident macrophages, and there is substantial heterogeneity of phenotype, which most probably reflects the specialization of individual macrophage population within their microenvironment. In addition to their role in replenishing tissue macrophages and dendritic cells in the steady state, monocytes are recruited to infected tissues and mediate direct antimicrobial activity at these sites. Thus, these cells can participate in the initial inflammatory response by releasing cytokines such as TNF-α and chemokines (Geissman, et al. 2003). However, there are poor studies of these cells in the last steps of inflammation.

Analysis of pro- and anti-inflammatory markers in IFN-γ and IL-4-stimulated enriched Ly-6C^{high} monocytes

Thus, to study the functional heterogeneity of enriched Ly-6C^{high} monocytes as described in macrophages, we performed different experiments. First, Ly-6C^{high} monocytes from *in vitro* differentiation were incubated with IFN-γ (300U/ml) for several times (3, 6 and 24 hours) as typical classical stimuli (Figure 4A). Second, Ly-6C^{high} monocytes were also incubated with IL-4 (10 ng/ml) as an alternative pathway stimulus (Figure 4B). Then, the expression by mRNA of proinflammatory cytokines (*tnf*α) and inducible nitric oxide synthase (*nos2*) was

measured in IFN-γ activated monocytes and the expression of mannose receptor (CD206) and arginase I in IL-4-activated macrophages (Figure 4).

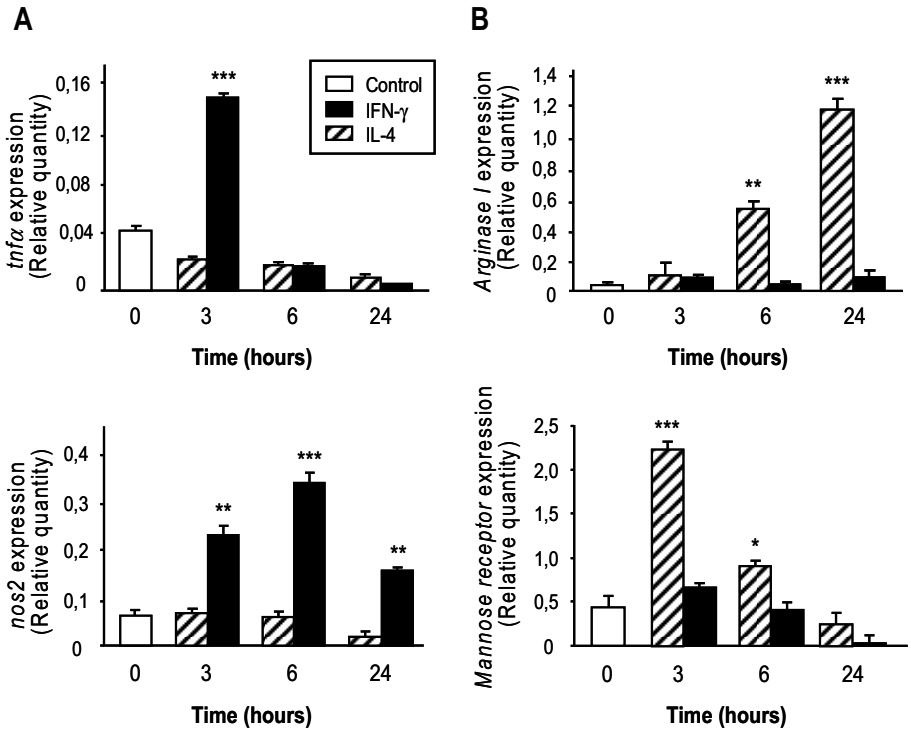

Figure 4. Plasticity of the Ly-6C^{high} monocytes to the environmental conditions. mRNA expression of different genes were evaluated in Ly-6C^{high} monocytes incubated by different stimuli in the indicated times. A) Ly-6C^{high} monocytes were incubated with IFN-γ (pro-inflammatory stimuli) and *tnfα* and *nos2* analysed. B) Ly-6C^{high} monocytes were incubated with IFN-γ (pro-inflammatory stimuli) and *arginage I* and *mannose receptor*. Results represent mean ± SD of three independent experiments. *P<0.05, **P<0.001 vs control (no stimulated).

Ly-6C^{high} cells stimulated with IFN-γ induced the mRNA expression of *tnfα* and *nos2* with a characterized kinetics similar to obtained with macrophages derived from bone marrow cells (Figure 4A). In addition, these Ly-6C^{high} cells increased *arginase I* and *mannose receptor* mRNA levels when they were

stimulated with IL-4 (Figure 4B). Thus, these in vitro results have shown the plasticity of the Ly-6C^{high} monocytes to the environmental conditions as previously described by macrophages and thus, suggest us that these cells can also adapt to a changing microenvironments as previously described by others (Gordon S., 2005; ; Biswas SK., et al. 2011).

PART 4: Assessment of *in vivo* homing capacity of *in vitro* generated Ly-6C^{high} monocytes in a skin inflammation model

Skin irritation can be developed due several externs agents like physical irritants and chemical irritants, such as DNFB (1-fluoro-2,4-dinitrobenzene) an hapten with low molecular weight, which in contact to skin endowed proinflammatory and antigenic properties (Rowland et al., 2001; Saint-Mezard et al., 2004). This chemical once in contact to the skin induce a irritant contact dermatitis (ICD), when applied in high doses, which closely reflect the characteristics of chronic T-cell-dependent inflammatory dermatoses as pronounced keratinocyte proliferation, strong hypervascularization, immune cell infiltration such as inflammatory monocyte and over expression of T cell and inflammatory cytokines (Röse L., et al. 2012; Bonneville M., et al 2007). For this reason, we decided to study the recruitment and the role of Ly-6C^{high}CD11b^{+} monocytes in the skin inflammatory model.

Study of recruitment of enriched Ly-6C^{high} in an acute inflammation in the DNFB-induced skin model in mice

Ly-6C^{high}CD11b^{+} monocytes are well known for their capability to migrate from the blood stream to the inflamed tissue (Drevets DA, et al. 2008). For this reason, the second aim of this Doctoral Thesis consisted in the evaluation of the migratory

capacity of the enriched Ly-6C^{high} cells obtained *in vitro.* For this propose we optimized an *in vivo* experimental model of acute inflammation of ear contact dermatitis (Ahmed AR., et al. 1983; Sakai S., et al. 2010). Contact dermatitis was induced in the right ear of a female Balb/C immunocompetent mouse by application of a solution containing 1%DNFB, in a mixture with acetone (10μM). Acetone was used as a control in the mice left ear (Figure 5).

In order to stain enriched Ly-6C^{high}CD11b^{+} monocytes we used the DiR (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide) a colorant with weakly fluorescent in aqueous phase, but highly fluorescent and photostable when incorporated into membranes or bound to lipophilic biomolecules (Pittet MJ, et al. 2006). These optical characteristics make them ideal for staining the cytoplasmic membranes of cells. Once applied to cells, these dyes diffuse laterally within the plasma membrane, resulting in staining of the entire cell. This fluorocrome has excitation and emission maxima in the near infrared region, where many tissues are optically transparent (Lassailly F et al, 2010). For this since, cells were stained with PBS with 10μM of DIR, and then fluorescent cells were injected intravenously in the mouse tail after induction of inflammation of the ear with DNFB. Cellular migration was appreciated through an in vivo image in the IVIS machine (Figures 5).

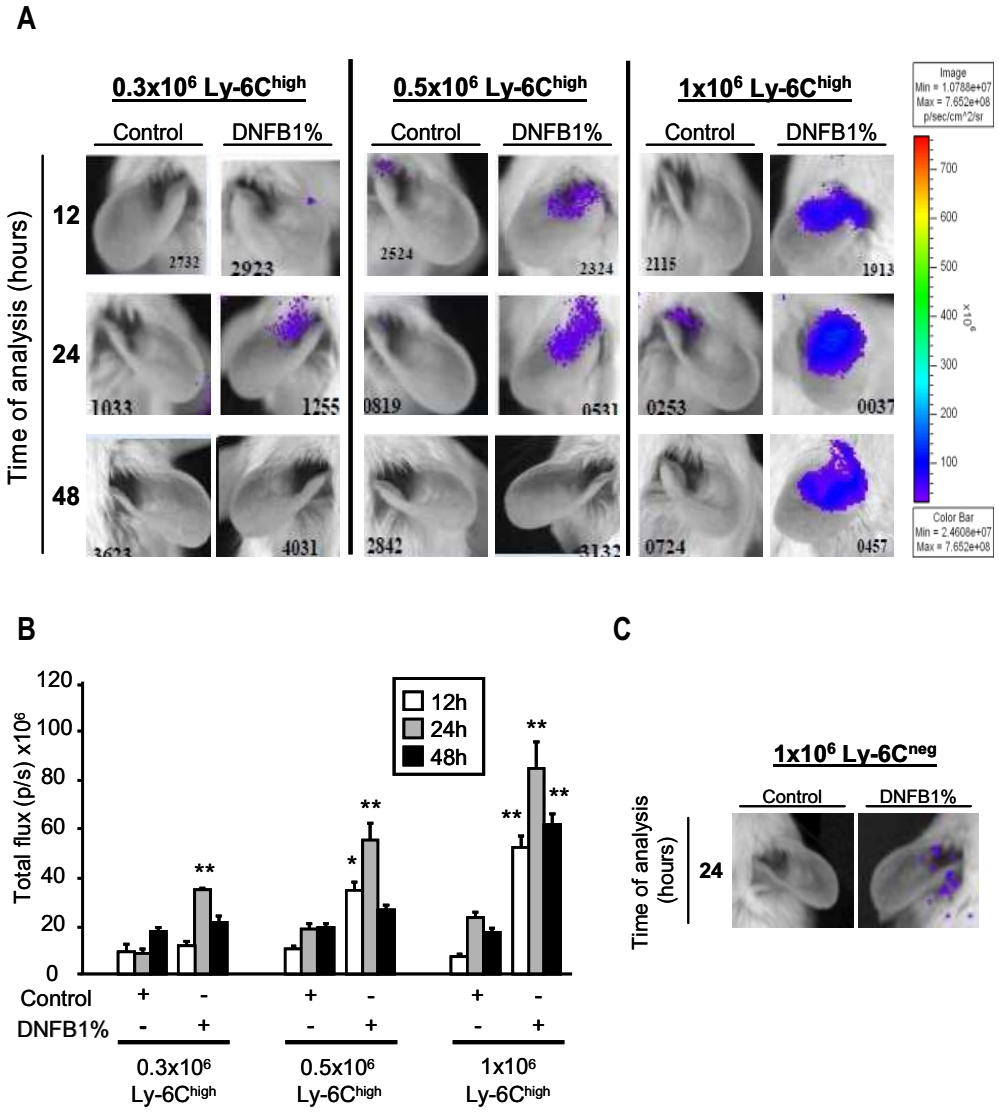

Figure 5. Enriched Ly-6C^{high} monocytes differentiated, in vitro, migrate to inflamed skin. The skin irritation was performed in the right ear of Balb/C mice by 1%DNFB (10μM), simultaneously an injection of different concentrations of enriched Ly-6C^{high} monocytes DiR stained were injected in the tails of mice. Acetone was used as a control in the mice left ear. A) Cellular migration of different amounts of enriched Ly-6C^{high} (0.3; 0.5 and 1 million) cells was appreciated through time in an in vivo image in the IVIS machine. B) Quantification of total flux (pixels/seconds) of in vivo images. C) One million of Ly-6C^{neg} monocytes were injected in the tail and were used as a negative control. Results from A and C are representative of at least three independent experiments. Results from B represent mean ± SEM of three independents experiments from at least 5 mice per experiment. *P<0.05, **P<0.001 vs control (no stimulated).

In the DNFB model different amounts of Ly-6C^{high}CD11b^{+} cells (0,3x10^{6}, 0,5x10^{6} or 1x10^{6}) were injected intravenously in the mouse tail after induction of inflammation. To evaluate the migratory capacity of these cells to the inflammatory foci in vivo image in an IVIS machine was performed after 12, 24 and 48 hours. The results demonstrated that the presence of monocytes in the inflamed tissue depend on the amounts of Ly-6C^{high} cells injected in the tails (Figure 5A). In this regard, low amounts of Ly-6C^{high} cells (0.3 x 10^{6}) were detected after 24 hours after inflammation as well as 0.5x10^{6} cells injected monocytes. However, 1x10^{6} of enriched Ly-6C^{high} monocytes are still in the inflammatory foci after 48 hours (Figure 5A). The quantification of the cells in the IVIS machine confirms these results (Figure 5B). For this reason we decided to work with 1x10^{6} Ly-6C^{high} monocytes in future experiments. Moreover, we also analyzed the capacity of migration of the enriched Ly-6C^{low} cells to the DNFB-inflamed ear. In this case, these cells were not able to migrate from the tail to the ear and almost all of fluorescent cells were maintained in the tail of the mice (Figure 5C).

Role of enriched Ly-6C^{high}CD11b^{+} monocytes during acute inflammation in the DNFB-induced skin model in mice

To analyse the function and phenotype of the enriched Ly-6C^{high} monocytes generated *in vitro* in the skin inflammation model, induced by DNFB, irritant contact dermatitis was induced and monocyte injection was carried, followed by the performance of an *in vivo* images. To analyse inflammation levels, ear was removed and weighed after 1, 2 and 4 days (Figure 6). The results showed the presence of monocytes at *in vivo* imaging from day 1 to day 4. In the acetone control group, no significant numbers of Ly-6C^{high} monocytes have been observed (Figure 6A). As a consequence of the inflammatory process, ear weight increased significantly at day 1 and then reached almost basal levels after 4 days of DNFB

induction (Figure 6B). The inflammatory infiltrate was also observed at histological level using hematoxiline&eosine staining where dermal edema and cell infiltration was observed (Figure 6C). Furthermore, analysis of full diameter of the ear (ear thickness) was made to compare injected animals versus uninjected animals, which demonstrated that until day 4 animals injected with enriched Ly-6C^{high} monocytes presented thicker ears than control mice, which could be due to the presence of large amounts of cells in the tissue (Figure 6C and D). In this regard, the histological analysis showed the presence of granulocyte (neutrophils) and phagocytes (monocytes and macrophages), in addition to lymphocytes infiltration in control and cell injected animal. However animals injected with Ly-6C^{high} cells exhibited larger amounts of cellular infiltrate when compared to control mice, at day 4 (Figure 6C). Thus, the results demonstrated that Ly-6C^{high} monocytes are still in the ear after 4 days of inflammation.

To determine the severity of inflammation, the *tnfα* expression as inflammatory marker and *mannose receptor* as repairing tissue marker in ears were evaluated in the DNFB irritant contact dermatitis model (Figure7). In the inflamed tissues due to the DNFB treatment (control group), the *tnfα* mRNA expression was significantly increased after 2 and 4 days (Figure 7). In addition, the mannose receptor mRNA expression was only detected after 4 days of ear inflammation these results as well as histological analysis suggest that after this period the tissue is initiating a repairing process.

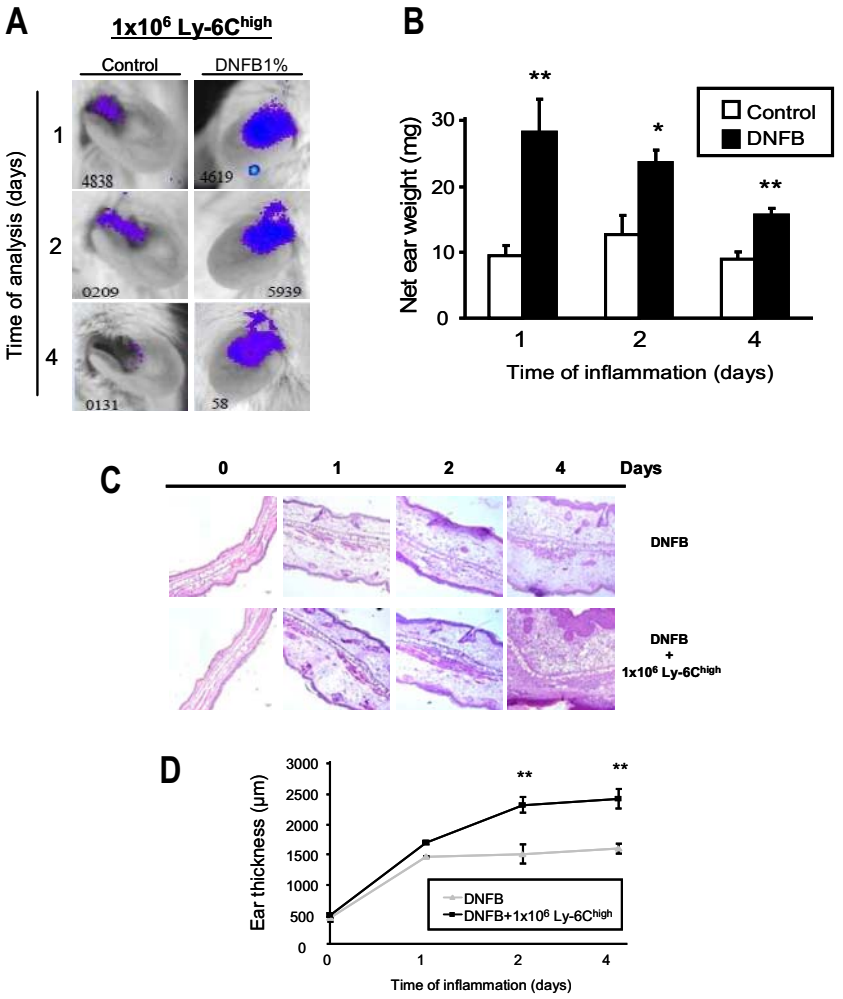

Figure 6. Role of recruited Ly-6C^{high} monocytes during acute inflammation. Enriched Ly-6C^{high} monocytes differentiated, *in vitro*, migrate to inflamed skin. The skin irritation was performed in the right ear of Balb/C mice by 1%DNFB (10µM), simultaneously an injection of different concentrations of enriched Ly-6C^{high} monocytes DiR stained were injected in the tails of mice. Acetone was used as a control in the mice left ear. A) One million of Ly-6C^{high} cells injection in DNFB-induced skin inflamed mice, *in vivo* IVIS image. B) Net ear weight in DNFB-treated+Ly-6C^{high} monocytes injected mice (weight of left ear was subtracted for the weight of right ear). C) Representative hematoxylin&eosin analysis of ear sections at various days after DNFB-treated (control group) and DNFB-treated+Ly-6C^{high} monocytes injected mice. Original magnification 10x. Bar, 50 µm. D) Evaluation of ear thickness was performed as previously described in material and methods. Results from A and C are representative from at least three independent experiments. Results from B and D represent mean ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs control (no stimulated) and #P<0.05, ##P<0.001 vs DNFB alone.

