

INTERACTION BETWEEN HAEMATOPOIETIC AND MESENCHYMAL STROMA

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A thesis submitted for the degree of Doctor of Philosophy in Imperial
College London

2013

*Alla mia famiglia,
mamma, papà e Patrizia*

DECLARATION OF ORIGINALITY

The research described in this thesis is the sole and original work of the author. Contributions by other persons have been acknowledged where necessary.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Francesco Dazzi, for his guidance and support throughout these years and for contributing to deepen my knowledge and strengthen my love for research. I am immensely grateful to my dear friend Ilaria, to whom I am indebted for the incredible amount of knowledge she offered me, for the time she dedicated to my project and for the endless support she gave me every step of the way. I am also deeply indebted for the friendship and affection of my lab mates, Antonio Galleu, Chun-Yin Wang, Andrea Guerra, and Yuan-Tsung Li, with whom I spent these amazing years. I would also like to thank every other single person in my group, in particular Ling, to whom goes my affection for having understood and accepted my temperament in these four years. I am thankful to Luigi Dolcetti, for proof-reading my thesis and making insightful comments and suggestions. I am incredibly indebted for the love and support of my beloved friends and flatmates, Joana and Rute, who encouraged me and suffered my whining every day and with whom I am still sharing an amazing life. I would like also to thank all the people in the Department, above all Tak, George, Tommaso, Valentina and Antonella, for all the support and time spent with me in the lab. I would not be where I am without the help of my previous supervisors, Lara Tickenbrock and Antonio Rosato, to whom I am hugely grateful for assisting my first steps in research. I owe a debt of appreciation to Leuka, who sponsored all my research, and to Prof. Vincenzo Bronte, Prof. Marina Botto, Dr. Jorge Caamano and Prof. Antonella Viola, for providing important tools for my research. Special thanks goes to Livio, for being there for me throughout all this period. I would like to particularly thank my parents and my sister Patrizia, to whom I am indebted for all the encouragement and love they entrusted me every day of my life.

ABSTRACT

Parenchyma and stroma represent the functional and structural units in every organ of the body respectively. Stromal cells of mesenchymal origin (MSC) have traditionally been associated with a structural support activity within the tissue, but it is only recently that more complex functions have been unveiled. Subsets of MSC have been shown to play a fundamental role in self-renewal and differentiation of haematopoietic stem cells (HSC). Recent findings show that MSC and bone marrow (BM) macrophages represent fundamental components in the niche, modulating egress and mobilization of HSC during normal or emergency myelopoiesis. Therefore, I have decided to investigate whether and how MSC contribute to the formation and function of myeloid cells. In an *in vitro* co-culture model I have observed that MSC have the ability to induce the expansion and differentiation of different subsets of mature myeloid cells from haematopoietic BM cells. Based on the differential expression of CD11b and Gr-1, three cell subsets recapitulating myeloid differentiation could be identified. MSC induced differentiation targets common myeloid progenitors (CMP) or granulocyte/macrophage progenitors (GMP) but not the primitive HSC. CD11b⁺ sorted cells obtained at the end of the co-culture exhibited a functional profile characterised by high levels of both anti- and pro-inflammatory markers, such as nitric oxide synthase 2 (NOS2) and arginase-1 (ARG-1). In order to identify the mechanisms involved in this phenomenon I have chosen to investigate a number of molecules involved in the regulation of haematopoietic differentiation by the microenvironment. I have demonstrated that whilst NOS2 and agrin, an ECM protein, play a key role in the differentiation of CD11b⁺ Gr-1⁻ F4/80⁺ cells, complement appears to be primarily involved in the generation of CD11b⁺ Gr-1^{int-low} F4/80⁻ cells. My studies have shown that MSC differentiating activity is not confined to the BM but can also be detected in MSC from other tissues like skin and kidney. Overall these results suggest a key role for stromal cells as regulators of myeloid differentiation. Further investigation is under way to assess the importance of such a function *in vivo*.

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ABBREVIATIONS

APC	Antigen Presenting Cell
BM	Bone Marrow
C/EBP	CCAAT/enhancer binding protein
CAR	CXCL12 Abundant Reticular (cell)
cDC	classical Dendritic Cell
CDP	Common DC progenitor
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
ConA	Concanavalin-A
COX2	Cyclooxygenase 2
CSF-1	Colony Stimulating Factor -1
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	Chemokine (C-X-C motif) receptor 4
DC	Dendritic Cell
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
GALC	galactosylceramidase
G-CSF	Granulocyte-Colony stimulating Factor
GD2	ganglioside GD2
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Granulocyte Macrophage Progenitor
GvHD	Graft versus Host Disease
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HLA	Human Leukocyte Antigen

HO-1	Heme-oxygenase 1
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplantation
HSPC	Haematopoietic Stem and Progenitor Cell
ICAM	intercellular adhesion molecule 1
IDO	Indoleamine-2,3- dioxygenase
IFN	Interferon
IL	Interleukin
IL1RA	Interleukin 1 Receptor Antagonist
IL7R α	Interleukin 7 Receptor alpha
LIN	Lineage
LNGFR1	low-affinity growth factor receptor-1
LPS	Lipopolysaccharide
LSK	Lineage- Sca-1+ c-Kit+ (cell)
LT-HSC	Long-term HSC
M-CSF	Macrophage Colony Stimulating Factor
MCP-1	Monocyte Chemotactic Protein 1
MDP	Macrophage/DC precursor
MDSC	Myeloid Derived Suppressor Cells
MEP	Megakaryocyte/Erythrocyte Progenitor
MHC	Major Histocompatibility Complex
MPP	Multipotent Progenitor
MSC	Mesenchymal Stromal Cells
NF- κ B	nuclear factor kappa B
NK	Natural Killer (cells)
NO	Nitric oxide
NOS	Nitric oxide synthase
pDC	plasmacytoid DC

PDGFR	Platelet Derived Growth Factor Receptor
PGE2	Prostaglandin E2
RANK-L	receptor activator of nuclear factor kappa B ligand
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
Sca-1	Stem Cell Antigen-1
SCF	Stem cell Factor
SLAM	Signalling lymphocytic activation molecule family
SNS	Sympathetic Nervous System
ST-HSC	Short-Term HSC
STAT	signal-transducer and activator of transcription protein
TAM	Tumour Associated Macrophage
TGF	Transforming Growth Factor
TipDC	TNF- α and iNOS producing DC
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
Tregs	Regulatory T cells
TSG-6	TNF- α stimulated gene 6
VCAM	vascular cell adhesion molecule 1
VSEL	Very Small Like Embryonic Cell
WT	Wild Type
α -DG	α -dystroglycan
α -SMA	α -Smooth Muscle Actin

CHAPTER 1.

INTRODUCTION

Tissues can be generally defined as consisting of two main components – parenchyma and stroma – that represent the functional and structural element, respectively, of an organ. Whilst parenchymal cells effect the specific function of the organ in which they reside, stroma comprises all other ‘accessory’ components, and thus includes cells of both mesenchymal and haematopoietic origin. Mesenchymal stromal cells (MSC) have been traditionally related to supportive functions, with macrophages being accounted for modulating inflammation. Such a notion has recently been challenged by numerous studies indicating that an interaction between MSC and macrophages might play a fundamental role in tissue homeostasis.

1.1 MESENCHYMAL STROMAL CELLS

1.1.1 Definition of mesenchymal stromal cells

The definition of mesenchymal stromal cells (MSC) according to the criteria outlined before present a substantial overlap with the traditional concept of tissue fibroblasts (**Figure 1.1**). However, the last decade has seen a renewed interest in MSC because, irrespective of the tissue from which they can be sourced, they contain a large proportion of cells capable of differentiating into mature mesenchymal tissues such as adipocytes, chondrocytes and osteoblasts [2, 3]. MSC can be isolated from the bone marrow (BM) and virtually all tissues, both in adult and foetal life [3-9]. They have been described to be capable of differentiating also into endothelial cells [10], neural cells [11] and cells of endodermal origin [12], but the evidence available is limited.

These findings have led to introducing the misleading terminology of ‘mesenchymal stem cells’. The same confusing terminology is used to include other ‘stromal cells’ [13, 14], like reticular cells, pericytes. Although the origin of such confusion is primarily semantic, there is a need to unravel the complex question of their developmental origin.

1.1.1.1 Source and ontogeny of MSC

Several studies have attempted to identify MSC progenitors that would possess embryonic stem cell (ESC) features. In this way, the existence of mesodermal progenitor cells (MPC) [15], and marrow-isolated adult multilineage inducible cells (MIAMI) [16] was discovered, augmenting the poor approaches used in the many attempts to define MSC. Very small embryonic like stem cells (VSEL) represent another population identified *in vivo* that expresses markers of pluripotent stem cells [17]. Furthermore, in the BM other cells with precursor characteristic were isolated, such as multipotent adult progenitor cells (MAPC), showing ability to differentiate *in vitro* into various lineages and to contribute to haematopoiesis upon transplantation [18].

A recent work from Vodyanik has suggested that MSC might share the same precursor with haematopoietic and endothelial cells, the so called ‘mesenchymoangioblast’ [19]. Indeed, it has been proposed that MSC are associated with blood vessels *in vivo* [20, 21]. Several reports suggest that pericytes, cells that are located on the abluminal side of blood vessels in close contact with endothelial cells, show great similarity with MSC *in vitro* [22], and may behave as tissue specific stromal cells *in vivo* [23, 24]. In line with these findings, transplantation of MCAM/CD146⁺ subendothelial cells from human BM stroma established a haematopoietic niche to heterotopic sites, through specific interactions with developing sinusoids [25].

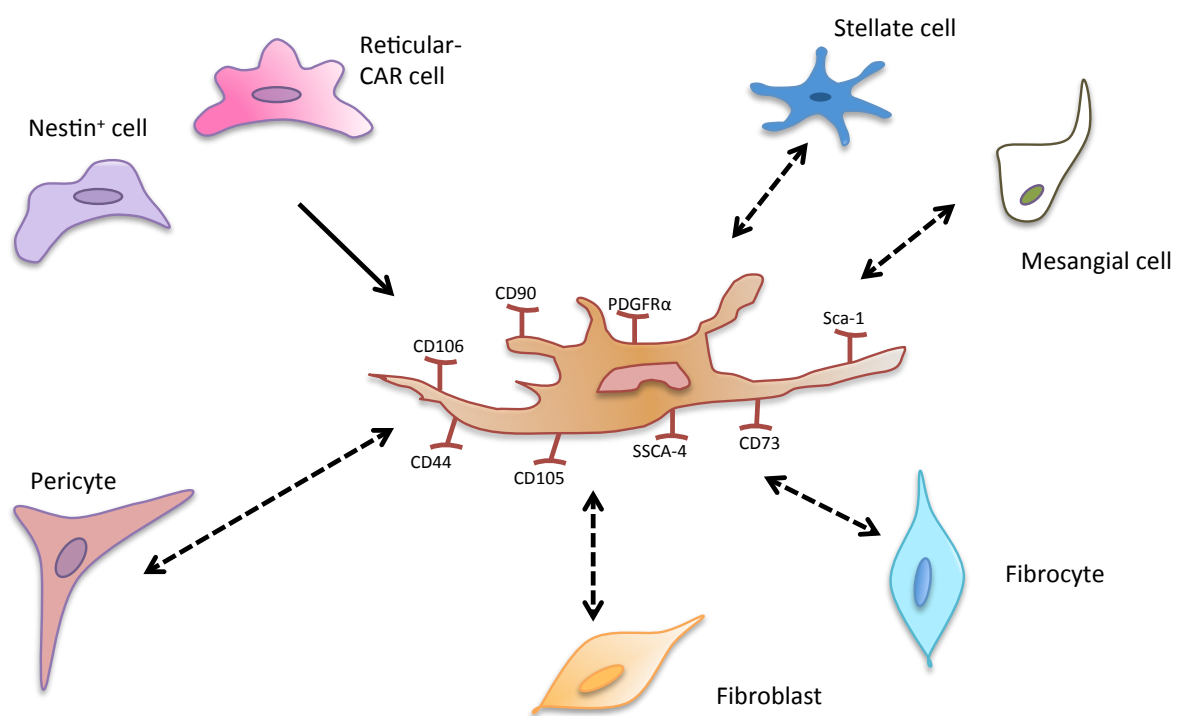


FIGURE 1.1. Mesenchymal stromal cells phenotype and related tissue-specific entities.

1.1.1.2 Phenotype of MSC

MSC are defined as plastic adherent cells expressing stromal markers like CD73, CD105, CD90, CD106 and CD44, and lacking haematopoietic and endothelial markers (CD45, CD11b, CD34, CD14 and CD31) (**Figure 1.1**) [4]. A trypsin resistant antigen, STRO-1, has been proposed as marker for human MSC [26], although its expression is shared by endothelial progenitors and is lost by MSC during culture [27]. Neuronal markers, such as low-affinity growth factor receptor-1 (LNGFR1) [28] and ganglioside GD2 [29], have been identified in human MSC. Indeed, the notion of a possible neuroepithelial ontogeny of MSC has been supported also by a work from Takashima *et al.* [30], thus confuting their broadly accepted mesodermal origin.

Different markers associated with an embryonic phenotype have been proposed to identify mouse MSC, like stage-specific embryonic antigen-1 (SSEA-1) [31]. More recently, Morikawa *et al.* suggested the expression of platelet derived growth factor receptor α (PDGFR α) and stem cell antigen-1 (Sca-1) in the absence of TER119 and CD45 to characterise the subset with higher differentiation potential [32]. This subset of cells could generate colonies *in vitro* at high frequency, and differentiate into haematopoietic niche cells, osteoblasts and adipocytes after *in vivo* transplantation [32].

Despite few differences in markers that identify them, human and mouse MSC share the expression of several receptors associated with specific functions. Growth factor receptors, adhesion molecules, cytokine and chemokine receptors can be detected on their cell surface, although expression varies with prolonged *in vitro* culture. Indeed, large *in vitro* expansion of MSC is consequential to the absence of markers that can unequivocally identify these cells *ex vivo* in their original tissues. PDGFR, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), insulin growth factor receptor (IGFR), hepatocyte growth factor receptor (HGFR), and transforming growth factor- β receptor I (TGF β RI) and TGF β RII have been detected on MSC [33, 34]. Pattern recognition receptors, such as Toll-like receptors (TLR), that detect microbial pathogen associated molecular

patterns, are expressed by both human (TLR1 to TLR10) and mouse MSC (TLR1 to TLR8) [35]. Cell adhesion molecules including intercellular adhesion molecule 1 (ICAM-1), ICAM-2, vascular cell adhesion molecule 1 (VCAM-1, also known as CD106), and lymphocyte function-associated antigen 3 (LFA-3) can also be detected on their surface [36, 37], as well as various integrin α and β subunits [38]. Chemotaxis assays showed that cultured MSC express a broad range of chemokine receptors that play a crucial role in their migratory abilities. The list of chemokine receptors includes chemokine (C-X-C motif) receptor 4 (CXCR4), as well as chemokine (C-C motif) receptor 1-5 (CCR1-5), CCR7, CCR9, CXCR3-6 and chemokine (C-X3-C) receptor 1 (CX3CR1) [34, 36, 39, 40].