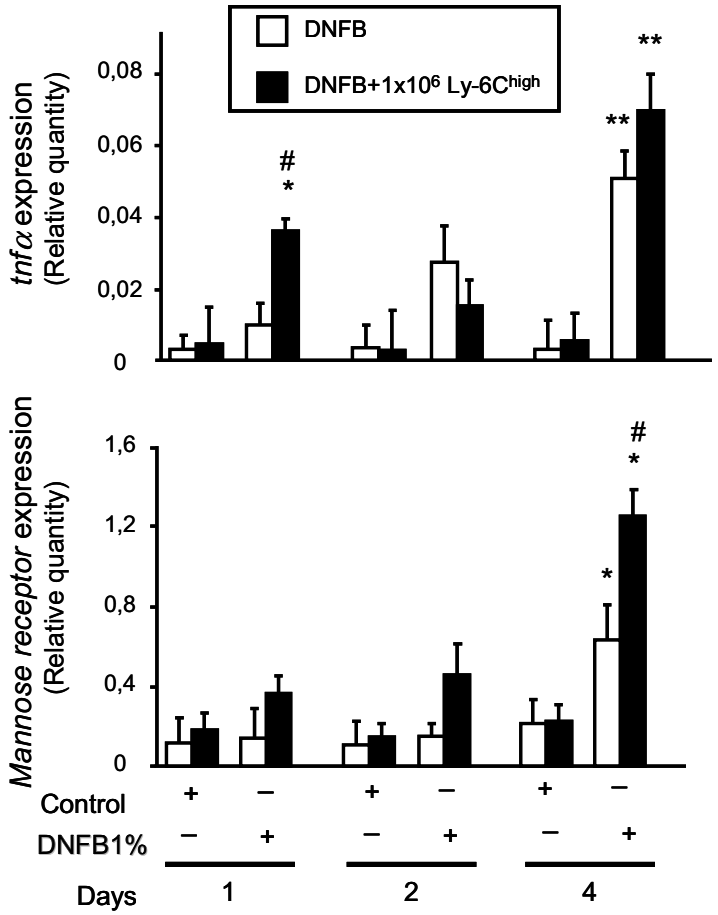

Figure 7. The injection of Ly-6C^{high} monocytes correlates with high levels of mRNA expression of *tnf* and *Mannose receptor*. Quantification of mRNA for tnfα (A) and *mannose receptor* (B) in the skin of DNFB-treated and DNFB-treated+Ly-6C^{high} monocytes injected Balb/C mice through time. Results represent means ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs control (no stimulated) and #P<0.05, ##P<0.001 vs DNFB alone.

Interestingly, the increased infiltration of Ly-6C^{high} monocytes in the ears leads to an increased *tnfα* expression after 1 day of inflammation, however, after this point, the levels of *tnfα* mRNA expression were similar with control DNFB mice (Figure 7A). In addition, the high amount of Ly-6C^{high} monocytes in the tissue did not increase significantly the mannose receptor expression until day 4 (Figure 7B).

Thus, the Ly-6C^{high} infiltration *in vivo* increases these two markers suggesting the plasticity of these cells in function of microenvironment.

Role of Ly-6C^{high}CD11b^{+} monocytes recruitment during chronic inflammation in the DNFB-induced skin model in mice

The observed results at histological levels in Figure 7C suggested to us that Ly-6C^{high} cells could be used as a cellular therapy since animals injected with Ly-6C^{high} monocytes showed an improvement in the inflamed tissue. To further investigate these results inflammation was observed from day 1 until 15 at histological level using hematoxiline&eosine staining, where dermis, epidermis and total ear thickness analysis (Figure 8) and at mRNA gene expression was evaluated (Figure 9). Histological results demonstrate that animals treated with Ly-6C^{high} monocytes presented a decrease of cellular infiltration after day 7, which did not occur to control animals that have cell infiltrate until day 15 (Figure 7A). Furthermore, almost complete recovery was notice 15 days after inflammation in animals injected with Ly-6C^{high} monocytes, what was not seen in control animals that still present cellular infiltrates 15 days past the induction of inflammation (Figure 8A). In addition, epidermis, dermis and total ear thickness indicate that until day 4 animals injected with the enriched monocytes, presented an abrupt inflammatory process when compared with the control mice. At day 7 where control mice values were higher than treated mice, however values in both individual decrease until day 15. At this point, injected animals presented a decrease in the values of epidermis, dermis and total ear thickness, showing a faster tissue improvement in these animals, on the contrary of the control group that presented a decrease in the values only at day 15 (Figure 8B).

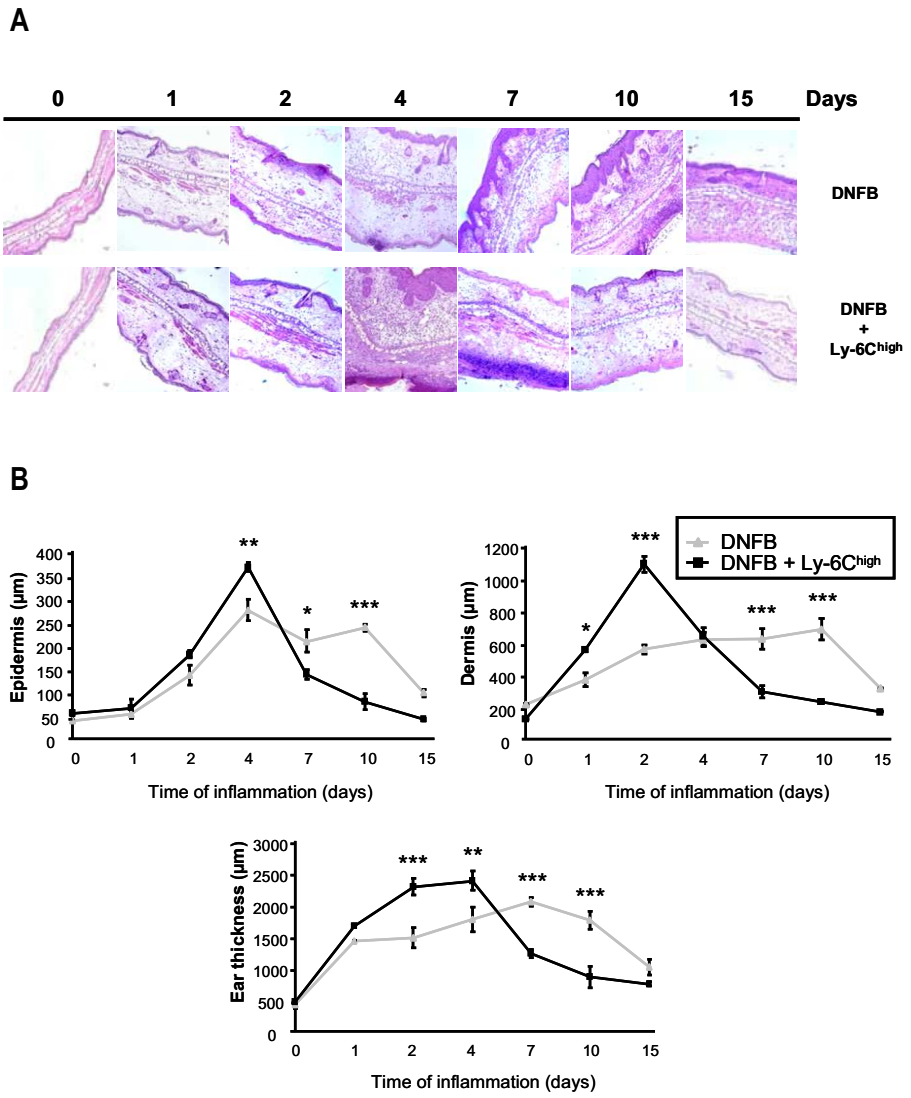

Figure 8. Improvement of the inflammatory process of the ear in Ly-6C^{high} monocytes injected mice. A) Representative hematoxylin&eosin analysis of ear sections at various days after DNFB-treated (control group), DNFB-treated and 1x10^6 Ly-6C^{high} monocytes injected mice. Original magnification 10x. Bar, 50 µm. Results are representative of at least three independent experiments. B) Epidermis, dermis and total ear thickness was evaluated as previously described in material and methods. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. #P<0.05, ##P<0.001 vs DNFB alone.

Furthermore, mRNA results in Figure 10, indicate that animals injected with enriched Ly-6C^{high} monocytes present low expression of pro-inflammatory genes (Figure 9A) on the contrary, anti-inflammatory genes such as mannose receptor and arginase I exhibit high increase at day 4 and 10, respectively, decreasing in the remaining days (Figure 9B). On the other hand, control group clearly presented a higher induction of *tnf*α and *il-1*β (Figure 9A), and a lower and later expression of arginase I and mannose receptor, respectively, when compared to injected animals (Figure 9B). These results together with histological results indicate that administration of enriched Ly-6C^{high} monocytes is beneficial to restore the inflamed tissue and suggest that Ly-6C^{high} monocytes can be used as a therapeutically tool under inflammatory conditions to decrease an inflammatory process.

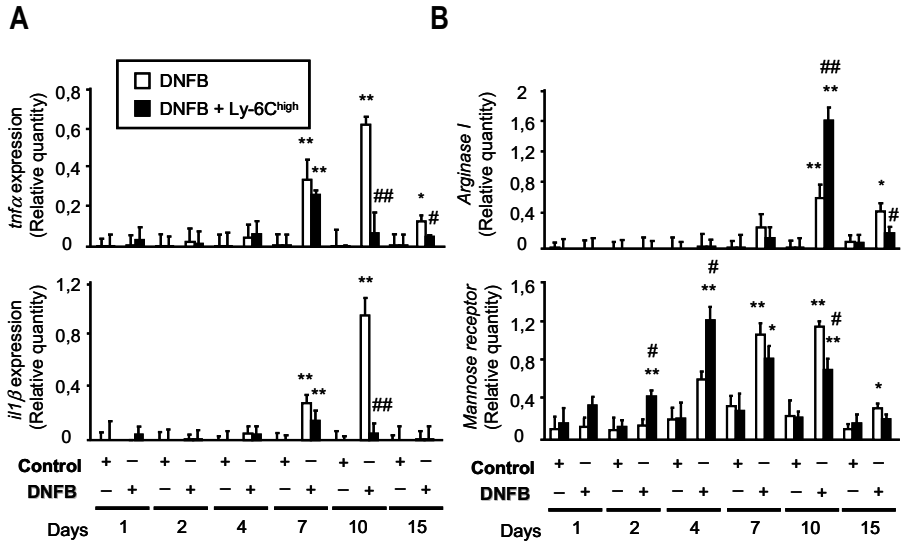

Figure 9. Ly-6C^{high} monocytes injected mice with DNFB are correlated with high levels of mRNA expression of anti-inflammatory genes. A) Detection of *tnf*α and *il1*β mRNA expression in the ear of DNFB control and DNFB-treated and Ly-6C^{high} monocytes injected mice for several days. B) Detection of *arginasel* and *Mannose receptor* mRNA expression in the ear of DNFB control and DNFB-treated+Ly-6C^{high} monocytes for several days. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs control (no stimulated) and #P<0.05, ##P<0.001 vs DNFB alone.

Involvement of IL-4 - STAT6 pathways in the generation of Ly-6C$^+$ monocytes from bone marrow

It is well known that STAT6 in response to cytokines and growth factors, are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein plays a central role in the IL-4 biological responses, since STAT6 knockout studies in mice demonstrated the involvement of this protein in T helper 2 (Th2), differentiation and class switch of immunoglobulins (Hou J. et al. 1994). Moreover IL-4 induces and activates the anti-inflammatory or M2 like phenotype in macrophage.

In order to determine if these important transcription factors, STAT6 (IL-4 signalling pathway), was involved in the Ly-6C$^+$ monocyte generation from bone marrow cells, we used wild type (WT) and knock-out (KO) mice of these proteins (Figure 10). In this regard, bone marrow cells from WT and STAT6 KO mice were incubated with the growth factor cocktail combination for 7 days, and monocytes population was analyzed as Ly-6C$^+$CD11b$^+$ cells (Figure 10A). Figure 2A shows that STAT6 deficient mice present a significative lower production of Ly-6C monocytes, with a 16% when cultured with M-CSF and a 34% when cultured with the growth factor cocktail (Figure 10A). On the other hand, WT mice present, a 22% when cultured with M-CSF and a 50% when cultured with the growth factor cocktail, indicating that the absence of this pathway alter the Ly-6C$^+$ cell production in the bone marrow (Figure 10A). Thus, in both cases, there is a reduction of Ly-6C production in comparation with wild type mice which suggest that STAT6 is important for the correct differentiation of Ly-6C$^+$ monocytes from bone marrow.

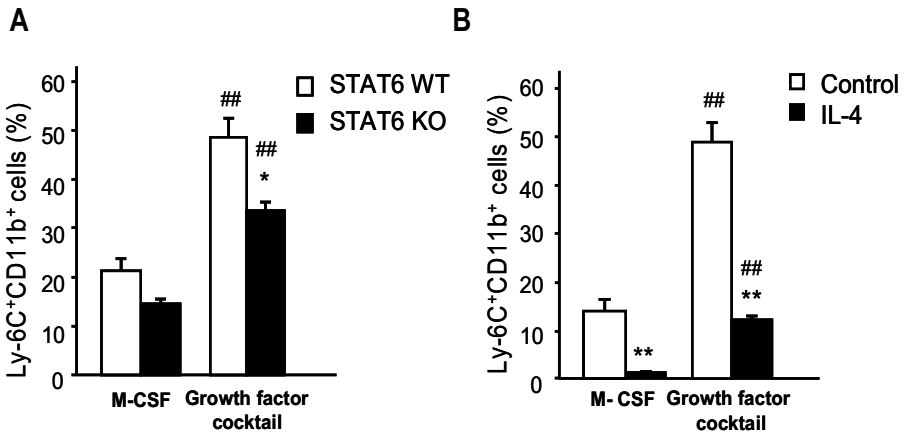

Figure 10. The presence of STAT6 and IL-4 are necessary to produce high amounts of Ly-6C$^+$ monocytes from bone marrow of mice. A) Bone marrow cells from wild type and STAT6 knockout mice were cultivated with M-CSF or growth factor cocktail during 7 days. B) Bone marrow cells from Balb/C mice were cultivated with M-CSF or grow factor cocktail in addition of IL-4 (10ng/ml) during 7 days. Cells were recollected and stained with FITC-conjugated anti-Ly-6C and PE-conjugated anti-CD11b and analysed by Flow Cytometer Galios™. Results represent mean ± SD of three independent experiments with poodle cell from 2 mice per experiment. *P<0.05, **P<0.001 vs group treated with M-CSF growth factor and #P<0.05, ##P<0.001 vs groups treated with growth factor cocktail.

On the other hand, bone marrow cells were also incubated with IL-4 cytokine (10 ng/ml) in addition of growth factors in order to obtain higher amounts of Ly-6C^{high}CD11b$^+$ monocytes (Figure 10). The results presented in figure 2B demonstrate that, bone marrow cells cultured in the absence of IL-4 induced high expression of Ly-6C$^+$CD11b$^+$ monocytes in presence of the grow factor cocktail (~50% of double-positive cells) when compared with cells generated with M-CSF (~13%). On the other hand, the addition of IL-4 (10ng/ml) to the cultured cells, induce a lower production of Ly-6C monocytes, with a ~3% of Ly-6C$^+$CD11b$^+$ cells when cultured with M-CSF and a ~13% with the grow factor cocktail (Figure 10B). These results suggest that IL-4 even inhibits the production and/or release of Ly-6C$^+$CD11b$^+$ cells in the bone marrow. Thus, it seems that hematopoietic steam cells (HSC) are not able to differentiate in to high amounts of monocytes under anti-

inflammatory environmental conditions, however, depends on the presence of the STAT6 transcription factors to differentiate.