1.1.2 Role of MSC in haematopoiesis

Haematopoietic stem cells (HSC) are the self-renewing, multipotent progenitors that give rise to all mature blood cells. They persist in adult life, and their maintenance and regulation of self-renewal and differentiation depends upon both cell-intrinsic and cell-extrinsic mechanisms. The contribution of a specific microenvironment to the regulation of HSC is a concept firstly introduced by Curry *et al.* in 1967 [41] and then by Schofield *et al.* in 1978 [42], who referred to a ‘haematopoietic inductive microenvironment’ and ‘stem cell niche’, respectively. Although such concept has been known for several decades, only recent work shed light to the structure and localization, and also to molecules and cell types involved in the formation of the HSC niche.

Currently, two models of niche are being proposed: the endosteal niche near bone surfaces, and the vascular niche associated with sinusoidal endothelium [43]. The endosteum, the cellular lining of bone surfaces separating the bone from the BM, has long been viewed as the environment regulating HSC. Osteoblasts, osteoclasts and other stromal cells of mesenchymal origin are associated with the endosteum. On the other hand, the vasculature has an important role for the formation and expansion of HSC during development. Sinusoidal endothelial cells, perivascular reticular cells, and other cells of mesenchymal origin are associated to the perivascular niche. Although it has been proposed that

endosteal components maintain the HSC in a quiescent, dormant state, whereas BM endothelial cells are responsible of dividing, self-renewing HSC [43], it is still unclear whether these two niches are physically and functionally separated or if they contribute to a ‘common’ niche influenced by both endosteal and perivascular stimuli [44].

1.1.2.1 The endosteal niche

The first study describing the relationship between haematopoiesis and bone development (osteogenesis) dates back to 1970s. After subcutaneous transfer of total BM, Patt and Maloney demonstrated that bone was forming before vascularized BM [45, 46]. In the same years, it was shown that HSC reside close to the bone surface [47]. It is well known that osteoblasts are directly implicated in HSC maintenance. For instance, mouse and human osteoblast cell lines can secrete a large number of cytokines that promote the maintenance of HSC *in vitro* [48].

The direct proof of the involvement of osteoblasts in HSC regulation came from two simultaneous *in vivo* studies. Both studies were aimed at increasing the number of osteoblasts. The first study utilised a constitutively active form of parathyroid hormone (PTH) or the PTH/PTH-related protein receptor (PPR) [49], whilst the other employed mice deficient for the bone morphogenetic protein (BMP) receptor 1A (BMPR1A), normally expressed on osteoblasts lining the endosteum. In both cases, the increase in osteoblast number was associated with parallel increment in HSC [50].

Amongst the various osteoblast populations, a subset expressing N-cadherin has been proposed to directly interact with HSC [51]. This notion was further supported by the finding that an overall increase in the number and function of osteoblasts without a concomitant increase in N-cadherin⁺ cells is not sufficient to enhance HSC numbers [52].

1.1.2.2 The vascular niche

Haematopoiesis and vasculature have an intimate relation during embryonic and foetal development. HSC are indeed able to self-renew and differentiate before the formation of BM cavities, and they undergo haematopoiesis in association with the blood vessels of placenta. Although it is arguable that such association could be present only during development and not in the adult, several studies suggest the existence of a perivascular niche.

Similar to osteoblasts, BM sinusoidal endothelial cells can promote the maintenance of HSC *in vitro*, constitutively expressing cytokines such as chemokine (C-X-C motif) ligand 12 (CXCL12, also called stromal-derived factor-1, SDF-1) and adhesion molecules like endothelial-cell (E)-selectin and VCAM-1 that are important for HSC mobilization, homing and engraftment [53]. Furthermore, endothelial cells are known for supporting the survival, proliferation and differentiation of myeloid and megakaryocyte progenitors, suggesting that development of these lineages could be initiated at the perivascular niche [54]. Apart from *in vitro* studies, it has been shown that reticular cells, specialised pericytes of BM sinusoids within the vascular medullary network, make contact with HSC and more mature blood cells [55]. Several studies have shown that, after myeloablation or stress, HSC are mobilized from their quiescent state near the endosteal niche and migrate towards the centre of the BM to the vascular zone, near the sinusoidal endothelium [53]. This notion of vascular BM niche as a site where HSC are dividing and highly proliferating has recently been confuted by the discovery of early precursors attached to the endothelium of BM sinusoids [56].

1.1.2.3 Contribution of mesenchymal cells to endosteal and perivascular niches

Mesenchymal progenitors have long been studied for regulating HSC (**Figure 1.2**). As previously discussed, several studies have demonstrated that transplantation of BM stromal cells under the kidney capsule results in the formation of bone-like structures, in which osteoblasts, adipocytes and reticular cells could be detected [57].

Recent findings, based on the adoptive transfer of BM derived mesenchymal precursors, showed that a human CD146⁺ subendothelial subset could give rise to haematopoietic bone upon transplantation in mice [25]. Furthermore, two main studies identifying mesenchymal derived candidate niche cells strengthen the concept of MSC as fundamental niche components. The first study from Sugiyama *et al.* [58] proposes that reticular cells expressing high levels of the chemokine CXCL12, called CAR (CXCL12 abundant reticular) cells closely interact with HSC. Deletion of CXCR4, receptor for CXCL12 in mice, determined a reduction in HSC numbers [58]; depletion of CAR cells also led to a reduction of HSC numbers, accompanied by a quiescent status and increased myeloid differentiation [59].

Another subpopulation of mesenchymal cells residing on the walls of BM sinusoids proposed as candidate niche cells has been characterised in mice by the expression of the cytoplasmic filament protein nestin [60]. Nestin-expressing MSC are subendothelial cells spatially associated with HSC and sympathetic nerve fibres, expressing HSC maintenance genes such as CXCL12 [60]. Their depletion results in an increased mobilization of HSC to the periphery, and poor homing of HSC after transplantation. Although it could be argued that CAR cells and nestin⁺ cells represent overlapping cell types, two theories are being diffused: nestin-expressing MSC might represent a functional subtype of CAR cells that are found in the perivascular location [61], or CAR cells might represent a more committed precursor regulating haematopoietic progenitors rather than HSC [62]. In fact, CAR cells depletion was lethal within 5 days, affecting the numbers of various haematopoietic cell types, whereas depletion of nestin⁺ cells only affected HSC numbers.

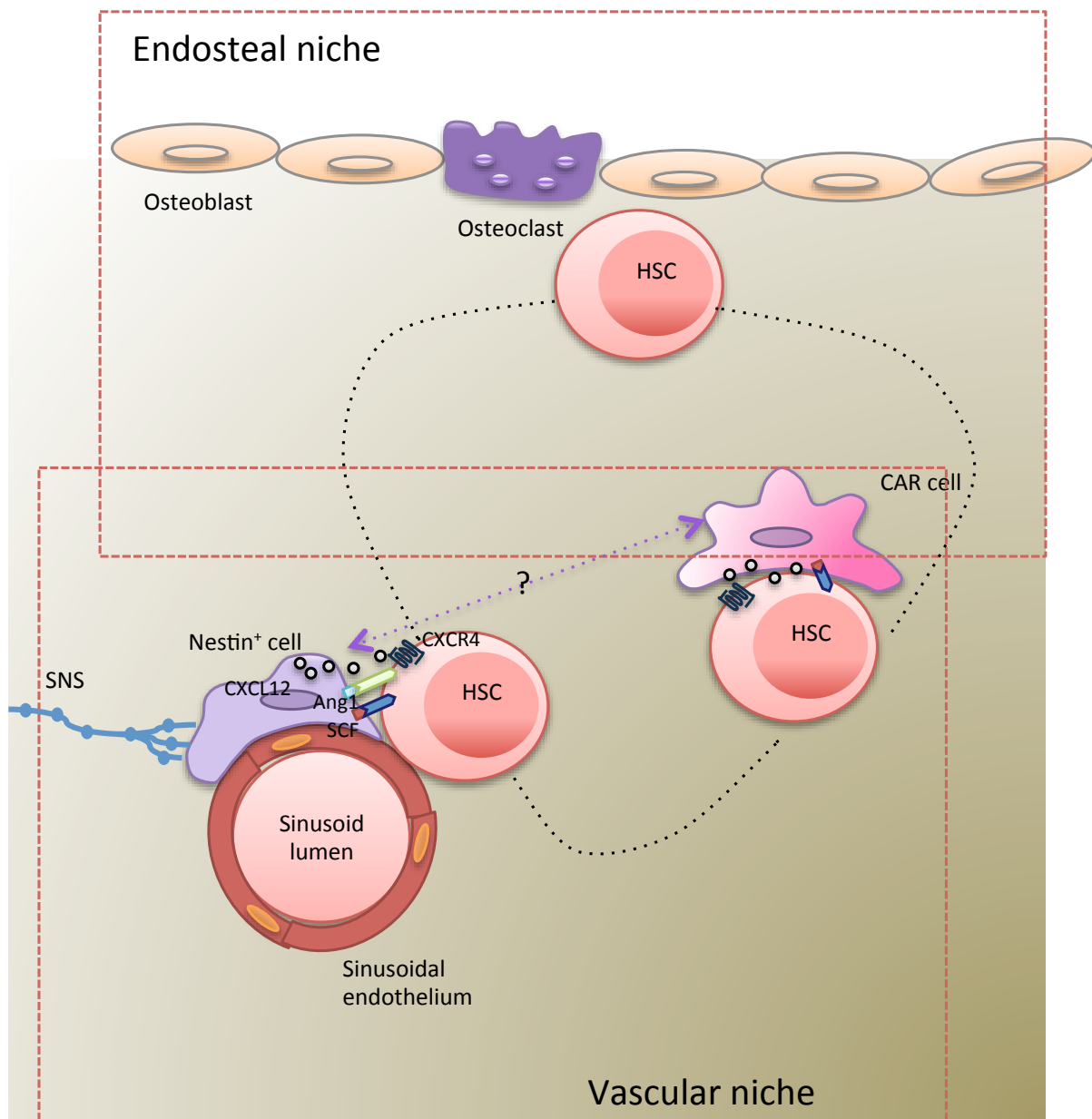


FIGURE 1.2. MSC in the haematopoietic niche. HSC can be found near the endosteal surface, in association with CXCL12 abundant reticular (CAR) cells, and near perivascular nestin⁺ cells. Each niche provides signals to support HSC functions, although there is no consensus yet about the specific activity of each niche (dotted arrow). Nestin⁺ MSC are innervated by SNS, which regulates their cell cycle, differentiation and expression of HSC retention genes. The relation between nestin⁺ cells and CAR cells is still under investigation. Ang1, Angiopoietin-1, CXCL12, chemokine (CXC motif) ligand 12; CXCR4, chemokine (CXC motif) receptor 4; SCF, Stem cell factor (Modified from Mercier et al.).

1.1.2.4 Role of MSC in HSC transplantation

Not only MSC progenitors have been studied for their ability to give rise to niche cells, but also for their possible role in favouring the engraftment of HSC. In fact, a large number of studies investigated the potential application of cultured MSC in the treatment of graft failure or in HSC transplantation. In several experimental mouse models [63, 64] the simultaneous injection of BM derived MSC and HSC determined acceleration and increase in the engraftment after total body irradiation. Such effect was shown to be MHC-restricted, as the formation of cobblestone colonies of HSC under MHC-mismatched stromal cells was demonstrated to be decreased in comparison with MHC-matched stromal cells [65]. These data prompted investigations also in clinical transplantation, although the results obtained were inconsistent [66, 67].

The first clinical trial on breast cancer patients undergoing autologous HSC transplantation in combination with culture-expanded MSC showed that haematopoietic recovery was accelerated [67]. However, this beneficial effect has not been confirmed in allogeneic HSC transplantation even when donor and recipient are HLA identical siblings [68]. Furthermore, there is no evidence of long-term engraftment of adoptively transferred MSC in the host stroma [69].

1.1.3 MSC and the immunomodulatory function

In recent years, much attention has been paid to a unique property of MSC, apparently unrelated to their primary function in regulating haematopoietic differentiation. MSC have been found to exhibit a potent immunosuppressive activity that can target virtually every cell of the immune system (**Figure 1.3**).

1.1.3.1 MSC and T lymphocytes

The first indications on the immunosuppressive action of MSC come from studies on humans [70-73], baboons [74], and mice [75, 76] demonstrating that MSC are able to suppress the activation and proliferation of T cells *in vitro*. This inhibition affects CD4⁺ and CD8⁺ T cells, independently of whether they are naïve or antigen-experienced [76], and regardless of the stimulus utilised, whether putative or allo-antigens [71-74], mitogens such as phytohaemagglutinin (PHA) [70, 71, 77], Concanavalin-A (ConA) [71, 75], and tuberculin [78], or CD3/CD28 agonists [73, 76, 79]. The effect is dose-dependent, as inhibition increases with increasing amount of MSC, but it has been proposed that low concentration of MSC may stimulate rather than inhibit mixed leukocyte reactions (MLR), although the experimental approaches utilised are questionable [71, 72, 78].

MSC have been reported to inhibit the cytotoxic effects of cytotoxic T cells (CTL) [72], although it seems to be related to their ability to suppress proliferation rather than inhibiting the effector function [80, 81]. Suppression of T cell proliferation is not MHC restricted, as similar effects are being observed whether MSC were autologous or allogeneic to the responder cells [76, 82, 83]. Interestingly, it appears that MSC inhibitory effect can overcome also species barriers [75].

MSC induce the arrest of T cell cycle in G0/G1 phase, strongly inhibiting cyclin D2 and up-regulating cyclin dependent kinase inhibitor p27^{kip1} [84]. Although it has been proposed that MSC can induce T

cell apoptosis by converting essential amino acid tryptophan into its breakdown products [85], this notion is still controversial [71, 77]. It is instead broadly accepted that MSC can induce T cell anergy. Whether anergy is the consequence of the absence of co-stimulatory molecules, such as CD80 or CD86, on MSC surface remains to be convincingly demonstrated [73, 75, 82]. In fact, a study by Klyushnenkova *et al.* [82] showed that up-regulating co-stimulatory molecules in MSC did not result in increased T cell proliferation. Several studies have shown that the unresponsiveness of T cells is transient and can be restored after removal of MSC [70, 76], whereas others suggest that T cell anergy is induced [86].