Therapeutic effects of Ly-6C^{high} monocytes differentiated from STAT6 knock-out mice in an acute inflammation in the DNFB-induced skin model in mice

As mentioned before STAT6 gene is involved in IL-4 signalling pathway and this cytokine is crucial for the switch from M1 to M2 macrophages (Hebenstreit D., et al. 2006; Jerrold MO. and Christopher KG. 2010). For this sense, enriched Ly-6C^{high} monocytes were generated from wild type (WT) and knock-out (KO) Balb/c STAT6 mice and were injected in immunocompetent female Balb/C mice, while DNFB1% was applied on the mice right ear. As a control acetone was applied in the left ear. The results showed that animals treated only with DNFB1% and animals injected with Ly-6C^{high} cells from WT mice respond in the same manner as control group, where injection of Ly-6C^{high} monocytes help to resolve the inflammatory process (Figure 11). On the other hand, injection with enriched Ly-6C^{high} cells generated from STAT6 KO mice worse ear inflammation, causing ulcerogenic lacerations in the right ear (Figure 11A). Histological images showed a rise of neutrophils, lymphocytes, monocytes and macrophages infiltration in the inflammatory focus in animals injected with treated STAT6 KO Ly-6C^{high} monocytes during the whole inflammatory process having its pick at day 7, remaining until day 15 (Figure 11A). In animals injected with STAT6 KO cells the regeneration process was not noticed, when compared with the other two conditions, where ear regeneration was noticed in day 15 (Figure 11A).

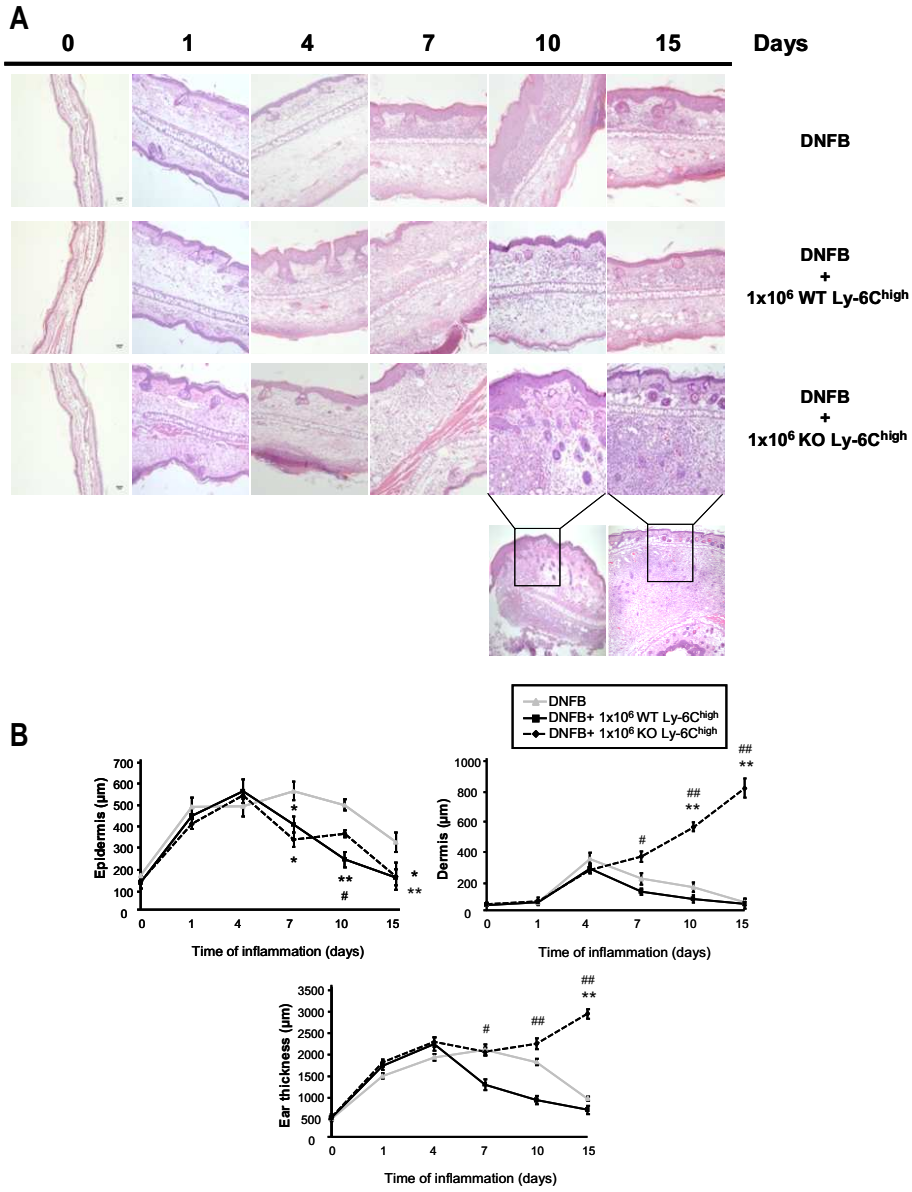

Figure 11. Evaluation of the inflammatory process in mice injected with Ly-6C^{high} monocytes from STAT6 knockout animals. A) Representative hematoxylin&eosin analysis of ear sections at various days after DNFB-treated (control group); DNFB-treated and Ly-6C^{high} monocytes injected mice; DNFB-treated and STAT6 knockout Ly-6C^{high} monocytes injected mice. Original magnification 10x. Bar, 50 µm. Results are representative of at least three independent experiments. B) Epidermis, dermis and total ear thickness was evaluated as previously described in material and methods. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs DNFB WT and #P<0.05, ##P<0.001 vs DNFB alone.

The ear, dermis and epidermis thickness analysis were performed in histological images (Figure 11B). The results showed that animals injected with STAT6 KO monocytes presented an increase in the epidermis, dermis and total ear thickness from day 7 until day 15, process that could be due to the result of the increase of inflammatory process through the cellular infiltration (Figure 11B). RNA expression corroborated the histological results showing a significant increase in expression of pro-inflammatory genes such as *tnfα* and *il-1β* at day 7 until day 10 (Figure 12). At day 15 levels of mRNA expression decreased although were still high when compared to the other groups (Figure 12A). On the other hand, expression of anti-inflammatory genes such as arginase I and mannose receptor in animals injected with enriched Ly-6C^{high} cells generated from STAT6 KO animals were lower or similar to the control group in all time analyzed (Figure 12B). These results suggests that in the absence of a STAT6 pathway the pro-inflammatory macrophages are not able to switch phenotype to an anti-inflammatory macrophage, leading to an increase of pro-inflammatory macrophages in the inflammatory loci, thereby increasing the inflammatory process, thus leading to a ear atrophy or necrosis.

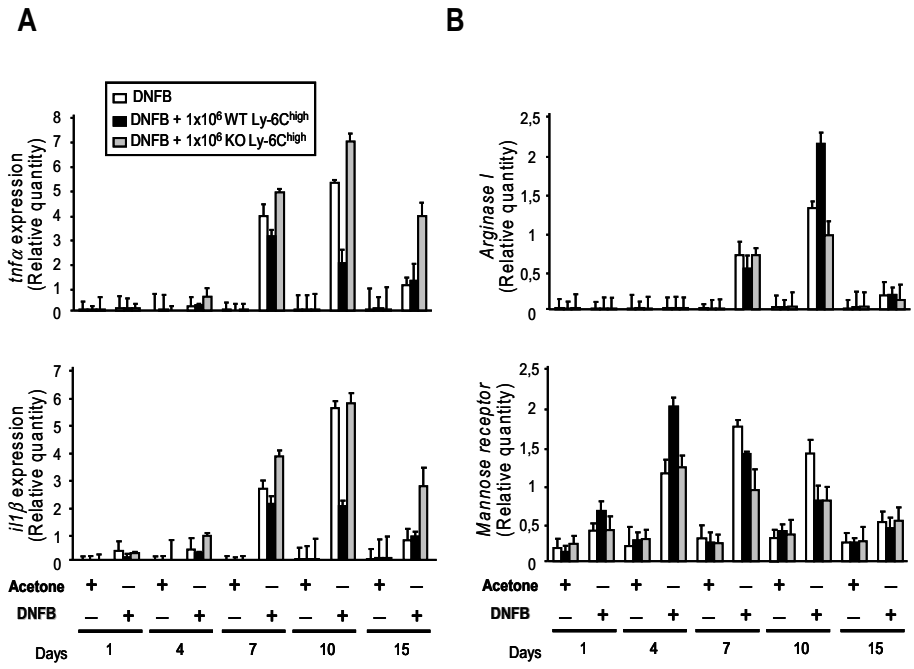

Figure 12. The Ly-6C^{high} monocytes from STAT6 KO mice injected in STAT6 wild type mice correlated with increased expression of pro-inflammatory genes. A) mRNA expression for *tnfα* and *il1β* in the ear of DNFB control and DNFB- treated and injected with WT or KO Ly-6C^{high} cells form STAT6 mice. B) mRNA expression of *arginase I* and *Mannose receptor* in the ear of DNFB control and injected with WT or KO Ly-6C^{high} cells form STAT6 mice. Results represent the means ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs DNFB WT and #P<0.05, ##P<0.001 vs DNFB alone.

Therapeutic effects of IL-4- pre-treated Ly-6C^{high} monocytes in an acute inflammation in the DNFB-induced skin model in mice

At this point one of the ambitious aims of this thesis is to use enriched Ly-6C^{high} monocytes as a therapeutic tool to solve acute inflammatory processes. To achieve this propose several experiments were performed. Enriched Ly-6C^{high} monocytes were incubated for 15 minutes with IL-4 (10ng/ml) before intravenous injection (Figure 13). After washing the cells with PBS1x for several times, one

million of IL-4 pre-treated and untreated monocytes was injected intravenous in mice at the same time as DNFB1% was applied in the mice ear. Analyses of histological images were performed during several days, from day 1 until day 15 (Figure 13A). Animals injected with IL-4 pre-treated cells presented a diminished inflammatory response when compared with mice injected with untreated Ly-6C^{high} monocytes, where at day 4 cellular infiltrations and ear swelling decrease almost until basal levels (Figure 13A).

To evaluate the therapeutic effect of animals treated with IL-4 pre-treated Ly-6C^{high} monocytes, epidermis, dermis and total ear thickness was analysed (Figure 13B) the results demonstrated that mice injected with IL-4 pre-treated cells presented a smaller increase in epidermis, dermis and total ear thickness when compared with the other conditions (Figure 13B). To corroborate these results mRNA expression was analyzed (Figure 14). The Ly-6C^{high} treated group with IL-4 showed a decreased *tnf*α and *il1*β expression in comparation of other injected and control groups (Figure 14A). On the contrary, increase of anti-inflammatory genes such as *mannose receptor* and *arginase I* were appreciated at day 1 and 7, respectively when compared with the Ly-6C^{high} cell injected and control group (Figure 14B). These results indicate that although administration of enriched Ly-6C^{high} monocytes is beneficial to restore the inflamed tissue, the use of cells pre-activated with anti-inflammatory cytokines such as IL-4, brings a better result, improving in the diminished of the inflammatory process.

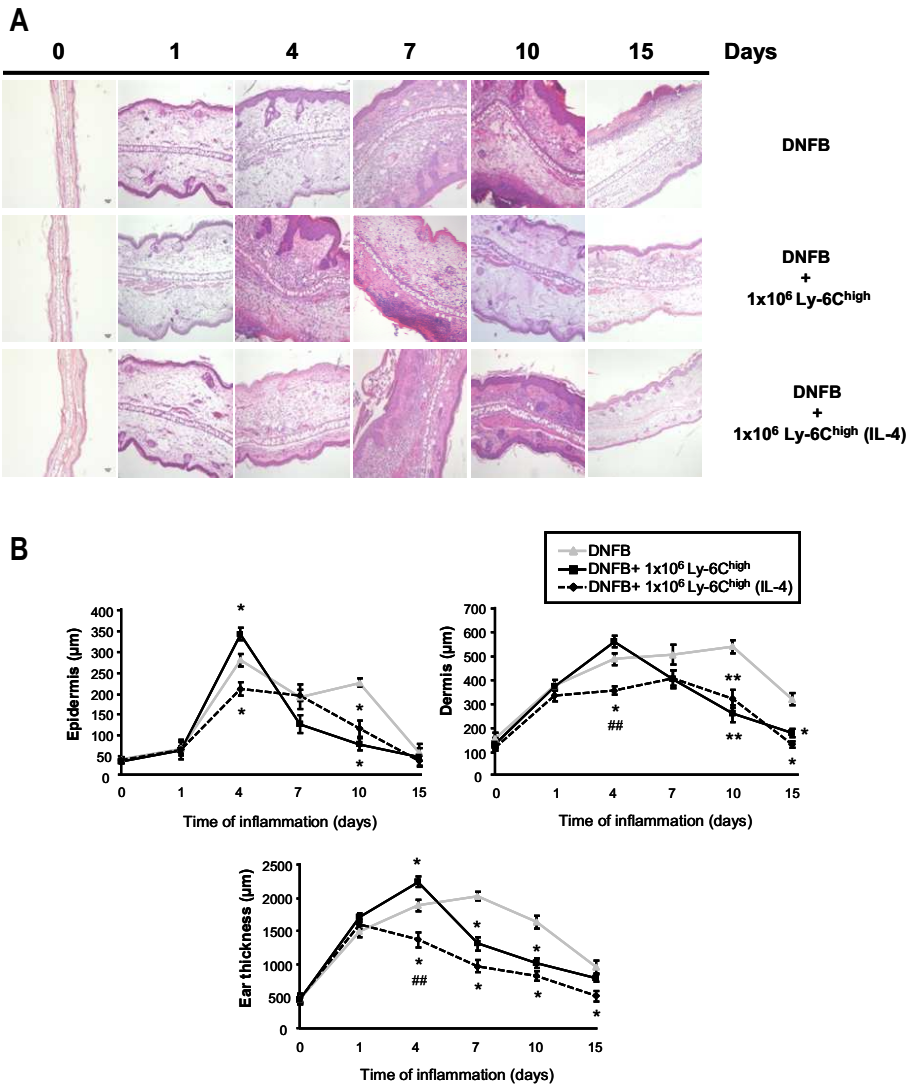

Figure 13. Evaluation of the inflammatory process in mice injected with IL-4 pre-treated Ly-6C^{high} monocytes. A) Representative hematoxylin&eosin analysis of ear sections at various days after DNFB-treated (control group), DNFB-treated and Ly-6C^{high} monocytes injected mice; DNFB-treated and pretreated Ly-6C^{high} monocytes injected mice. Cells were pretreated during 15 minutes with IL-4 (10ng/ml) previously injections in mice, then no pretreated or IL-4 treated Ly-6Chigh monocytes were injected in DNFB- treated mice. B) Ear, dermis and epidermis thickness were evaluated as described in material and methods. Original magnification 10x. Bar, 50 µm. Results are representative of at least three independent experiments. B) Epidermis, dermis and total ear thickness was evaluated as previously described in material and methods. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs DNFB IL-4 and #P<0.05, ##P<0.001 vs DNFB alone.

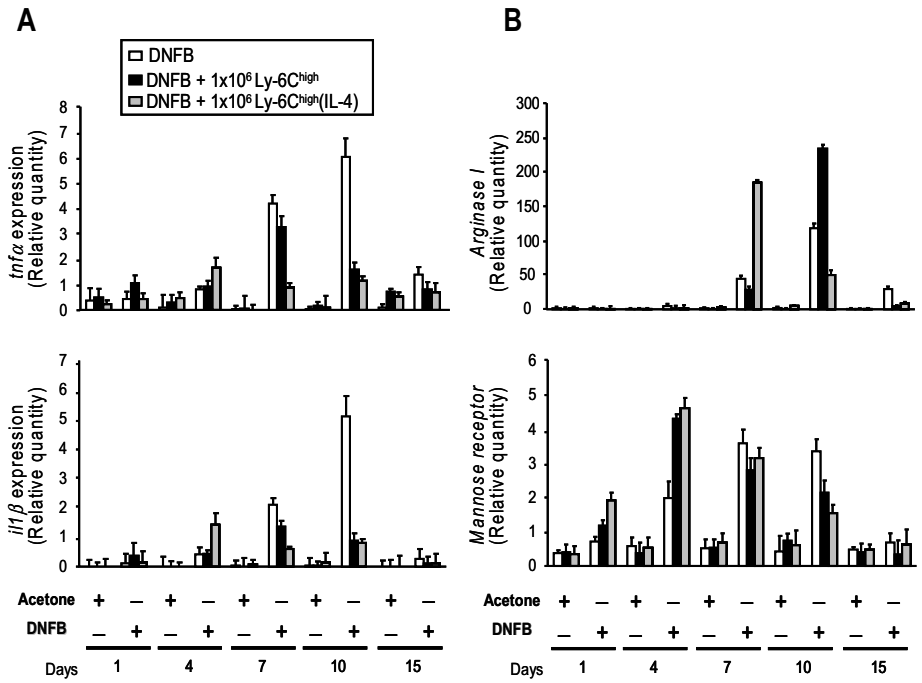

Figure 14. Increase of anti-inflammatory genes in ears of IL-4 pre-treated Ly-6C^{high} monocytes. A) mRNA expression for *tnfα* and *il1β* in the ears of DNFB control and DNFB- treated mice and injected with no pretreated or IL-4 treated Ly-6C^{high} cells. B) mRNA expression for *Arginasel* and *Mannose receptor* in the ears of DNFB-treated (control group), of Ly-6C^{high} monocytes no pretreated and IL-4 treated DNFB-mice. Results represent the means ± SEM of three experiments. *P<0.05, **P<0.001 vs DNFB IL-4 and #P<0.05, ##P<0.001 vs DNFB alone.