1.1.3.2 MSC and B cells

T cells are not the only target of MSC mediated immunosuppression. Consistent with the non-specific anti-proliferative activity also the proliferation of pokeweed mitogen stimulated B cells can be inhibited by murine MSC [87]. Spleen derived B cells stimulated with T cell dependent stimuli, such as CD40 and IL-4, can also be suppressed by MSC [84]. In an experimental model of human systemic lupus erythematosus (SLE), BXSB mouse, allogeneic MSC can inhibit the proliferation, activation and immunoglobulin G (IgG) secretion of B cells [88]. Also human MSC have been described for their inhibitory effect on B cells previously activated either with anti-Ig antibodies, soluble CD40 ligand or cytokines. Not only were they able to suppress the proliferation of B cells, but MSC also affected the differentiation, antibody-production and chemotactic activity in response to CXCL12, CXCL13, and chemokine (C-C motif) ligand 19 (CCL19) [89]. In a murine model of multiple sclerosis, the production of antigen specific antibodies was inhibited by the infusion of MSC, as well as T cell responses [90]. However, other *in vitro* studies showed that MSC support the proliferation and differentiation into Ig secreting cells of naïve B cells, either isolated from healthy donors or from pediatric SLE patients [91].

1.1.3.3 MSC and natural killer cells

MSC can modulate also the activity of natural killer (NK) cells. MSC can inhibit IL-2 or IL-15 driven proliferation of NK cells, and also their cytokine [interleukin-10 (IL-10), interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α)] production [79, 92]. The cytotoxic activity of resting [93] and IL-2 activated NK cells is also reduced in culture with MSC [94]. Furthermore, a short-term culture with MSC reduces NK cell cytotoxicity against HLA class I expressing tumours, but not on HLA class I negative tumours [92].

As MSC express very low levels of HLA class I molecules, they can be lysed by IL-2 activated NK cells [92]. MSC also express several ligands for activating NK cell receptors, such as UL16-binding proteins, polio virus receptor (PVR) and nectin-2 [93]. Nevertheless, IFN- γ exposed MSC are not a target of NK mediated lysis, as IFN can up-regulate the expression of HLA molecules on MSC surface [93]. Adoptively transferred MSC could therefore be protected from lysis by activated, IFN- γ producing NK cells during inflammatory conditions.

1.1.3.4 MSC, monocytes and dendritic cells

Antigen presenting cells (APC) are also a target of MSC. *In vitro* studies have demonstrated that MSC can inhibit the differentiation of DC from monocytes [95, 96] or from IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) induced CD34⁺ precursors [97] [98]. As a result of this, DC ability to induce allogeneic T cell responses is impaired [79, 95-99], as MSC induce a down-regulation of MHC molecules of class II, CD11c, CD83, a decrease in the production of TNF- α and IL-12 and an increase in the production of IL-10 [79, 95, 96, 98, 99].

It has recently been shown that MSC-treated DC are unable to form immune synapses with T cells because of a rearrangement of actin distribution [100]. As for T cells, monocytes are inhibited to enter

the cell cycle, as they are retained all in G0 phase, due to a down-regulation of cyclinD2 and p27^{kip1} [96]. Recently, it was observed that the adoptive transfer of MSC decreased the migration of subcutaneously administered ovalbumin-pulsed DC to the draining lymph nodes. This effect could thus explain the decreased number of CFSE-labelled DC in draining lymph nodes and the impaired priming of ovalbumin specific CD4⁺ T cells [101].

1.1.3.5 MSC and macrophages

MSC have been shown to stimulate the entry of monocytes into the bloodstream during infections [102]. An elegant work by Shi *et al.* demonstrated that BM MSC progenitors and CAR cells expressed monocyte chemotactic protein 1 (MCP-1) after sensing microbial pathogens through their TLR, thus inducing CCR2-dependent monocyte entry into the bloodstream. Human and mouse MSC are able to recruit monocytes and macrophages in inflamed tissues also through the release of chemokines CCL3, CXCL12 and CCL12 [103].

Monocytes co-cultured with human or mouse MSC are turned into M2 type of macrophages, increasing the production of anti-inflammatory cytokines like IL-10, and down-regulating TNF- α and IFN- γ [104-106]. The *in vitro* data have been confirmed by *in vivo* studies. In a sepsis model, the injection of BM derived MSC effectively improved survival. The mechanisms responsible for the therapeutic efficacy have been related to the ability of MSC to temporarily reprogram monocyte and macrophage functions, inducing IL-10 production from M2 macrophages that were able in turn to reduce neutrophils infiltration in tissues [107]. Further evidence has been provided by the work of Gupta *et al.*, in which the intrapulmonary administration of MSC in an endotoxin-induced lung injury model reduced TNF- α and CXCL12, and increased the production of IL-10 by alveolar macrophages [108]. In a zymosan peritonitis model, human MSC produce the anti-inflammatory protein TNF- α stimulated gene 6 (TSG-6), which interacts with resident macrophages to decrease zymosan-mediated nuclear translocation of nuclear factor- κ B (NF- κ B) [109]. Not only MSC can recruit monocytes and

induce M2 macrophages, but also they produce interleukin 1 receptor antagonist (IL1RA), that prevents the release of TNF- α and IL-1 from activated macrophages [110].

1.1.3.6 MSC and neutrophils

Naïve or TLR3-triggered MSC are able to promote the survival of resting or IL-8 activated neutrophils *in vitro*. The recent work of two Italian groups suggests that MSC protect neutrophils of the storage pool in the BM niche from apoptosis, through an IL-6, IFN- β and GM-CSF dependent mechanism [111, 112]. Neutrophils co-cultured with MSC up-regulate the expression of anti-apoptotic factors, such as myeloid cell leukaemia sequence 1 (MCL1), and down-regulate pro-apoptotic factors, such as BCL-2 associated X protein (BAX) [111]. Also stimulation with lipopolysaccharide (LPS), a TLR4 ligand, induces human MSC to increase the production of IL-6, IL-8 and macrophage migration inhibitory factor (MIF), thus attracting neutrophils and enhancing their lifespan and pro-inflammatory activity [113]. These *in vitro* results were confirmed in a work in which LPS activated human MSC embedded in Matrigel and subcutaneously injected in a mouse model were able to recruit neutrophils *in vivo* [114].

1.1.3.7 Mechanisms involved in immunomodulation

The mechanisms underlying MSC anti-proliferative effect are only partially known, although the secretion of soluble factors is now widely accepted as fundamental for MSC immunosuppressive ability. Contact-dependent mechanisms and soluble factors are likely to act in concert and/or in alternate fashion. For instance, suppression of T cell, B cell and NK cell proliferation can be mediated by soluble factors as demonstrated by transwell experiments [70, 73, 81] [89] [92]. Only supernatants coming from co-cultures of MSC and lymphocytes are able to deliver the same immunosuppressive effect [75, 78], whereas human or mouse MSC conditioned media are not [72, 77, 78, 87], suggesting that the suppressive factor requires a dynamic cross-talk between MSC and T cells. Similarly, the

inhibitory effect of MSC on DC differentiation is mediated by soluble factors produced only upon interaction between MSC and monocytes [98].

It is arguable that a concert of molecules is responsible for the inhibitory effect, as selective inactivation of only one molecule does not have any restoring effect. TGF- β 1, hepatocyte growth factor (HGF) [70], indoleamine 2,3-dioxygenase (IDO) [115], prostaglandin E₂ (PGE₂) [79], nitric oxide (NO) [116], heme oxygenase-1 (HO-1) [117], and insulin-like growth factor binding proteins [118] are some examples of candidate factors proposed to be involved in MSC mediated immunosuppression. IDO seems to play an important role in human MSC, whereas NO is more important in the mouse system and rat MSC use preferentially HO-1.

IDO is one of the mechanisms believed to control T cell responses to autoantigens and alloantigens [119, 120], and has been implicated in the inhibition of proliferation of T cells [115], NK cells [93] and B cells [94] mediated by MSC. Its activity causes tryptophan depletion and synthesis of toxic catabolites like kynurenine, thereby inhibiting the growth and function of immune cells [121]. IDO is not constitutively expressed by MSC, as it is produced only after exposure to inflammatory conditions, such as IFN- γ [94].

Prostaglandin E₂ (PGE₂) is a synergistic partner of IDO [79, 93]. It has been shown that MSC-derived PGE₂ is involved in skewing an inflammatory environment into an immunological tolerance state, as shown by the down-regulating effect on TNF- α and IFN- γ production by DC and T cells [79]. MSC constitutively express PGE₂, but upon co-culture with PBMC they up-regulate it [73]. PGE₂ has also been studied as a mechanism involved in MSC-macrophage interaction, with MSC-derived PGE₂ binding to the prostaglandin receptors EP2 and EP4 on macrophage surface [105, 107].

Nitric oxide (NO) is another important mediator of MSC properties, although its role seems to be confined in the mouse system. It is synthesized by the inducible isoform of the NO synthase (NOS2, NOS2), which is induced in MSC by contact with activated CD4⁺ or CD8⁺ lymphocytes [116], mainly

due to the presence of IFN- γ or the combined presence of TNF- α , IL-1 α and IL-1 β [122], and inhibits the proliferation of T cells via the phosphorylation of Stat5 [123]. Interestingly, NOS2^{-/-} MSC are less effective in suppressing T cell proliferation than wild type MSC, and this is demonstrated also by the blocking effect of N-nitro-L-arginine methyl ester, a specific inhibitor of NOS2, on MSC immunomodulatory potential [116].

Other cytokines that have been studied for their role in the immunomodulatory properties of MSC are represented by IL-6, IL-11, IL-10, CXCL12, receptor activator of NF- κ B ligand (RANK-L) and osteoprotegerin (OPG). Di Nicola *et al.* have shown that IL-6 and IL-11, although up-regulated by MSC, are not important to deliver immunosuppression [70]. IL-10 is not secreted by MSC, but it has been investigated because its levels are always up-regulated in the co-cultures of immune cells with MSC [82, 99, 124]. Blocking IL-10 partially restores the inhibitory effect on cytokine release and proliferation of T cells [99]. CXCL12 has been investigated for its known activity in BM and thymic stroma [125]. Low doses of CXCL12 are chemoattractive, but high doses repel T cells [126]. Even though low levels of CXCL12 are detected in culture, blocking antibodies do not have any effect on the MSC mediated immunomodulation. RANK-L and OPG were studied for their role in promoting survival and function of DC, T cell activation and communications between them, but neutralization had no effect [77].

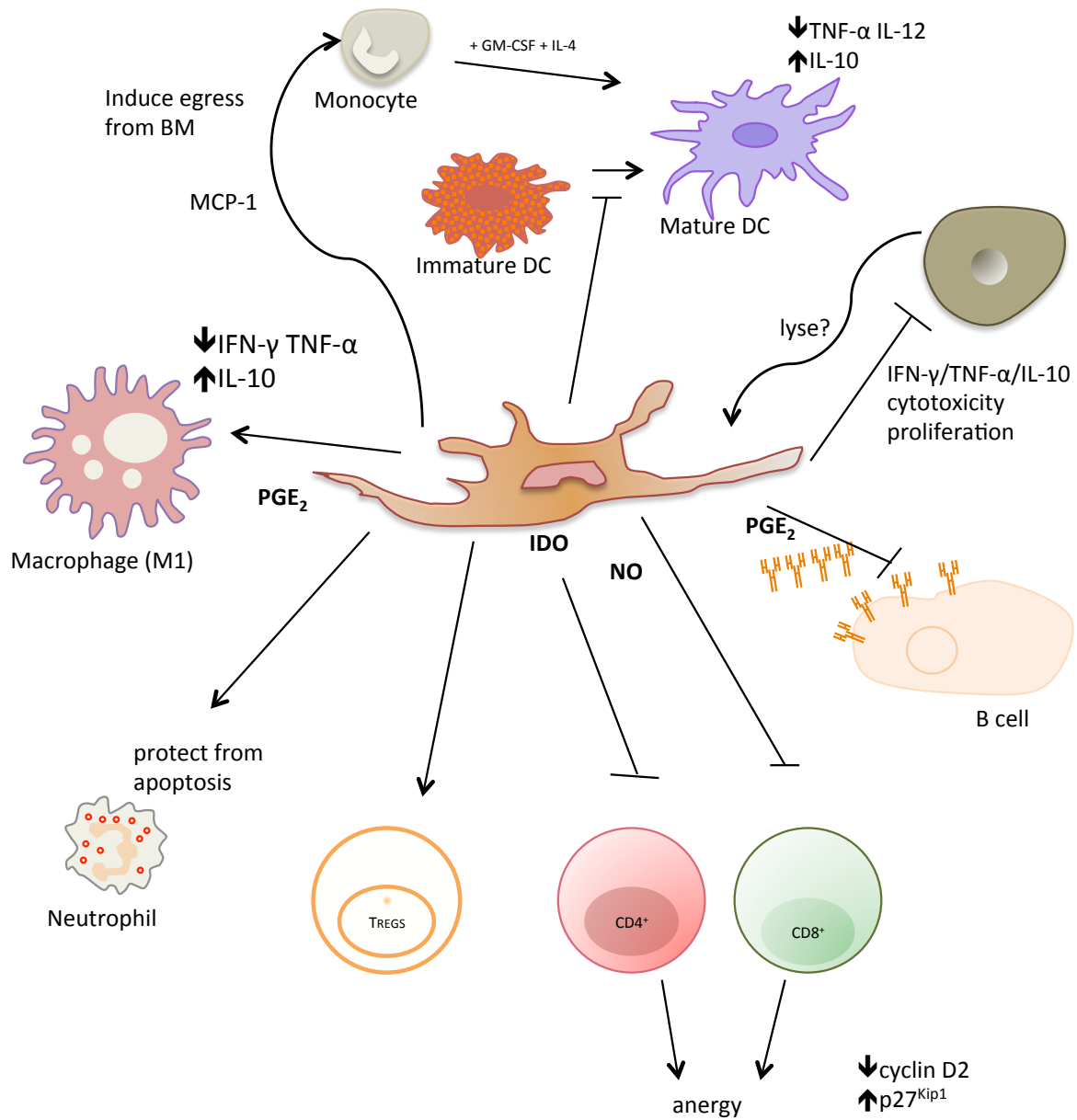


FIGURE 1.3. MSC immunomodulatory activity. DC, dendritic cell; IDO, indoleamine-2,3-dioxygenase; IFN, interferon; IL, interleukin; MCP-1, monocyte chemotactic protein-1; NK, natural killer; NO, nitric oxide; PGE₂, prostaglandin E₂; Tregs, regulatory T cells; VEGF, vascular endothelial growth factor.