PART 6: Assessment of *in vivo* homing capacity of *in vitro* generated Ly-6C^{high} monocytes in a muscle model of inflammation

The next step consisted in the demonstration of the highly versatile phenotype of these cells, depending on the inflammatory microenvironment. So, the aim of this specific objective consisted in the functional analysis of enriched Ly-6C^{high} monocytes in an *in vivo* model of inflammation, of skeletal muscle injury, in which macrophages play an important role in the muscle regeneration. Macrophage

function participating on myogenic cell growth through mitogenic and antiapoptotic activities. In this model, Ludovic and co-workers demonstrated that inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis (Ludovic A., et al. 2007).

Study of recruitment of enriched Ly-6C^{high} in an acute inflammation in the Notexin-induced muscle model in mice

First we evaluate the migratory capacity of the enriched Ly-6C^{high} cells during the skeletal muscle injury (Ludovic A., et al. 2007). To induce the skeletal muscle injury, 10 µl of Notexin (NX) at 25 µg/ml in PBS, a myotoxic phospholipase**,** from *Notechis scutatus* snake venom, was administrated into tibialis anterioris (TA) of an immunocompetent female Balb/C mice, causing extensive damage that is largely specific to muscle fibers, without causing damage to the basal lamina, blood vessels, or the regenerative capabilities of satellite cells (Karlsson E., et al. 1972; Halpert J et al. 1975 ;Mollier P et al. 1989; Chwetzoff S et al. 1990). In this model, injection of PBS1x was used as a control in the left TA (Figure 15).

As mentioned before, enriched Ly-6C^{high}CD11b^{+} monocytes were stained with DIR and were injected intravenously in a different concentration, 1x10^{6} and 2x10^{6}, in the mouse tail after induction of inflammation of TA muscle injury with Notexin (Figure 15).To evaluate the migratory capacity of these cells to the inflammatory foci *in vivo* image in an IVIS machine was performed after 24 and 48 hours. The results indicated that bout amounts of Ly-6C^{high} cells were detected 48 hours after inflammation (Figure 15A). Quantification of cells in the IVIS machine is in agreement with the results obtained from the *in vivo* images (Figure 15B). However, Ly-6C^{high} monocytes can be sensed in the inflammatory foci 24 hours after inflammation but also in the spleen or peritoneal cavity, which is understandable since the liver is a common reservoir of these cells (Figure 15A).

Due to this reason, and in order to avoid other systemic effects induced by these monocytes injection, we decided to work with 1 million of Ly-6C^{high} monocytes which was considered less aggressive. As a control of migration, 1x10^{6} enriched Ly-6C^{low} cells were injected in animals treated with Notexin (Figure 15C). After 48 hours low amount of fluorescence was sensed in the right TA, which means that this Ly-6C^{low} cell were not able to migrate from the tail to the inflammatory loci (Figure 15C).

The analysis of inflammatory status using the mRNA expression of several pro-inflammatory markers was evaluated *in vivo* during 24 and 48 hours (Figure 16). At 24 hours after the Notexin treatment the *tnf*α expression was increased in mice in which Ly-6C^{high} monocytes were injected and *il1*β expression was similar when compared to control group and injected mice (Figure 16A).

The *arginase I* and *mannose receptor* as markers of alternative pathway were evaluated in order to determine the involvement of repairing process in this model (Figure 16B). Interestingly, although the intravenous injection of Ly-6C^{high} monocytes was able to increase in a significant manner these anti-inflammatory markers at 24 hours, the Ly-6C^{high} injected animals had the same RNA expression as the control group at 48 hours (Figure 16B).

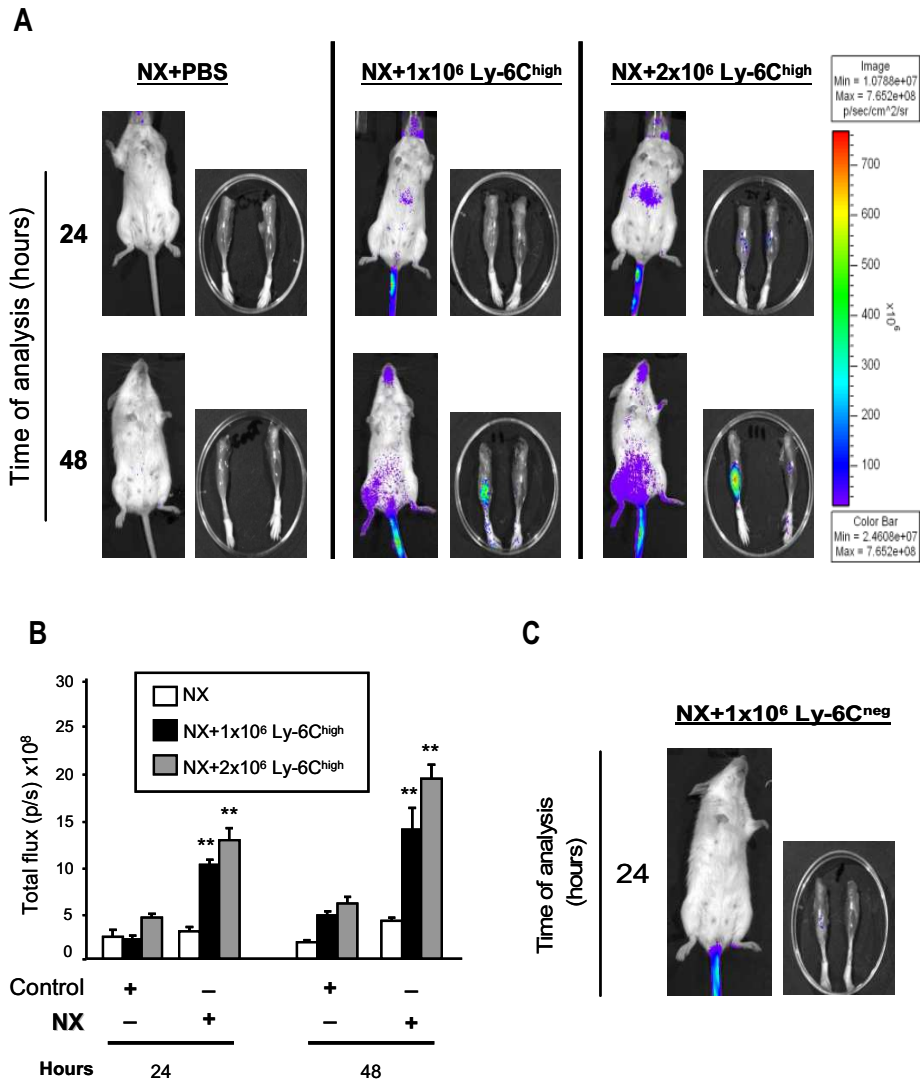

Figure 15. Enriched Ly-6C^{high} monocytes differentiated *in vitro* migrate to inflamed muscle. Induction of muscle inflammation was performed in right tibialis anterioris of Balb/C mice by 25 µg/ml Notexin (NX) and then cell injection of enriched Ly-6C^{high} monocytes stained with DiR was performed as described in material and methods. A) Cellular migration of different amounts of enriched Ly-6C^{high} (1x10^6 and 2x10^6) cells were appreciated through time in an *in vivo* image in the IVIS machine. B) Quantification of total flux (pixels/seconds) of *in vivo* images. C) Ly-6C^{neg} monocytes migration were evaluated as in A) and used as control. Results from A and C are representative of at least three independent experiments. Results from B represent mean ± SEM of three independents experiments from at least 5 mice per experiment. #P<0.05, ##P<0.001 vs NX alone.

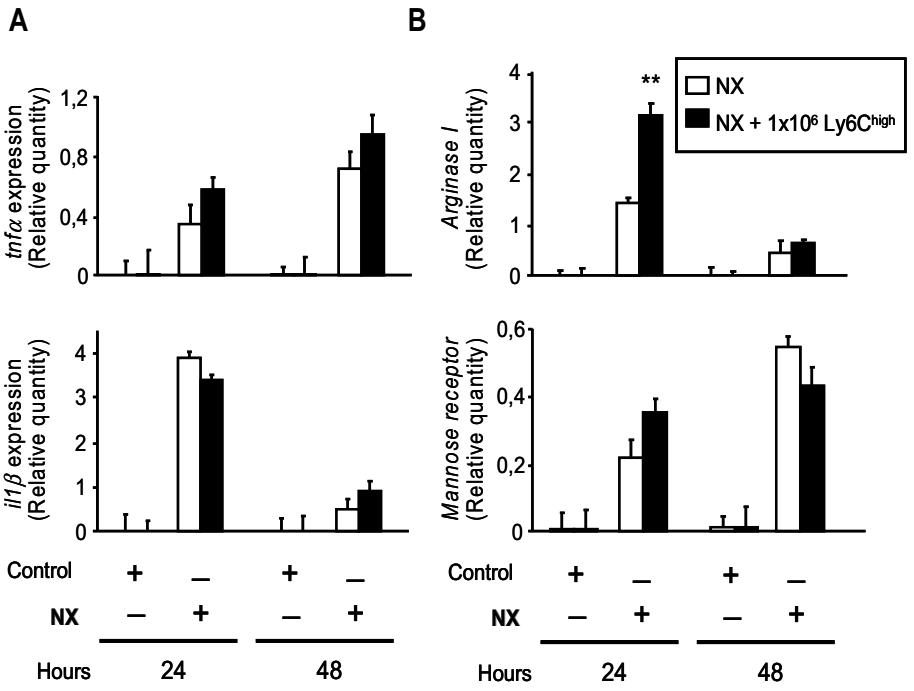

Figure 16. The injection of Ly-6C^{high} monocytes correlates with high levels of mRNA expression of *arginase I*. A) mRNA expression for tnfα and il1β in the muscle of NX-treated and NX-treated and 1x10^6 Ly-6C^{high} monocytes injected mice. B) mRNA expression for *Arginase I* and Mannose receptor in the muscle of NX-treated and NX-treated+Ly-6C^{high} monocytes injected mice. Results represent mean ± SEM of three independents experiments from at least 5 mice per experiment. . #P<0.05, ##P<0.001 vs NX alone.

Role of Ly-6C^{high}CD11b$^+$ monocytes recruitment during chronic inflammation in the Notexin-induced muscle model in mice.

After the characterization of cell migration to the in vivo model of muscle injury, one million of enriched Ly-6C^{high} monocytes differentiated *in vitro* were injected intravenous simultaneously as the induction of injury in the tibialis anterioris (TA) of mice with Notexin (Figure 17). The analysis of inflammatory status was performed using muscle slides stained with hematoxilin&eosin (Figure 17A). The

quantitative analysis of muscle necrosis and regeneration (Figure 17B) and mRNA myogenin levels were also performed in this study (Figure 17C).

Histological analysis demonstrated that levels of infiltrating cells were reduced at day 7 in Ly-6C^{high} monocytes injected mice when compared to control group, which still have a cellular infiltration (Figure 17A). Furthermore, reductions in necrotic cells were appreciated in the inflamed tissue (Figure 17B) and increased in the formation of myofibers were detected at day 10 in animals which Ly-6C^{high} monocytes were injected, although in control group the same profile was observed with decreased values (Figure 17B). In the analysis of myogenin RNA expression it has not been observed a significative increase at day 4 in injected animals when compared to untreated mice, however at day 7, an increased myogenin mRNA expression levels were appreciated in animals injected with monocytes (Figure 17C).

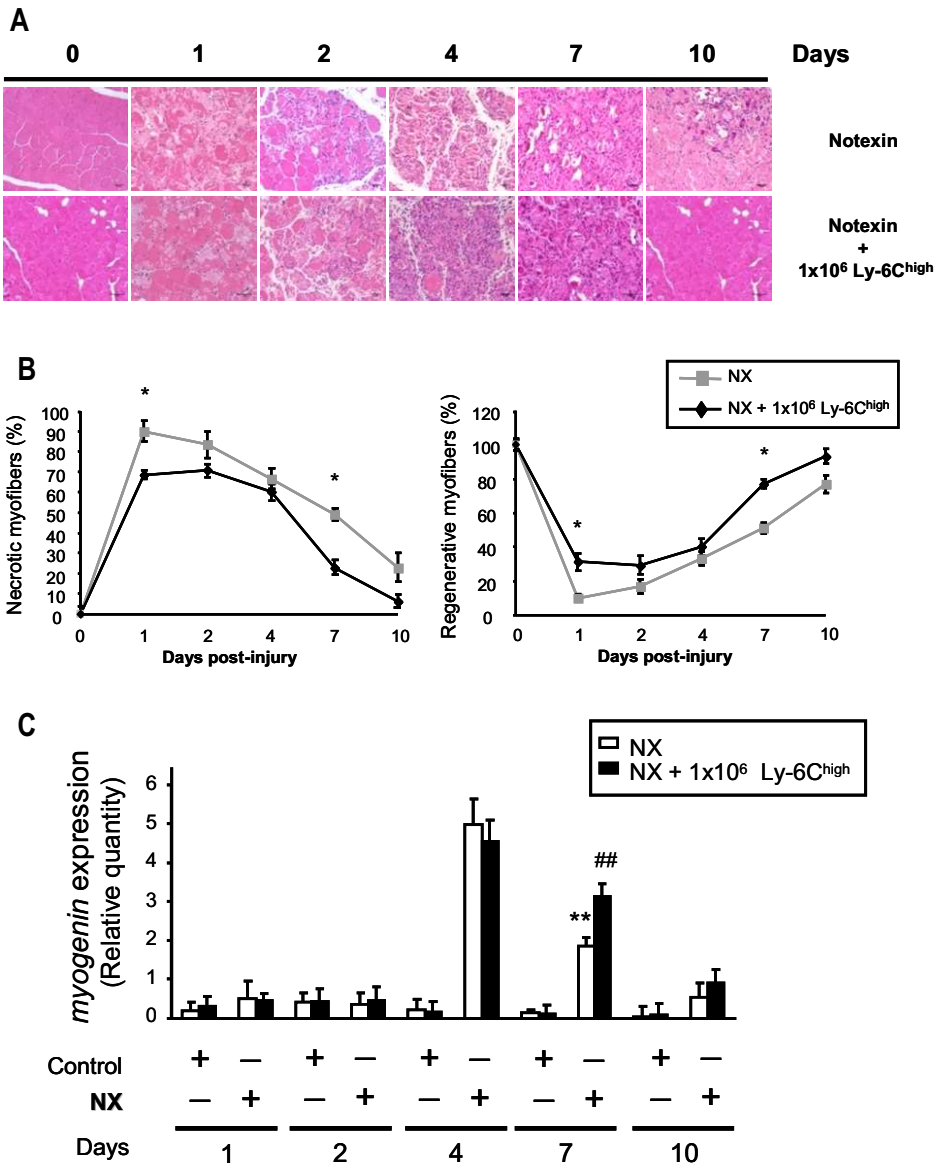

Figure 17. Improvement of the inflammatory process in mice injected with Ly-6C^{high} monocytes after NX-induced muscle injury. A) Representative hematoxylin&eosin analysis of muscle sections at several days after NX-treated (control group) and NX-treated and 1x10^6 Ly-6C^{high} monocytes injected mice. Original magnification 20x. Bar, 50 μm. Results are representative of at least three independent experiments. B) Ear, dermis and epidermis thickness were evaluated as described in material and methods. C) Expression of myogenin was evaluated by real time PCR. Results from B and C represent mean ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs control (no stimulated) and #P<0.05, ##P<0.001 vs NX alone.

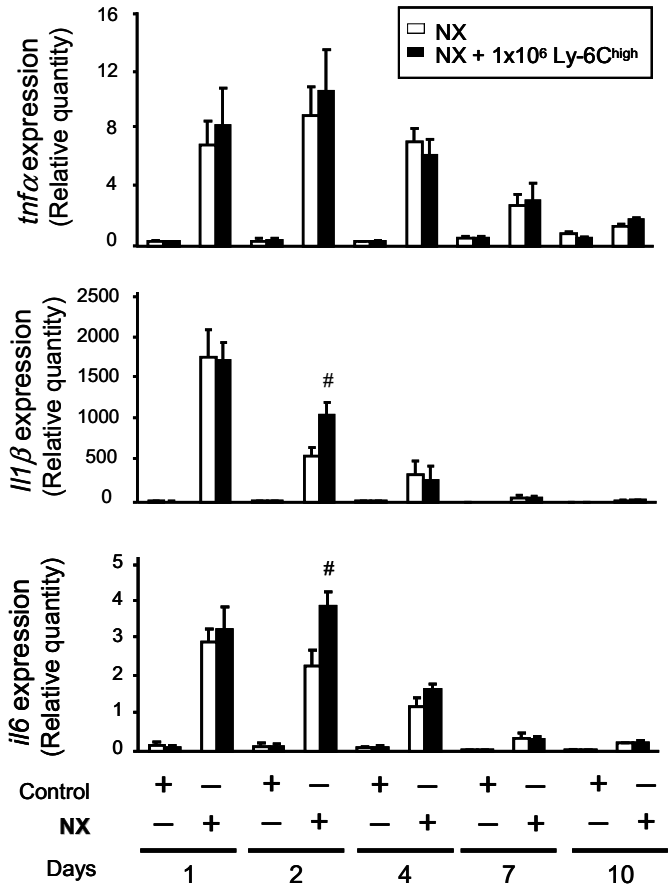

Figure 18. The injection of Ly-6C^{high} monocytes does not increase mRNA expression levels of pro-inflammatory genes. Detection and quantification of mRNA for *tnfα*, *il1β* and *il6* in the muscle of NX-treated and NX-treated (control group) and 1x10^6 Ly-6C^{high} monocytes injected mice. Results are representative of three independent experiments. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. *P<0.05, **P<0.001 vs control (no stimulated) and #P<0.05, ##P<0.001 vs NX alone.