1.1.3.8 Induction and polarization of MSC immunomodulatory properties

The immunosuppressive properties of MSC are not constitutive, but they can be acquired after exposure to the inflammatory environment. The concept of ‘inflammatory MSC licensing’ was initially reported in the context of IFN- γ treatment [94]. Other pro-inflammatory cytokines that can license MSC are TNF- α , IL-1 α , or IL-1 β , and they can also induce an up-regulation of HLA class I molecules, ICAM-1, VCAM-1 and *de novo* expression of HLA class II [122]. Although these changes might promote an immunostimulatory function of MSC, they are also critical for their immunoregulatory activity [83]. In fact, IFN- γ can enable MSC to acquire APC properties and stimulate the generation of cytotoxic CD8⁺ T cell *in vivo* when present at low concentrations. High concentrations of IFN- γ instead induce immunosuppressive properties of MSC [127, 128]. However, further investigation is required in order to clarify the physiological relevance of these studies.

Recently it has been proposed that TLR may control MSC ‘plasticity’. Human and mouse MSC express TLR on their surface, and their expression can be up-regulated under hypoxia [129] or inflammatory conditioning [130]. TLR2 and TLR4 are stimulated by bacterial components, such as LPS; TLR1, TLR2 and TLR6 by lipoproteins; TLR3 by double stranded RNA; TLR9 by bacterial or viral unmethylated CpG DNA. Different TLR agonists influence differently MSC survival, proliferation and differentiation, as well as their migratory and immunoregulatory functions. The stimulation with LPS protects human MSC from oxidative stress by enhancing the expression of cellular antioxidants [131, 132]. TLR2 or TLR4 ligands promote cell division, whereas TLR9 agonists induce cell cycle arrest in MSC [35]. TLR activation is known to increase antigen presentation and stimulation of immune cells, but apparently this does not apply to MSC. Although MSC up-regulate the expression of MHC molecules, they probably maintain their immunoprivileged status. However, long term engraftment of MSC after transplantation is not supported by the literature. TLR3 triggering is known to stimulate the migration of MSC, which is a prerequisite for an efficient function of transplanted MSC [133]. TLR signalling has also been investigated for the immunosuppressive functions of MSC. As an example, TLR2 has been shown to up-regulate the secretion of T cell

suppressive protein galectin 3 on human MSC [134]; TLR2, TLR3 and TLR4 signalling promotes the production and release of IL-6 [134], which is involved in neutrophils modulation and inhibition of DC maturation [112]. Nevertheless, the relationship between TLR stimulation and MSC properties is still open to further investigations. It has been proposed, in analogy with the M1 and M2 macrophages paradigm, that also MSC can be induced by TLR signalling into a pro-inflammatory MSC1 and an anti-inflammatory MSC2 phenotype [135]. In this model, TLR4 activation would promote the generation of an IL-6, IL-8 and TGF- β producing pro-inflammatory phenotype, whereas TLR3 would favour the up-regulation of IDO, cyclooxygenase 2 (COX2), IL-4 and IL1RA, typical of an anti-inflammatory phenotype. It remains to be determined if these populations exist *in vivo*.

1.1.4 MSC therapeutic applications

The immunosuppressive properties and tissue repair function of MSC have prompted investigations into many disorders in the last decade. Indeed, MSC are a promising population of cells for the treatment of immune-mediated diseases, promotion of tissue repair, delivering anti-cancer agents, and facilitating the engraftment of HSC transplantation.

Bartholomew and colleagues were the first to investigate the ability of MSC to modulate immune responses *in vivo*, showing that injection of donor MSC prolonged allogeneic skin-graft survival in primates [74]. This result has been later confirmed also in a murine model of skin graft, where infusion of syngeneic MSC reduced the rejection of allogeneic skin transplant [136].

Another study on allogeneic islet transplantation tested MSC in a rat model of streptozotocin-induced diabetes for the induction of chimerism and islet allograft tolerance [137]. The effect of *in vivo* administration of MSC has been successfully tested also in autoimmune diseases. MSC have been shown to ameliorate experimental autoimmune encephalomyelitis (EAE), decreasing inflammation

and demyelination in the central nervous system due to the induction of peripheral T cell tolerance [86].

MSC could affect the capacity of encephalitogenic T and B cells directed against the immunizing antigen to adoptively transfer the disease into naïve recipients [90]. MSC have also been investigated for their effect in collagen induced arthritis (CIA), a model of rheumatoid arthritis; although initially it was shown that MSC did not have a beneficial effect for curing arthritis [138], it has recently been demonstrated that a single injection of MSC in a CIA model can prevent the damage to bone and cartilage [139]. Sepsis [107] and colitis [140], for their inflammatory nature, have been tested for the effect of MSC, showing that activated MSC can induce *in vivo* the production of higher amounts of IL-10 from macrophages [107, 140] by releasing PGE₂, and prevent neutrophils from migrating into tissues and causing oxidative damage [107].

Besides their effects on immune responses, MSC are known for their ability to promote tissue repair. Rather than trans-differentiating into parenchymal cells, the anti-proliferative and anti-apoptotic activity of MSC on parenchymal cells [141] and the anti-inflammatory activity might effect a cytoprotective action that preserves residual stem cells from destruction, thus favouring their recovery and spontaneous tissue repair. In a streptozotocin-induced diabetes model, MSC were able to promote endogenous repair of pancreatic islets and renal glomeruli [142]. As far as their cytoprotective action is concerned, it has been demonstrated that the co-infusion of MSC and BM cells inhibited the proliferation of antigen specific T cells, restored insulin levels and, importantly, induced recipient-derived pancreatic β cell regeneration [143].

MSC orchestrate the resolution of acute inflammation, which is crucial for tissue repair. Although several studies have demonstrated that MSC mediate a direct immunosuppressive effect, it is more probable that they act through the release of cytokines and/or in concert with other cells in order to resolve inflammation. As shown in a model of ischaemic renal failure, MSC ameliorate tissue injury by reducing the expression of IL-1 β , TNF- α , IFN- γ and NOS2, and up-regulating anti-inflammatory

cytokines [144]. Similarly, MSC can protect from bleomycin-induced lung fibrosis by releasing IL1RA [110]. As previously discussed, MSC favour the generation of anti-inflammatory macrophages, which are equally important to promote tissue repair [145]. Also, endothelial cells and myofibroblasts are recruited from MSC at the injury site to help the tissue repair through the release of several growth factors [146].

Graft-versus host disease (GvHD), a severe condition that develops after allogeneic haematopoietic stem cell transplantation (HSCT), has been the most extensively studied application for MSC in the clinical setting. The use of MSC in GvHD has produced different outcomes. After the anecdotal report [147], a phase II trial involving 55 patients [148] suffering with steroid resistant grade IV acute GvHD, demonstrated the clinical efficacy of MSC in improving the overall survival. In contrast, in a multicentre phase I/II clinical trial, the infusion of MSC at the time of HSCT did not impact on the frequency of developing GvHD, as no difference was observed between the group receiving MSC and the controls [68]. The findings in the preclinical studies can possibly explain these discrepancies. A single infusion of MSC given at the time of the transplant did not prevent GvHD in MHC-mismatched donor-recipient pairs [149]. Instead, when given in multiple doses, MSC could fully prevent the development of GvHD [150]. Furthermore, an interesting study by Polchert *et al.* suggested that MSC could significantly increase the survival rate when administered at day +2 or +20, when IFN- γ levels are at their peak [151].