Furthermore, in addition of histological images, pro-inflammatory markers were also evaluated in this muscle inflammation model (Figure 18). The results indicated that Ly-6C^{high} cells injection together with Notexin did not increase significantly any of the pro-inflammatory markers (*tnfα*, *il-1β* and *il-6*) in the first days, as well as in the last days of inflammation, having the same expression levels

as control group (Figure 18). On the contrary, anti-inflammatory markers involved in the tissue remodelling were evaluated, in which an increase in *arginase I* and *il-10* mRNA expression was observed from day 1 until day 4 when compared with uninjected animals (Figure 19). Although, when mannose receptor mRNA expression was evaluated no significative increase was appreciated when compared to control group (Figure 19). All of these results together suggest than enriched Ly-6C^{high} monocytes in a chronic inflammatory models, can contribute to the faster restauration of the tissue.

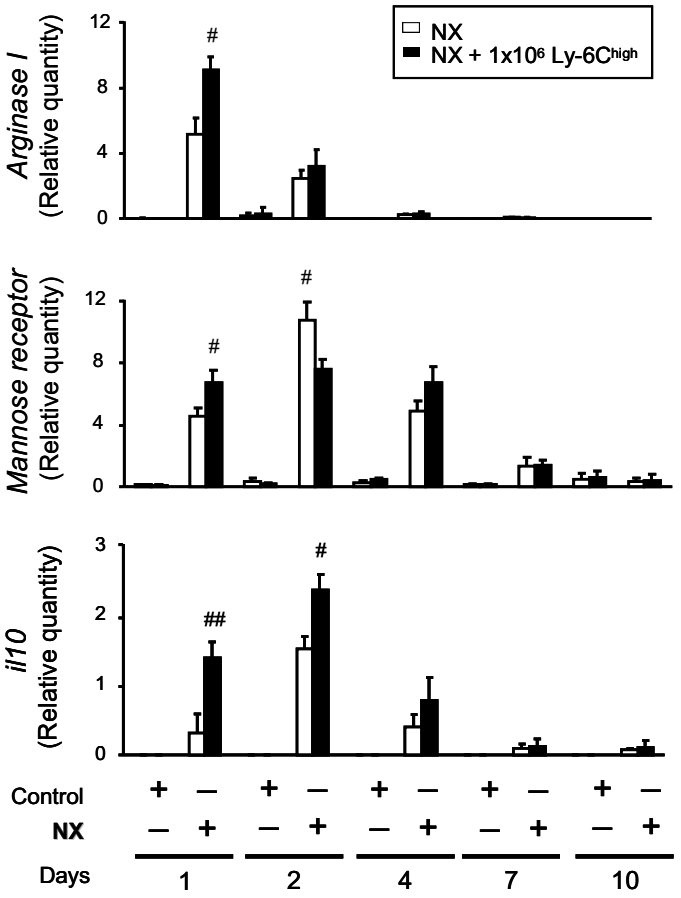

Figure 19. The injection of Ly-6C^{high} monocytes increases in mRNA expression levels of anti-inflammatory genes. Quantification of mRNA for *Arginase I, Mannose receptor* and *il10* in the muscle of NX-treated (control group) and NX-treated and Ly-6C^{high} monocytes injected mice. Results represent the means ± SEM of three independent experiments with at least 5 mice per group. *P<0.05, **P<0.001 vs control (no stimulated) and #P<0.05, ##P<0.001 vs NX alone.

Role of the enriched Ly-6C^{high} monocytes recruitment at different time of skeletal muscle inflammation.

In order to evaluate if the Ly-6C monocytes could accelerate the de-activation of inflammation and thus increase in time and effectiveness of the tissue repairing, we injected enriched Ly-6C^{high} monocytes after 24 hours of induction of

muscle inflammation by Notexin (Figure 20). Histological analysis demonstrated that infiltrating cells were reduced at day 4 in $1x10^6$ Ly-$6C^{high}$ monocytes injected mice (Figure 20A). Moreover in the inflamed tissue a reduction of necrotic fibers were also observed (Figure 20B) when compared with un-injected animals and with animals injected with enriched Ly-$6C^{high}$ monocytes at the same time of the Notexin-induction inflammation. The injection of these cells induced an increase in the myogenin mRNA expression after 2 days of induced inflammation by Notexin (Figure 20C), showing higher levels than control group. Although, this expression being to react at basal levels after 7 days of inflammation in both groups.

Moreover the Ly-$6C^{high}$ injected mice increased the *tnfα* and *il1β* mRNA expression at 2 days of inflammation (Figure 21). Furthermore, important differences were observed when anti-inflammatory markers were analyzed in the same samples (Figure 22). The injection of monocytes increased the mannose receptor and arginase I expression at short time points after inflammation (Figure 22). In the case of mannose receptor the expression was maintained until day 4 (Figure 22) suggesting to us that a more pronounced M2 alternative pathway was activated in an early point and thus, it could contribute to the faster restauration of the tissue as previously observed in histological analysis, where at day 7 the muscle damage was almost repaired (Figure 22). These results are in agreement with those obtained analysing the IL-10 expression (Figure 22) although no significative results were obtained with TGF-β expression (data not shown).

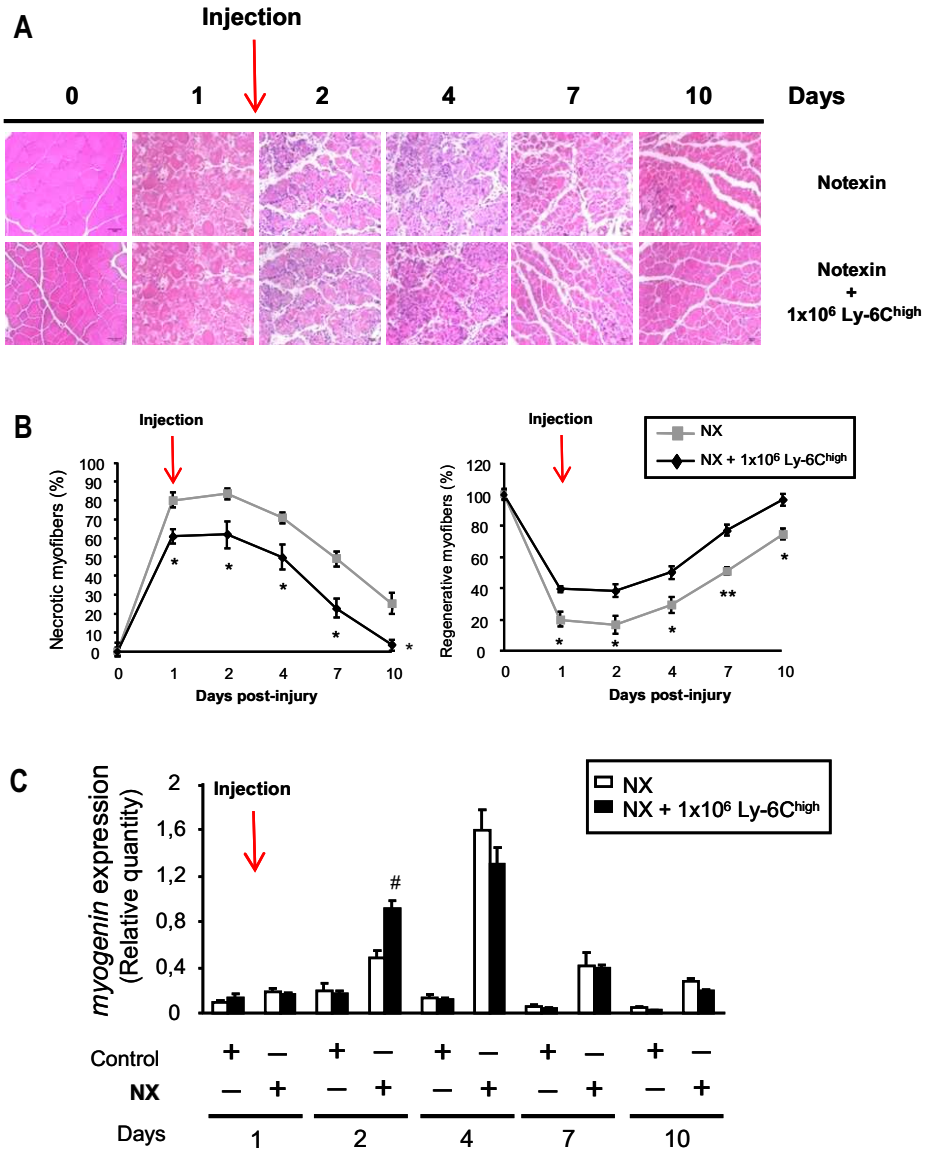

Figure 20. Faster improvement of the inflammatory process in mice injected with Ly-6C^{high} monocytes after 1 day of NX-induced muscle injury. The muscle inflammation was induced at day 0 in tibialis anterioris of mice by Notexin injection and after 1 day of inflammation induction, mice received an injection in the tails anterioris of 1x10^6 Ly-6C^{high} monocytes. A) Representative hematoxylin&eosin analysis of muscle sections at several days after NX-treated (control group) and NX-treated and 1x10^6 Ly-6C^{high} monocytes injected mice. Original magnification 20x. Bar, 50 µm. Results are representative of at least three independent experiments. B) Ear, dermis and epidermis thickness were evaluated as described in material and methods. C) Expression of myogenin was evaluated. Results from B and C represent mean ± SEM of three independent experiments with at least 5 mice per experiment. #P<0.05, ##P<0.001 vs NX alone.

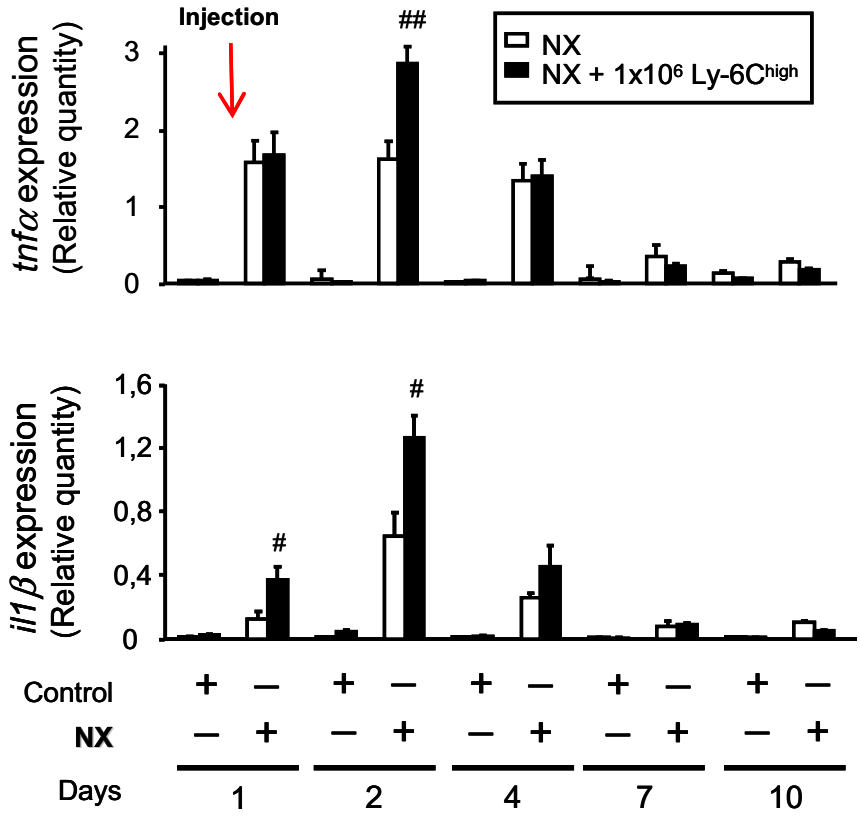

Figure 21. Ly-6C^{high} monocytes injection after 1 day of NX-induced muscle injury increase mRNA expression levels of pro-inflammatory genes in the first days of inflammation. mRNA expression for *tnfα*, *il1β* and *il6* in the muscle of NX-treated and NX-treated and 1x10^6 Ly-6C^{high} monocytes injected mice, after 1 day of inflammation. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. #P<0.05, ##P<0.001 vs NX alone.

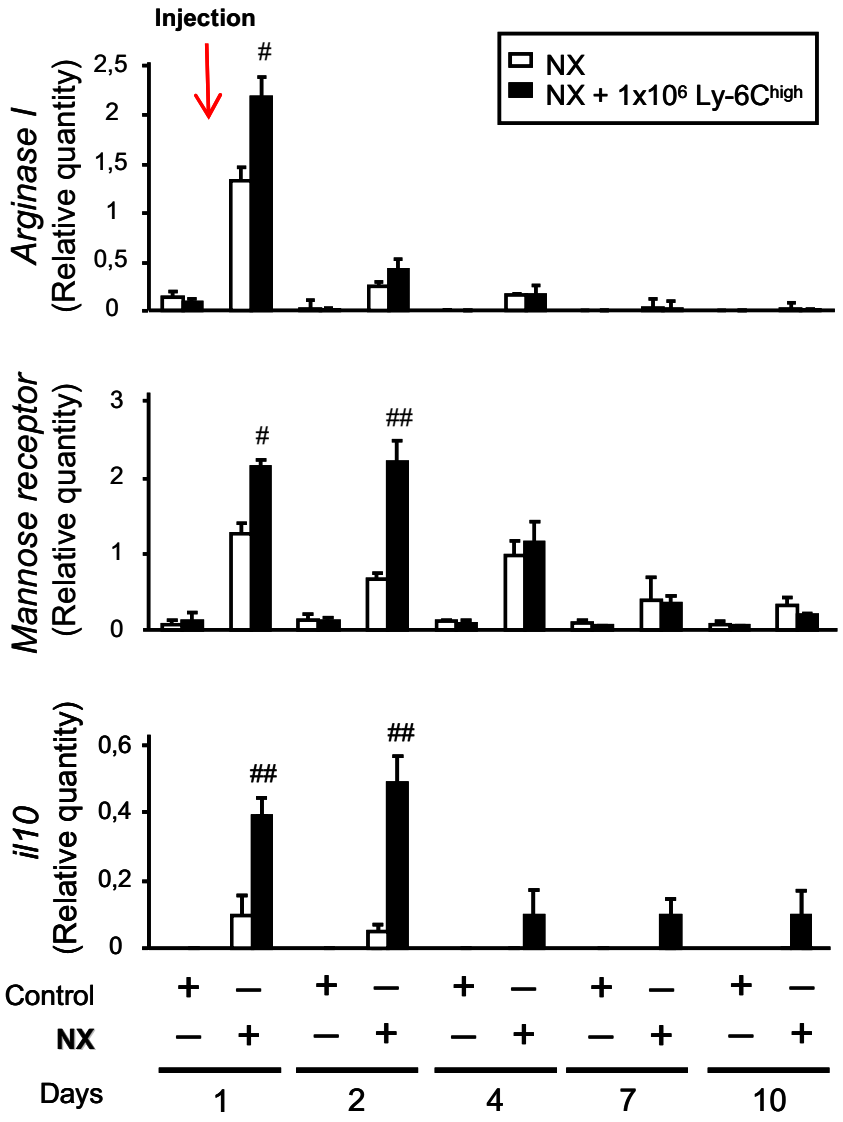

Figure 22. Ly-6C^{high} monocytes injection after 1 day of NX-induced muscle injury induces high levels in mRNA expression of anti-inflammatory genes in the first days of inflammation. mRNA expression for a*rginase I, Mannose receptor* and *il10* in the muscle of NX-treated and NX-treated and 1x10^6 Ly-6C^{high} monocytes injected in mice, after 1 day of inflammation. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. #P<0.05, ##P<0.001 vs NX alone.

Therapeutic effects of IFN-γ and IL-4- pre-treated Ly-6C^{high} monocytes in a skeletal muscle inflammation induced by notexin in mice

As demonstrated in figure 5 monocytes present as well as macrophages high heterogenicity and plasticity depending on the stimuli received and Ly-6C^{high} monocytes can differentiate in to different macrophage phenotype in function of the environment. In a final objective of this thesis, we decided to test the hypothesis if the previously treatment of enriched Ly-6C^{high} monocytes differentiated to a pro-inflammatory (by IFN-γ stimuli) or to a repairing (by IL-4) phenotype could collaborate in the resolution of the muscle inflammation.

The experiments were performed with 1x10^6 Ly-6C^{high} monocytes pre-incubated with IFN-γ (300 U/ml) for 15 minutes before mice injection at the same time of the inflammation induction by Notexin (Figure 23). The expression of pro-inflammatory genes (*nos2*, *tnfα* and *il1β*) increased significantly after 2 days of muscle injury (Figure 23A). Moreover no significative differences in the expression of the anti-inflammatory markers were appreciated in IFN-γ-pre-treated Ly-6C^{high} monocytes group compared to control (Figure 23B). In counterpart, *arginase I* expression did not increase (Figure 23B), suggesting that animals injected with pre-treated cell can present an earlier pro-inflammatory response when compared with untreated cells.

The injection of Ly-6C^{high} monocytes previously activated by IL-4 (10 ng/ml) during 15 minutes were not able to induce an increase in the expression of pro-inflammatory genes, such as *il1β*, *tnfα* and *nos2*, when compared to the other groups (Figure 24A). Interestingly, mRNA expression of anti-inflammatory markers such as *mannose receptor* and *arginase I* increased (Figure 24B). All these results together, suggest that pre-incubation with anti-inflammatory cytokines could be a good alternative in order to reduce chronic inflammation. However more studies

with other models and with different periods of injection have to be done in a future in order to corroborate the previous results.

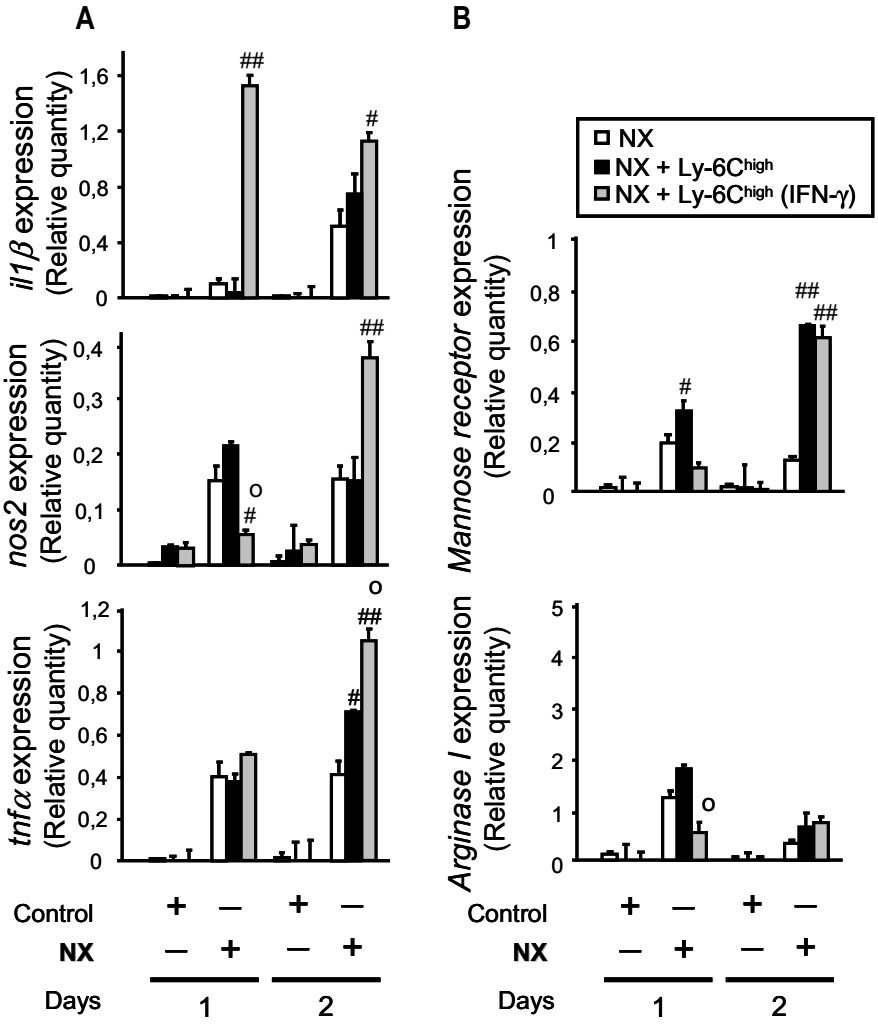

Figure 23. The injection of IFN-γ pre-treated Ly-6C^{high} monocytes in mice induces high levels in mRNA expression of pro-inflammatory genes. The Ly-6C^{high} monocytes were pretreated with IFN-γ (300 U/ml) for 15 minutes previously injection in mice. Then pre-treated Ly-6C^{high} with IFN-γ and no- treted cells were injected to the tail of Notexin mice. Muscle inflammation was previously described in material and methods. Then mRNA expression of *tnfα*, *il1β* and *nos2* (A) and a*rginase I* and *Mannose receptor* (B) was evaluated in the tibialis anterioris of mice groups. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. #P<0.05, ##P<0.001 vs NX alone and ºP<0.05, ººP<0.001 vs NX (IFN-γ).

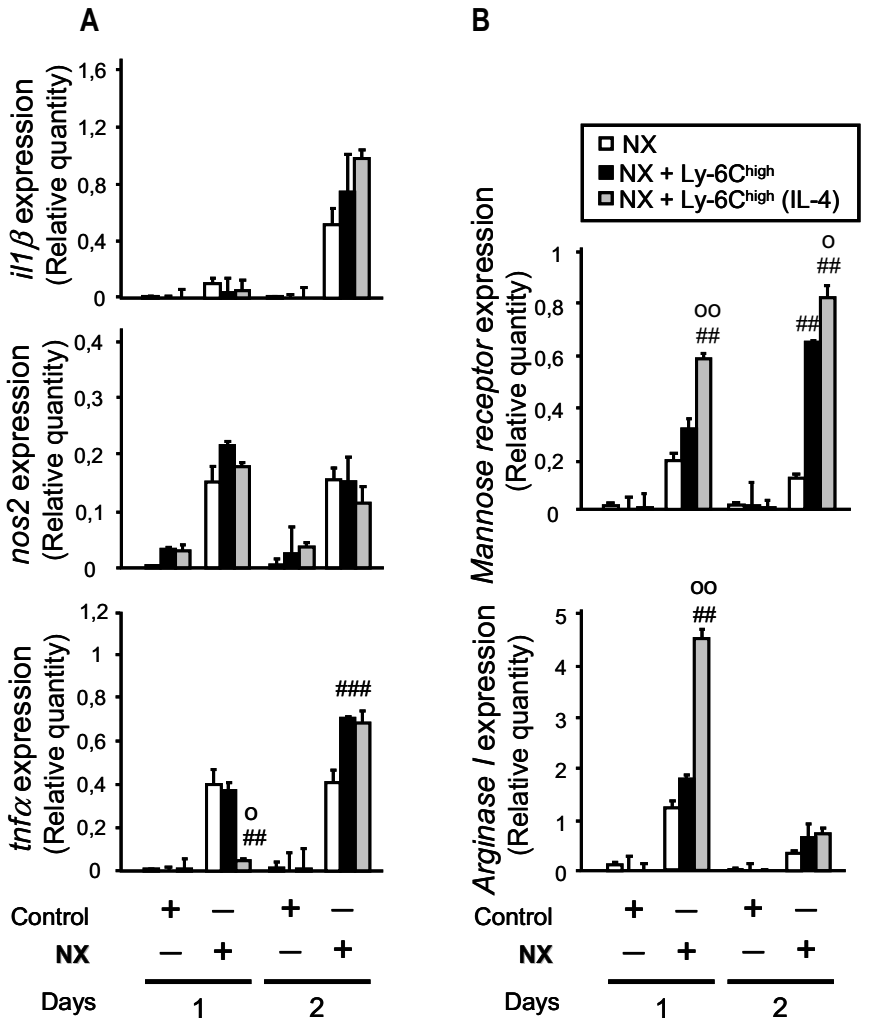

Figure 24. The injection of IL-4 pre-treated Ly-6C^{high} monocytes induces high levels in mRNA expression of anti-inflammatory genes. The Ly-6C^{high} monocytes were pre-treated with IL-4 (10ng/ml) for 15 minutes previously injections in mice. Then pre-treated Ly-6C^{high} with IL-4 and no-treted cells were injected to the tail of Notexin mice. Muscle inflammation was previously described in material and methods. Then mRNA expression of *tnfα*, *il1β* and *nos2* (A) and *arginase I* and *Mannose receptor* (B) was evaluated in the tibialis anterioris of mice groups. Results represent mean ± SEM of three independent experiments with at least 5 mice per experiment. #P<0.05, ##P<0.001 vs NX alone and ºP<0.05, ººP<0.001 vs NX (IL-4).

DISCUSSION

V. DISCUSSION

Monocytes, have been described by Geissmann F. and coworkers in 2003 to be divided in two main subsets: a short-lived "inflammatory subset" also known as Ly-6C^{high} monocytes in mice (CD14^{high}CD16^{-} in human) (Passlick B., et al. 1989), that homes to inflamed tissue, where it can trigger immune responses to acute injury or infection (Drevets DA., et al. 2004; Nahrendorf M., et al. 2007; Serbina NV., et al. 2006; Tsou CL., et al. 2007). And a "resident subset", Ly-6C^{low} cells in mice (CD14^{low}CD16^{+} in human), with a longer half-life, that homes to non inflamed tissues where they will differentiate in resident macrophages to regulate the tissue integrity and homeostasis (Gordon S. and Taylor PR. 2005; Auffray C, et al. 2007). These cells are not (or to a lesser extent) recruited during inflammation.

Monocytes become a very important part for the acute needs of the immune system once they are responsible to repopulate the tissue macrophages and are also responsible to phagocyte invading agents in the blood flow and to produce some cellular mediators (Auffray C., et al. 2007; Liu K., et al. 2007). In the tissue, they can differentiate to pro-inflammatory, participainting in pathogens elimination (M1) or anti-inflammatory repairing the tissue (M2). Several studies indicate that Ly-6C^{high} monocytes are selectively recruited to the site of inflammation and infection in many models of inflammatory diseases such as inflammatory bowel disease (Zigmond E, et al. 2012), colitis (Waddell A., et al. 2011), artherosclerosis (Tacke F., et al. 2007; Swirski FK., et al. 2007; Swirski FK., et al. 2009; Woollard KJ., et al. 2010), rheumatoid arthritis (Bruhl H., et al. 2007), central nervous system (CNS) infections such as experimental autoimmune encephalomyelitis (EAE) (King I., et al. 2009; Milder A., et al. 2009) and other, in which differentiate into macrophages with distinct phenotypes according to the

environmental state. So, due to their heterogenity monocyte could be potentially candidates for cell therapy of various diseases, especially in the innate immunity where macrophages plays a key role in the maintenance of tissue homeostasis in addition to phagocytosis and antigen presentation for other cells from the immune system, such as T lymphocytes (Gordon S. and Taylor PR. 2005).

In fact, in the last few years, due to the special features of the Ly-6C^{high} monocytes in the inflammatory response, these cells have acquired great importance among various groups of investigation. However, due to the low levels of the Ly-6C^{high} cells in the mice blood and the difficulty for their acquisition, currently, all studies performed with monocytes are done in transgenic models (i.e. CCR2$^{-/-}$; GPF-CX3CR1 models) (Serbina NV., et al. 2006; Holt MP., et al. 2008; Mildner A., et al. 2009; Donnelly DJ., et al. 2011), or with expensive techniques, such as clodronate to study and acquire the maximum number of Ly-6C^{high} cell possible from mice. Up until now, clodronate is one of the most used protocols to study monocytes in murine and human models (Rossi L., et al. 2005; Getts DR., et al. 2008; Kreisel D., et al. 2010), this pharmacological treatment has been demonstrated to induce specific apoptotic death in monocytes and phagocytic macrophages (Summan M., et al. 2006). The clodronate-loaded liposomes eliminate monocytes and macrophages in peripheral blood and spleen, although after a certain time, their reappearance in the circulation consists only of Ly-6C^{high} monocytes, which are acquired through a blood extraction followed by positive selective sorting (Sunderkötter C., et al. 2004, Tacke F., et al. 2006). Although all the techniques mentioned above, for the acquisition of large numbers of Ly-6C^{high} monocyte require a great number of animals due to the low obtention of these cells from each mice, increasing the values and delaying experiments results, which impair the study of these cells. So, due to the difficulties and the high costs of the current protocols for the acquisition of high amounts of Ly-6C^{high} monocytes through the same ones, both fate and function of these populations remain unclear, in mouse. Although, up until know any protocol for the generation of Ly-6C^{high}

monocytes *in vitro* has been reported. Therefore one of the main objectives of this Doctoral Thesis was to establish a novel model to generate large amounts of Ly-6C^{high} monocytes from bone marrow of mice *in vitro,* procedure that can replace the current expensive techniques.

Results obtained in this thesis demonstrate a new method of generating Ly-6C^{high} monocytes from bone marrow of mice, with a cocktail of growth factors created by us. However, since this cocktail is currently under patent we were not able to display all experiments performed for the optimization of this protocol. The novel protocol enable an improvement in the efficiency of Ly-6C^{high} monocyte production in almost 100 times more, when compared with the current obtention from these cells from the blood flow from mice, which is around 2-3x10^5 cells/ml per mice. Our model, in contrast to the current protocols used for the acquisition of Ly-6C^{high}, cells needs a reduced number of animals for its accomplishment, which makes it a less expensive mechanism capable to generate large quantities of Ly-6C^{high} cells. Thus making this protocol a valuable and necessary tool for the study of these monocytes. Our experimental model for the generation and enrichment of Ly-6C^{high} monocytes was able to produce a heterogeneous cell population composed by the presence of 42-48% of cells Ly-6C^{high}, 29% of Ly-6C^{int} and 26% of Ly-6C^{low} cells, with a CD11b+CD62L+CCR2+F4/80+, and CD11c^{low}CX3CR1^{low} predominant phenotype, which also enabled us to use the other populations in our studies. This heterogeneous population was also described by Geissmann F and co-workers in the blood flow of mice and by several studies in humans (Sulicka J, et al. 2012; Lee HW, et al. 2012). So, in order to work only with the Ly-6C^{high} monocytes, we sorted the cells that have the highest levels of the Ly-6C marker. The population obtained by us through the sorting technique presented a CD11b$^+$ (98%) CD62L$^+$ (69%) CCR2$^+$ (70%) F4/80$^+$ (79%) $CX_3CR_1^{low}$ (5%) phenotype, same phenotype described from cells obtained from the mice peripheral blood circulation (Geissmann F., et al. 2003; Sunderkötter ,C. et al. 2004; Mack M., et al. 2001; Biburger M., et al, 2011), which means that these cells may have the same

functionality from the Ly-6C^{high} cells of murine models. So, the acquisition of these Ly-6C^{high} monocytes with a 98% of purity allows the study of this cell population in an easy manner in further experiments, solving in this way the problems related to the obtention of these cells with other techniques.

It is known that Ly-6C^{high} monocytes from peripheral blood form mice display a substantial heterogeneous phenotype, which reflects the specialization of individual macrophage population within their microenvironment (Varol C., et al. 2007; Yona S, et al. 2010). Our group decided to study the ability to switch phenotype of these cells *in vitro*, which will allow us to further investigate their role in different animal models of inflammation. For this purpose these cells were activated *in vitro* under pro- and anti-inflammatory conditions with IFN-γ or IL-4 stimuli, cytokines known to be responsible for the activation of macrophages in a classical (M1) or alternative (M2) manner, respectively (Gordon S. and Taylor PR. 2005; Gordon S. 2003).

Different activation states have been described *in vitro* for monocyte/macrophages, each being associated with a particular phenotype and function (Gordon S. and Taylor PR. 2005; Gordon S. 2003). Classical activation, play an essential role in the protection against intracellular pathogens and inflammation, eliminating infected cells and cellular debris to eradicate inflammation (Gordon S., 2003). However, the persistence of inflammatory processes has been described to results in tissue damage, which induces the activation of an anti-inflammatory mechanism to repair tissue injury (Gordon S., 2003). This alternative activation is responsible to protect organs and surrounding tissues against immune responses promoting in this sense tissue remodeling (Comalada M., et al. 2012). It is not known up until know in what point these macrophage phenotypes switch, pro- to an anti-inflammatory *in vivo* and whether macrophages destination is determined forever or whether it remains constantly malleable (Gordon S. and Taylor PR. 2005), although several *in vitro* studies have shown macrophage adaptation to a

changing microenvironment (Noel W., et al. 2004; Stout R.D., et al. 2004; Porcheray F., et al. 2005; Stout R.D., et al. 2005). To answer the question if monocyte/macropahges are able to switch phenotype from pro- to an anti-inflammatory *in vivo*. Thus we first analyzed the ability of the Ly-6C^{high} generated *in vitro* to adapt to a changing microenvironment *in vitro*, to further investigate their function in vivo.

Results obtained in our work demonstrated that enriched Ly-6C^{high} monocytes generated *in vitro* present the ability to switch phenotype according to the environmental conditions. In our work we could appreciate the ability of these cells to express pro-inflammatory markers such as *nos2* and *tnf*α when stimulated with IFN-γ or to express anti-inflammatory markers such as mannose receptor and *arginase1* when stimulated with IL-4. Thus, our Ly-6C^{high} monocytes generated *in vitro* act in the same way as macrophages and monocytes from the peripheral blood of mice, adapting to the environmental changes as previously described (Gordon S. and Taylor PR. 2005; Biswas SK. and Mantovani A. 2010; Rivollier A., et al. 2012).

The presence of CD11b^{high}, CD11c^{low}, F4/80^{high}, CCR2^{high}, CD62L^{high} and CX3CR1^{low} markers in the Ly-6C^{high} cells generated *in vitro,* provides a tool to investigate the migratory capacity of these cells to the inflamed loci in different *in vivo* models since some of this markers are responsible for these cells migration (Geissmann, F. 2003, Sunderkötter, C. et al. 2004, Mack, M. et al. 2001). In our days much is known about leukocyte migration, in which a coordinated activity of adhesion and chemotactic receptors, signaling molecules, and cytoskeleton, process cell movement (homing) from the blood to the tissues through the blood vessels favoring leukocyte activity in inflammation and accumulation of extracellular matrix (Ley K. 2003; Vicente-Manzanares., et al. 2004; Bandinelli F., et al. 2012). However, the chemotaxis capacity of the Ly-6C^{high} monocytes began to be studied over the past years when the different subtypes of these cells were described,

where several studies demonstrate the role of CCR2 and other chemokine receptor such as CCR7, CCR5 and others, in the Ly-6C^{high} monocytes traffic from the blood stream to the sites of inflammation (Geissmann F., et al. 2003, Auffray C., 2007; Drevets DA., et al. 2008). As we mention before enriched Ly-6C^{high} cells generated *in vitro* present the same chemokines receptor as the ones obtained from the mice blood flow suggesting that these cells present the capacity to migrate to the inflammatory loci like the others cells from the blood. Although in an *in vivo* level, the ability of migration (homing) or the damaged tissue functionality of the Ly-6C^{high} monocytes generated *in vitro* has not been described. For this reason, an important aim of this Doctoral Thesis consisted to confirm *in vivo* the migratory capacity of these enriched Ly-6C^{high} monocytes generated *in vitro* into *in vivo* inflammation models and to study the action of these cells in the site of inflammation. For this propose we used two *in vivo* experimental models. The animal models selected by our group were the DNFB (1-fluoro-2,4-dinitrobenzene) irritant contact dermatitis model and the Notexin muscle model.

The DNFB irritant contact dermatitis model induces ear inflammation and is wildly used to investigate the functions of the immune cells in the skin (Bonneville M., et al. 2007; Nosbaum A., et al. 2009). In the currently studies, haptens endowed with proinflammatory properties induce severe skin inflammation. This model is well known to induce immune cell from the acquired immunity into the inflamed loci where macrophages present antigens to effector T lymphocytes (Ahmed AR., et al. 1983; Sakai S., et al. 2010). In the *in vivo* model optimized by our group a single application of a high concentration of a hapten (DNFB) was applied in mice ear, inducing an irritant contact dermatitis that causes direct damage to skin without prior sensitization, leading to the production of several cytokines and signaling molecules by the skin cells (i.e. keratinocytes), responsible for the vasodilatation, increased vascular permeability and cell activation, which enable the recruitment of leukocytes to the inflammatory loci (Bonneville M., et al. 2004; Slodownik D., et al. 2008; Nosbaum A., et al. 2009). In addition to the ear irritation in our experimental

model, enriched Ly-6C^{high} injection was carried out. The DNFB model optimized by us differs from the previous models since we injected Ly-6C^{high} cells generated in vitro and use a single application of a higher concentration of the irritant, with no previous sensitization. In this model the single application of the DNFB as previously mentioned, was able to induce adhesion and chemotaxis conditions to the injected Ly-6C^{high} cells to migrate to the inflammatory focus, thus being an ideal model to study the acute and chronic inflammation in the skin, in which macrophage are involved in the proliferation of local cells, reepithelialization, neovascularization and remodelling of the inflamed tissue (Mahdavian Delavary B., et al. 2011).

In addition to the DNFB model, to further investigate the migratory capacity of the Ly-6C^{high} monocytes and to demonstrate the high versatile phenotype of these cells, depending on the inflammatory microenvironment, a skeletal muscle injury model (Notexin muscle model) was used, in which tibialis anterioris of mice were injected with myotoxic venom (Notexin), inducing myofiber necrosis. Muscle injury lead to Ly-6C^{high} monocyte recruitment from the peripheral blood to the site of inflammation, were these cells differentiate into macrophages, cells widely described to be essential for the muscle repair due to their ability to promote muscle growth through the myogenesis and fiber growth stimulation (Ludovic A., et al. 2007; Brunelli S. and Rovere-Querini P. 2008). Thus, the Notexin muscle model is highly dependent of the Ly-6C^{high} monocytes efflux from the blood into the tissue (Benoit PW., et al .1970; Gutiea`rrez JM., et al 2003; Harris JB., et al 2000; Harris JB., et al. 2003; Rosenblatt JD., 1992; Ludovic A., et al. 2007). The study of the Ly-6C^{high} monocyte in the Notexin muscle model by us was conducted to investigate the homing capacity and functionality of the enriched Ly-6C^{high} cells generated *in vitro* injected in the muscle inflammatory context.

Results obtained in this thesis reveal that the injection of enriched Ly-6C^{high} monocytes (stained with DiR as a fluorescent cell tracker) generated *in vitro*, were

capable to migrate into the inflamed tissues in both animal models. Cells injections were performed at the same time as the induction of the inflammatory process. Although a difference in the homing profile between these models can be appreciated at the moment that these cells reach the inflammatory site, suggesting that the intensity of the inflammation and the complexity of the tissue influence in its emigration into the tissue. In addition to the study of the phenotype switch of these cells *in vitro* and the study of the migratory capacity *in vivo,* we also decided to demonstrate their function in the inflamed tissue *in vivo* in the DNFB and Notexin model.

In orther to investigate the impact of *in vitro* generated and injected Ly-6C^{high} monocyte in the DNFB and in the Notexin model, tissue damage and gene expression was analyzed. In response to the injection of these cells in the DNFB model of inflammation, the results showed clearly, a faster improvement in the ear repair in the latter face of the inflammatory process, which corroborate with the results presented by the gene expression where an pro-inflammatory followed by an anti-inflammatory profile was appreciated in the DNFB model. Although the Notexin model suggest an improvement in muscle repair what was not clear when the gene expression level was evaluated, suggesting that the muscle injury present a more complex mechanism against the inflammatory response, when compared with the inflammation induced by DNFB. Another difference between both models is clearly appreciated in the kinetics of gene expression in which the Notexin have an earlier response when compared with the DNFB model, which in other hand seems to repair the tissue damage in a shorter period of time. All these results indicate that the administration of enriched Ly-6C^{high} monocytes could be beneficial to restore the inflamed tissue in our study, unlike the others, enriched Ly-6C^{high} cells generated *in vitro* are injected into immunocompetent animals, in which the integrity of the cells presented in the blood is maintained, making in this sense, our mechanism most reliable for the study of the resolution of inflammation. These results together with the ability of these cells to switch phenotype once activated *in*

vitro suggest that the adoptive transfer of pre-activated Ly-6C^{high} monocytes generated *in vitro* can be a potential therapeutically tool to decrease an inflammatory process.

Taking into account all the previously results, together with the fact that previously studies done in our laboratory indicate that bone-marrow-derived macrophages incubated with IL-4 block proliferation (Arpa L, et al. 2009), that Stat6-deficient cells are unable to differentiate into Th2 cells and have a impaired proliferative responses (Kaplan MH., *et al*., 1996; Shimoda *et al*., 1996; Takeda *et al*., 1996) and that IL-4 and its signaling pathway (STAT6) are involved in the differentiation of class switch in macrophages (Hou J. et al. 1994), we decided to study if bone marrow cells under IL-4 stimuli and bone marrow from wild type and knockout for STAT6 animals, were able to generate large amounts of Ly-6C^{high} monocytes *in vitro*. Results obtained in this thesis reveal the involvement of the IL-4/STAT6 pathway in the differentiation of Ly-6C^{high} monocytes, where bone marrow cells in the presence of IL-4 or with deficiency of STAT6 pathway were not able to differentiate into large numbers of Ly-6C^{high} cells, suggesting to us that hematopoietic stem cells are not able to differentiate in to high amounts of monocytes under anti-inflammatory environmental conditions, however, depends on the presence of the STAT6 transcription factors to differentiate. So, in addition to the study of the differentiation of these cells *in vitro* we also have demonstrated their capacity to migrate as well as their function in an inflammation model *in vivo*.

To further investigate the involvement of IL-4 in the inflammation process *in vivo*, the next objective of this Doctoral thesis was to use the enriched Ly-6C^{high} monocytes generated *in vitro* as a tool to control and restore inflamed tissue. Previously studies described that adoptive transfer of macrophages modified or stimulated ex vivo into patients with diabetes, tumours, atherosclerosis and other infection and inflammatory diseases, were effective in improving inflammatory processes (Burke B. 2003; Parsa R., et al. 2012). Although the results of the

studies performed with injections of pre-treated macrophages can not be entirely reliable due to their stationary or limited potential to migrate from the blood into the inflamed tissue (Auffray C., et al. 2007), role played by the Ly-6C^{high} monocyte (Woollard KJ. and Geissmann F. 2010; Gautier EL., et al. 2009; Zhang D., et al. 2012; Kim YG., et al. 2011). So, to accomplish an effective therapy able to improve inflammation the use of Ly-6C^{high} monocytes is essential, due to their capacity to migrate from the blood to the inflamed site. In this sense, to accomplish this aim Ly-6C^{high} cells generated *in vitro* were stimulated *in vitro* with IL-4 (see section 4.4 in material and methods) before injection and used into both animal models followed by induction of inflammation either by DNFB or Notexin The stimulation with IL-4 was performed to establish a previous anti-inflammatory profile in these cells. So, these cells could respond quickly repairing the tissue against an inflammatory process, which makes them a tool to avoid inflammation in an easier manner.

In the results obtained by the injection of IL-4-pretreated enriched Ly-6C^{high} monocytes generated *in vitro* into both mice model, a significant improvement in the inflamed tissue was appreciated. In this sense, a reduction in inflammatory infiltrate was observed in IL-4 per-treated mice were at the histological level the regeneration of the tissue integrity was observed in the last stages of inflammation and an increase in the gene expression of marker such as arginase 1 and mannose receptor was also appreciated, indicating that these cells have an anti-inflammatory profile. These results have given us a new reliable mechanism, unlike the previously mentioned one, to treat illnesses related to the inflammatory process or autoimmune disease, dependents on an anti-inflammatory response to be solved. Although, further investigation is need to be done in other animal models of inflammation, before the use of these cells as a therapeutic tool in autoimmune disease and in humans.

As mentioned before STAT6 gene is involved in IL-4 signalling pathway, which is responsible for the switch of pro- to anti-inflammatory macrophages

(Hebenstreit D., et al. 2006; Jerrold M O, et al. 2010). So, to understand the role played by this gene (STAT6) facing the inflammatory process, in vitro generated Ly-6C^{high} monocytes from wild type and knockout animal where used in the DNFB model. Results from experiments with Ly-6C^{high} cells from STAT6 wild type mice presented the same kinetic as cells from normal Balb/c mice, an expected result since the wild type and knockout STAT6 mice have a Balb/C background. However, Ly-6C^{high} cells derived from STAT6 knockout mice where not able to restore the ear tissue integrity, which suggest that the STAT6 pathway is essential for the activation of an anti-inflammatory response to decrease the inflammatory response. These results, suggest also that STAT6 knockout cells remain in a pro-inflammatory stage due to a lack of an active mechanism to perform the phenotype switch to an anti-inflammatory state, indicating that these cells present a constant pro-inflammatory profile. The inability from these cells to change their pro- to a anti-inflammatory phenotypes can be of great assistance, as mentioned before, in the study of disease such as cancer since anti-inflammatory macrophages are responsible to suppress tumor immunity and promote tumor survival and pro-inflammatory macrophages are well known to be involved in the resolution of inflammation (Biswas SK., et al. 2010; Lee HW., et al. 2012). Knowing that STAT6 knockout Ly-6C^{high} cells are able to migrate to the inflammatory loci and that these cells are unable to switch the phenotype in the STAT6 knockout animals suggest that these cells could be used as a therapeutic approach in cancer to suppress tumor growth (Kaplan MH., *et al*., 1996; Shimoda *et al*., 1996; Takeda *et al*., 1996). Moreover, we show that Ly-6C^{high} monocytes from STAT6 knockout mice were able to migrate to the site of inflammation where they were able to delay ear repair, suggesting to us that these pathway are important in the activation of the switch from the pro-inflammatory in to an anti-inflammatory phenotype in macrophage.

According to the knowledgement previously described in this work, enriched Ly-6C^{high} monocytes *in vivo* present a predominant anti-inflammatory profile when it reaches the site of inflammation in a DNFB model, although in the

Notexin model that is not clear. Thus, the knowledge that invading macrophages reach their highest concentration after 24 hours following the onset of some form of muscle cell injury or reloading (St Pierre BA, et al. 1994; Toumi H., et al. 2006), the next aim of this work was to analyze if the administration of the enriched Ly-6C^{high} cells generated *in vitro* 24 hours after the myoinjury modify the panorama of the inflammatory response, accelerating the de-activation of inflammation and thus increasing in time and effectiveness the tissue repair. The results achieved at this point suggest to us that a more pronounced anti-inflammatory alternative pathway was activated, which could contribute to the faster restoration or repairing of the muscle, thus suggesting that Ly-6C^{high} monocytes are able to adapt to different environment in order to solve inflammation. These results obtained by us are in accordance with previously described claims that monocytes/macrophages promote fiber regeneration in a later stage of the muscle injury when these cells present an anti-inflammatory profile, which promote the release of mitogenic growth factors and chemokines (Mantovani A., et al. 2005; Mantovani A., et al. 2007; Brunelli S, et al. 2008).

Therefore, due to the enriched Ly-6C^{high} monocytes in a myoinjury environment have an anti-inflammatory profile and that these cells can be stimulated previous injection, according to ours needs, the next objective of this Doctoral thesis consisted in investigate the role of enriched Ly-6C^{high} monocytes the myoinjury. So to accomplish this aim these cells were stimulated *in vitro* with IFN-γ a pro-inflammatory mediator or with IL-4 a cytokine that induce anti-inflammatory cell activation (see section 4.5 or 4.4 in material and methods) before injection into the Notexin animal model. The reason to stimulate these cells with IFN-γ or IL-4 was to establish whether these cells act in the same way as the DNFB model, exacerbating or repairing tissue damage, even knowing that the kinetics of damage and tissue repair works in a different way. The results obtained by our group have shown a new mechanism to increase or control the inflammatory response from illnesses that are related to the muscle inflammation.

Thus, our results have also shown that pre-treated Ly-6C^{high} monocytes with IL-4 were able to increase muscle or ear repair, in two experimental models, suggesting to us that these cells present a therapeutic potential facing the inflammatory process. Furthermore, we have shown also that pre-treated Ly-6C^{high} monocytes with IFN-γ were able to delay muscle repair, suggesting to us that these cells can respond distinctively due to the treatment or due to the environment in which they are found, suggesting that these cells can be a good therapeutic tools for other types of diseases, such as cancer, in which the presence of pro-inflammatory macrophages is beneficial to control tumor growth (Biswas SK. and Mantovani A. 2010; Movahedi K., et al. 2010).

In summary, the results obtained in this Doctoral Thesis reveal a new method to in vitro generate Ly-6C^{high} monocytes from bone marrow of mice, with an improvement in the efficiency of cell production, which facilitate the study of these cells *in vitro* and *in vivo*. In addition we also have demonstrated the capacity of the Ly-6C^{high} cells to change phenotype *in vitro* stimulation and the capacity to migrate as well as the functional heterogeneity in two models of inflammation *in vivo*, indicating that these cells actuate in the same way as the cells from the peripheral blood. Furthermore, we have shown that Ly-6C^{high} monocytes can be pre-treated with cytokines in order to delay or increase tissue repair (IFN-γ or IL-4), respectively. All these results together suggest that the Ly-6C^{high} monocytes generated by us *in vitro* are functional cells that can be used as a therapeutic tool to treat inflammatory diseases.

CONCLUSIONS

VI. CONCLUSIONS

1. We established a novel *in vitro* protocol to generate Ly-6C^{high}CD11b^{+} monocyte obtained from bone marrow of Balb/C mice.

2. The cells generated *in vitro* have the same phenotype of the Ly-6C from blood flow.

3. Cells Ly-6C^{high}CD11b^{+} monocyte present high plasticyty.

4. Ly-6C^{high}CD11b^{+} monocytes generated *in vitro* migrate *in vivo.*

5. Injection in acute and chronic *in vivo* inflammatory models of Ly-6C^{high}CD11b^{+} monocytes generated *in vitro,* display an improvement in the site of inflammation through the presentation of a more anti-inflammatory profile.

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VII. REFERENCES

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ANNEX

INTRODUCCIÓN

Inflamación es la primera respuesta del sistema inmune contra cualquier tipo de infección. Los signos clínicos de un proceso inflamatorio son los siguientes: enrojecimiento, hinchazón, calor y dolor, todos estos síntomas son causados por el incremento del flujo sanguíneo en el tejido afectado. El proceso inflamatorio se lleva a cabo por las moléculas de señalización (eicosanoides) y citocinas, que son liberadas por las células dañadas o infectadas. Las citoquinas y quimioquinas son responsables de la comunicación entre células blancas de la sangre, la quimiotaxis y tienen efectos anti-virales, tales como el cierre de la síntesis de proteínas en la célula huésped. Los factores de crecimiento y factores citotóxicos también pueden ser liberados durante el proceso inflamatorio. Estas citoquinas y otros productos químicos reclutan células del sistema inmune al sitio de la infección para promover el reparo del tejido dañado después de la eliminación de los patógenos.

Para funcionar correctamente, el sistema inmune debe detectar una amplia variedad de agentes, desde virus hasta parásitos y distinguirlos del tejido sano del mismo organismo. El sistema inmune innato es la primera línea de defensa del organismo que reconoce patógenos, iniciando así una respuesta inmune la cual activa la respuesta adaptativa del sistema inmune. Una interacción entre los dos sistemas es necesaria para la erradicación de las enfermedades. Sin embargo, el sistema inmune tiene que estar bien regulado pues un trastorno en una respuesta inmune puede resultar en la destrucción de los tejidos, en enfermedades autoinmunes, en enfermedades inflamatorias y en el cáncer.

En el contexto de la inmunidad innata, en los últimos años, el sistema mononuclear fagocítico, que se define como una familia de células que comprende células progenitoras de la médula ósea, en los cuales los monocitos sanguíneos y

macrófagos tisulares está adquiriendo una gran importancia en el estudio de diferentes patologías y particularmente en las funciones de los monocitos/macrófagos. A este respecto, estudios recientes demuestran que los monocitos presentan una población heterogénea de células innatas. Estos hallazgos permiten aumentar el interés de comprender esta población de células. En los últimos años, la investigación sobre los diferentes subtipos de monocitos ha llevado a cabo en sistemas experimentales, tales como los procesos inflamatorios y enfermedades autoinmunes.

Además, una plasticidad enorme en monocitos se encontró que conduce a las poblaciones de células diferentes con diversos subtipos con distintas funciones. Es importante destacar la condición de que determinado el fenotipo de los monocitos es microambiente del tejido y la condición de acogida, estando en un estado estacionario o durante un proceso inflamatorio. Dos tipos de monocitos de la sangre se identificaron recientemente en ratones. Monocitos "residentes", que presentan un fenotipo Ly-6C^{low}CD11b+CCR2^{low}CX3CR1^{high}, son capaces de migrar a los tejidos no lesionados rápidamente después de la emigración de la médula ósea, donde se diferencian en macrófagos residentes y células dendríticas. En cambio, un subconjunto distinto de monocitos fue descrito, los monocitos "inflamatorios", estas células presentan un fenotipo Ly-6C^{high}CD11b+CCR2^{high}CX3CR1^{low}, migran para el tejido infectado donde contribuyen en el desarrollo de la inflamación.

Monocitos es una parte muy importante del sistema inmune una vez que son responsables para la repoblación de los macrófagos tisulares, por fagocitar agentes invasores y por producir algunos mediadores celulares. En el tejido, estas células pueden diferenciarse en pro-inflamatoria, participando así en la eliminación de patógenos (M1) o anti-inflamatorios, siendo responsable por reparar el tejido (M2). Varios estudios indican que monocitos Ly-6C^{high} son selectivamente reclutados al sitio de la inflamación y infección en muchos modelos de

enfermedades inflamatorias tales como enfermedad inflamatoria del intestino, la colitis, arterosclerosis, artritis reumatoide, sistema nervioso central (SNC) tales como la encefalomielitis autoinmune experimental (EAE) y otros, en que se diferencian en macrófagos con fenotipos distintos según el estado del medio ambiente. Así, debido a su gran heterogeneidad monocitos poden ser considerados candidatos ideales para la terapia celular de diversas enfermedades, especialmente en la inmunidad innata donde los macrófagos desempeñan un papel clave en el mantenimiento de la homeostasis del tejido, además de la fagocitosis y la presentación de antígenos para otras células del sistema inmune, tales como los linfocitos T.

Algunas vías de señalización, también se pueden clasificar en M1/M2, un buen ejemplo de estas vías son transductor de señales y activador de la transcripción (STAT) 1 y STAT6. STAT1 es descrita como una vía de señalización de IFN que conduce a un fenotipo de macrófagos M1 y STAT6 es una vía de señalización inducida por IL-4 e IL-13 y es reconocida por inducir el fenotipo de macrófagos M2. En este estudio algunos de estos marcadores se utilizaron para caracterizar el fenotipo M1 y M2.

Actualmente, todos los estudios realizados con monocitos son hechos en modelos transgénicos (es decir, modelos CCR2-/-; GPF-CX3CR1) o con técnicas costosas como la del clodronato, para estudiar y adquirir un número máximo de células Ly-6C^{high} de la sangre periférica de ratones. Los monocitos constituyen entre 2-8% (450 monocitos/l) de los leucocitos presentes en la sangre humana y alrededor de 2% (100cells/µl) de la reserva de sangre periférica total de leucocitos en ratones, donde sólo 1-5% son monocitos Ly-6C^{high}. Lo que hace difícil para estudiarlo una vez que las técnicas utilizadas son ineficaces y costosas, lo que torna el estudio de estas células.

Sin embargo, los monocitos son una parte crucial de la defensa del huésped durante la infección y la cicatrización de heridas, ya que pueden ser perjudiciales, provocando o exacerbando enfermedades tales como osteomielitis, aterosclerosis, esclerosis múltiple y otras enfermedades inflamatorias. Por lo tanto, es importante tener un conocimiento detallado de las funciones de los monocitos en procesos inflamatorios, para que sea posible desarrollar fármacos y vacunas contra infecciones bacterianas y enfermedades de encontrar dianas terapéuticas.

OBJETIVOS

Dado el bajo número de monocitos Ly-6C^{high} existente en la sangre periférica de ratones y la baja cantidad obtenida de estas células mediante diversas técnicas (por ejemplo clodronate), el objetivo general de esta tesis consiste en la generación de un nuevo protocolo para generar altos niveles de monocitos Ly-6C^{high} in vitro e investigar su papel en un ámbito inflamatorio in vitro e in vivo. Para esto, nuestros estudios se han centrado en lo siguiente:

1. Desarrollo de un modelo in vitro que permita generar grandes cantidades de monocitos Ly-6C^{high} a partir de médula ósea de ratones.

2. Caracterización del fenotipo de los monocitos Ly-6C^{high} generados in vitro.

3. Analizar funciones de los monocitos Ly-6C^{high} generados in vitro tras su activación in vitro.

4. Estudio de la capacidad migratoria de los monocitos Ly-6C^{high} generados in vitro en dos modelos de inflamación.

- Piel (modelo de DNFB en oreja).
- Músculo (modelo Notexin muscular).

5. Analizar el efecto terapéutico de la inyección de monocitos Ly-6C^{high} generados in vitro en la resolución de la inflamación en dos modelos experimentales de inflamación.

METODOLOGÍA Y RESULTADOS

Para lograr el primer objetivo de nuestro trabajo, las células de la médula ósea fueran cultivadas con diferentes factores crecimiento, como M-CSF, coctel de factores de crecimiento o M-CSF + coctel de factores de crecimiento, en concentraciones distintas. Los resultados de estos estudios demostraran que M-CSF o coctel de factores de crecimiento solos no era capaz de producir grandes cantidades de células Ly-6C^{high}, pero que la combinación de ambos factores era capaz de inducir la diferenciación celular generando grandes cantidades de monocitos Ly-6C^{high}. Por lo tanto, con el fin de producir grandes cantidades de estas células, la médula ósea de un ratón Balb/C fue incubada con M-CSF + coctel de factores de crecimiento y FCS a 37 ºC en una atmósfera humidificada con 5% CO_2. Después de 7 días de cultivo, la población de células flotantes fue adquirida y teñida con Ly-6C y el marcador CD11b. Esta población se clasifica para la adquisición de los enriquecidos Ly-6C^{high}CD11b+ células.

Con el fin de poder caracterizar el fenotipo de los monocitos Ly-6C^{high} enriquecidos generados in vitro, diferenciados por MCSF/coctel de factores de crecimiento, se tiñeron con varios marcadores celulares. Nuestros resultados demuestran que esta población Ly-6C^{high} enriquecido es CD11b+CD62L+CCR2+F4/80+CX3CR1low, presentando el mismo fenotipo de las células presenta en la circulación.

Para estudiar la funcionalidad de los monocitos Ly-6C^{high} enriquecidos generados *in vitro* y analizar se estas células presentan la capacidad de cambiar su fenotipo como lo que ya se fue descrito en los macrófagos, hemos realizado experimentos en que los monocitos Ly-6Chigh generados in vitro se incubaron con IFN-γ como un estímulo de la vía clásica y IL-4 como un estímulo de la vía alternativa. Entonces, la expresión del RNA de TNF y NOS2 fue utilizados como marcadores clásicos y como marcador alternativos arginasa-1 y receptor de mannosa fueron observados. Células Ly-6C^{high} incubadas con IFN-γ fueran capaces de inducir la expresión de TNF y NOS2 con una cinética similar a los macrófagos. Sin embargo, estas células Ly-6C^{high} aumentarán los niveles de expression de arginasa-1 y de receptores de mannosa cuando fueron estimuladas con IL-4. Así, los resultados in vitro han demostrado la plasticidad y la heterogeneidad de los monocitos como se describió previamente para los macrófagos y por lo tanto, nos sugieren que estas células generadas in vitro también puede adaptarse a un cambio en el microambiente (Gordon S. 2005).

Además, para observar la capacidad de migración y la funcionalidad de estas células in vivo, hemos optimizado dos modelo experimentáis de inflamación aguda y crónica. En el primer modelo, la irritación de la piel de la oreja del ratón fue inducida con 1% de DNFB en una mezcla con acetona. En el modelo de inflamación muscular fue desarrollado por la inyección de Notexin en la anterioris tibialis. En ambos modelos la inflamación fue inducida y células Ly-6C^{high} enriquecidas generadas in vitro teñidas con un fluorocrome infrarrojos fueron inyectadas por vía intravenosa en ratones. En ambos modelos, la migración se observó por imagen in vivo durante diferentes días. La capacidad migratoria de estas células Ly-6Chigh para los tejidos inflamados fue apreciada en ambos modelos.

Para analizar el efecto terapéutico de la inyección de los monocitos Ly-6C^{high}CD11b+ generados in vitro pre-tratados o no ex vivo con citocinas pro- o anti-inflamatorias en la resolución de la inflamación, RNA y cortes histológicos fueron obtenidos de ambos modelos animales en distintos días del proceso inflamatorio. Los resultados mostraron que ratones inyectados con monocitos Ly-6C^{high} generados in vitro expresaran mayores niveles de genes anti-inflamatorios en ambos los modelos inflamatorios, lo que corrobora con las imágenes histológicas donde los animales tratados con células Ly-6C^{high} recuperarse antes del proceso inflamatorio que los animales no tratados.

Sin embargo células pré-tratadas con citocinas anti-inflamatoria presentan un incremento en su capacidad de reparar el daño tissular, lo que no ocurre cuando estas células son pré-incubadas con citocinas pro-inflamatorias. Células incubadas con IFN-γ o provenientes de animales STAT6 (con fenotipo M1) una vez inyectadas en modelos inflamatorios in vivo exacerban la inflamación.

DISCUSIÓN

Los resultados obtenidos en esta tesis demuestran un nuevo método para generar monocitos Ly-6Chigh a partir de médula ósea de ratones, con un cóctel de factores de crecimiento creadas por nosotros. Este nuevo protocolo permitir una mejora en la eficiencia de la producción de monocitos Ly-6Chigh en casi 100 veces más, en comparación con la obtención actual de estas células desde el flujo de sangre de los ratones, que es alrededor de 2-3x105 células/ml por ratón. Nuestro modelo, en contraste con los actuales protocolos utilizados para la adquisición de células Ly-6Chigh, necesita un número reducido de animales para su realización, lo que lo hace un mecanismo menos costoso capaz de generar grandes cantidades de células Ly-6Chigh. Tornando así, este protocolo una herramienta valiosa y necesaria para el estudio de estos monocitos. Nuestro modelo

experimental para la generación y enriquecimiento de monocitos Ly-6Chigh fue capaz de producir una población celular heterogénea compuesta por la presencia de células Ly-6Chigh, Ly-6Cint y Ly-6Clow. Así, con el fin de trabajar sólo con los monocitos Ly-6Chigh, este cultivo fue sorteado y apenas los más altos niveles del marcador Ly-6C fue seleccionado. La población obtenida por nosotros a través de la técnica de clasificación presenta un fenotipo CD11b+CD62L+CCR2+F4/80+CX3CR1low, mismo fenotipo descrito a partir de células obtenidas de la sangre periférica los ratones (Geissmann F., et al 2003;. Sunderkötter, C. et al 2004;. Mack M., et al 2001;. Biburger M., et al, 2011), lo que significa que estas células pueden tener la misma funcionalidad de las células Ly-6Chigh de modelos murinos. Por lo tanto, la adquisición de estos monocitos Ly-6Chigh generados in vitro permite el estudio de esta población celular de una manera fácil en otros experimentos, la solucionando de este modo los problemas relacionados con la obtención de estas células con otras técnicas como la técnica del clodronato.

En nuestro trabajo también fuimos capaces de demostrar que monocitos enriquecidos Ly-6Chigh generados in vitro a presentan la capacidad de cambiar de fenotipo de acuerdo con las condiciones ambientales. Expresando marcadores pro-inflamatorias tales como NOS2 y TNF α cuando son estimuladas con IFN-γ o expresando marcadores anti-inflamatorios tales como el receptor de mannosa y arginase1 cuando son estimuladas con IL-4. Por lo tanto, nuestros monocitos Ly-6Chigh generado in vitro son capaces de actual de la misma manera que los macrófagos y los monocitos de la sangre periférica de los ratones, adaptandose a los cambios ambientales como descrito previamente (Gordon S. Taylor y PR 2005; Biswas SK and Mantovani A. 2010; Rivollier A., et al 2012).

La capacidad de los monocitos Ly-6Chigh generados in vitro de actuar como las células provenientes de sangre periférica nos llevo a confirmar la capacidad migratoria y funcional de estos monocitos enriquecidos Ly-6Chigh

generado in vitro en modelos de inflamación in vivo. Para ello se utilizó dos modelos experimentales in vivo: el modelo DNFB (1-fluoro-2 ,4-dinitrobenceno) y el modelo Notexin muscular.

La inyección de monocitos Ly-6Chigh enriquecidos generados in vitro, estas células fueran capaces de migrar hacia los tejidos inflamados en ambos modelos animales. Aunque la diferencia en el perfil de migración entre estos modelos se pudo apreciar en el momento en que estas células alcanzaran el sitio de la inflamación, lo que sugiere que la intensidad de la inflamación y de la complejidad del tejido Influencia en su emigración al tejido.

Para investigar en profundidad la participación de los monocitos Ly-6Chigh el proceso de inflamación in vivo, el próximo objetivo de esta tesis doctoral fue estimular estas células ex vivo con citocinas pro o anti-inflamatorias (IFN-γ o IL-4) para utilízalas como herramientas para exacerbar o controlar y restaurar el tejido inflamado. En estudios descritos anteriormente la transferencia adoptiva de macrófagos modificados o estimuladas ex vivo en pacientes con diabetes, los tumores, la aterosclerosis y otras enfermedades inflamatorias y infecciones, fueron eficaces en la mejora de los procesos inflamatorios (B. Burke 2003; Parsa R., et al 2012).

Por lo tanto, nuestros resultados han demostrado que monocitos Ly-6Chigh pre-tratados con IL-4 fueron capaces de aumentar el potencial de la reparación del músculo o de la oreja, en ambos modelos experimentales, lo que sugiere que estas células presentan un potencial terapéutico frente al proceso inflamatorio. Entretanto, se ha demostrado también que monocitos Ly-6Chigh pre-tratados con IFN-γ fueron capaces de retrasar la reparación del tejido, lo que sugiere que estas células pueden responder claramente por el tratamiento o por el entorno en el que se encuentran, dando a

entender que estas células pueden ser una herramienta terapéutica válida para otros tipos de enfermedades, como el cáncer, en el que la presencia de macrófagos pro-inflamatorias es beneficiosa para controlar el crecimiento del tumor (SK Biswas y A. Mantovani 2010;. Movahedi K., et al . 2010).

En resumen, los resultados obtenidos en esta Tesis Doctoral revelar un nuevo método para generar in vitro Ly-6C^{high} monocitos de médula ósea de ratones, con una mejora en la eficiencia de la producción celular, que facilitan el estudio de estas células in vitro e in vivo. Además, también han demostrado la capacidad de las células Ly-6C^{high} para cambiar el fenotipo de la estimulación in vitro verdadera y la capacidad de migrar, así como la heterogeneidad funcional en dos modelos de inflamación in vivo, lo que indica que estas células accionar de la misma manera como se las células proveniente de la sangre periférica. Además, hemos demostrado que Ly-6C^{high} monocitos pueden ser pre-tratados con citoquinas en orther para retrasar o aumentar la reparación de tejidos (IFN-γ o IL-4), respectivamente Todos estos resultados juntos sugieren que los monocitos Ly-6C^{high} generado por nosotros in vitro son células funcionales que se pueden utilizar como una herramienta terapéutica para tratar enfermedades inflamatorias.

CONCLUSIONES

1. Hemos establecido un nuevo protocolo in vitro para generar Ly-6C^{high}CD11b + monocitos procedentes de la médula ósea de ratones Balb/C.

2. Las células generadas in vitro tienen el mismo fenotipo de la Ly-6C del flujo de sangre.

3. Las células Ly-6C^{high}CD11b+ monocitos plasticyty alta actual.

4. Ly-6C^{high}CD11b+ monocitos generados in vitro a migrar in vivo.

5. Inyección en monocitos agudas y crónicas en modelos in vivo inflamatorias de Ly-6C^{high}CD11b+ generados in vitro, muestran una mejora en el sitio de la inflamación a través de la presentación de un perfil más anti-inflamatoria.