These findings confirm and strengthen the concept of MSC ‘licensing’, showing that not only *in vitro* but also *in vivo* MSC need the appropriate inflammatory environment in order to exert their immunomodulatory properties. Inflammation can also facilitate the recruitment of MSC at the site of lesion, as shown by *in vivo* studies [144, 150, 152]. The IFN- γ produced by the inflammatory event causes the accumulation of antigen-specific T cells by inducing MHC molecule expression on the endothelium [153], thus enabling MSC and effector T cells to get in close proximity [154].

1.2 HAEMATOPOIETIC STROMA

1.2.1 Development of myeloid lineages

In murine haematopoiesis, the multipotent activity resides within a small fraction of BM cells, lacking the expression of lineage-associated surface markers (Lin) but expressing Sca-1 and c-Kit [155], termed 'LSK' fraction. In this population, the most primitive self-renewing HSC having long term reconstituting ability (LT-HSC) can be isolated. [156].

LSK population comprises also cells with transient reconstituting activity, called short-term HSC (ST-HSC) or multipotent progenitors (MPP). LT-HSC can be identified in the $CD34^- CD38^+$ fraction of LSK [157]. ST-HSC and MPP reside instead in the $CD34^+ CD38^-$ fraction of LSK [158]. More recently, cell surface receptors of the signalling lymphocyte activation molecule (SLAM) family were discovered to selectively distinguish primitive HSC from non-self-renewing MPP. HSC were defined as $CD150^+ CD244^- CD48^-$ cells while MPP were $CD244^+ CD150^- CD48^-$ and most restricted progenitors (myeloerythroid and B cell progenitors) were $CD48^+ CD244^+ CD150^-$ [56]. In contrast with such clear definition, there is still no phenotypic and functional distinction between ST-HSC and MPP. It is instead widely accepted that LT-HSC give rise to ST-HSC, which in turn give rise to committed progenitors that differentiate into end stage blood cells [156].

The lineage-committed progenitors are the common lymphoid progenitor (CLP) and myeloid progenitors (CMP). The CLP population is defined within the $Lin^- Sca-1^{low} c-Kit^{low}$ population by the up-regulation of interleukin 7 receptor α (IL-7R α), and it has clonogenic T, B and NK cell potential, but lacks of myelo-erythroid differentiation ability [159]. In contrast, the CMP population, is defined as $Lin^- Sca-1^- c-Kit^+ IL-7R\alpha^-$, and possesses myelo-erythroid colony forming activity. This subset of cells can be further fractionated upon the expression of Fc gamma receptor II/III (Fc γ R, CD16/32), and CD34: Fc γ R ^{low} $CD34^+$ CMP, Fc γ R ^{low} $CD34^-$ megakaryocyte/erythrocyte progenitors (MEP), and Fc γ R ^{high} $CD34^+$ granulocyte/macrophage progenitors (GMP) [160]. CMP differentiate into GMP and

MEP, and can generate all types of myeloid colonies, whereas GMP and MEP produces only granulocyte/macrophage and megakaryocyte erythrocyte colonies, respectively [161, 162].

Among the myeloid precursors, macrophage/DC progenitor (MDP) has been identified as a subset of BM cells sharing the same phenotype of GMP, lacking the expression of Lin, Sca-1 and IL-7R α , but positive for Fc γ R and c-Kit [160], and that specifically expresses colony stimulating factor 1 receptor (Csf-1R, also called CD115) and the chemokine receptor CX3CR1 [163, 164]. Monocytes, several macrophages subsets and the two main subsets of dendritic cells, splenic ‘classical’ dendritic cells (cDC) and plasmacytoid dendritic cells (pDC), are generated from the MDP. cDC are generated without a monocytic intermediate [164], whereas monocytes give rise to other types of DC, such as inflammatory DC or mucosal DC [165]. The chemokine receptor CX3CR1 is firstly detected on MDP, but not on CMP or GMP, thus it is strictly associated with the commitment of myeloid progenitors to the monocyte/macrophage/DC lineage.

Adding complexity to the origin of monocytes and DC, it has been reported that another progenitor, termed common DC precursor (CDP), is able to generate cDC and pDC but not monocytes [166]. CDP shares the same phenotype of MDP, but its existence is still controversial (**Figure 1.4**).

1.2.1.1 Transcription factors controlling myeloid development

Lineage commitment and differentiation of multipotent cells involves the selective activation and silencing of a number of genes. Cell intrinsic and extrinsic signals, or the combination of both, can trigger cell fate decisions, which are ultimately controlled by transcription factors. Transcription factors, in turn, can activate specific lineage commitment on the basis of their relative expression, timing, and collaborative or competitive relations with other transcription factors.

1.2.1.1.1 Transcription factors involved in formation of HSC

Several transcription factors involved in the formation of HSC are also required at later stages within the differentiation of individual blood lineages. Therefore, such a distinction is arbitrarily used for discussion purposes. Mixed lineage-leukaemia gene (MLL), runt-related transcription factor 1 (Runx1), ets variant gene 6 (TEL/ETV6), T cell acute lymphocytic leukaemia 1 (SCL/tal1) and LIM domain only 2 (LMO2) are some of the transcription factors discovered for being fundamental for the formation of HSC as they account for leukaemia-associated translocations.

Transcription factors SCL/tal1 and LMO2 are essential for the development of both primitive and definitive haematopoiesis [167]. A deregulated expression of the locus of these two transcription factors has been identified in T cell acute leukaemia.

MLL and Runx1 are essential for the generation of HSC in the aorta-gonad-mesonephros (AGM) region [168]. In the absence of *Runx1*, no hematopoietic clusters form in the dorsal aorta in mice. Mouse and zebrafish models show that Notch is required for aortic HSC production [169], and in zebrafish Runx1 lies downstream of Notch signalling. MLL instead seems to be fundamental for maintaining the expression of HOX genes, and in particular lies upstream of HOXB4, one of the most extensively studied HSC transcription factors [170].

1.2.1.1.2 Transcription factors controlling granulocyte and monocyte lineage commitment

One of the most important transcription factors controlling myelopoiesis is PU.1. Not only PU.1 induces myeloid commitment in ST-HSC [171] and generates CMP [172], but also is engaged in antagonistic interactions with other transcription factors. Its inhibitory interaction with GATA binding protein 1 (GATA-1) shuts down the megakaryocytic/erythroid pathway, and antagonizing GATA-2 blocks mast cell development [173].

PU.1 interaction with CCAAT/enhancer-binding protein α (C/EBP α) is instead critical for inducing monocyte development at the expense of the granulocyte compartment [174]. A current model proposes that C/EBP α activates Gfi-1, indispensable for granulocytic differentiation, whereas PU.1 activates Egr transcription factors to induce monocyte/macrophage differentiation [175]. Nevertheless, this model is far from being complete, and might involve other transcription factors. For instance, IFN consensus sequence binding protein/IFN regulatory factor 8 (ICSBP/IRF-8) [176] and Krueppel-like factor KLF4 [177] have been also demonstrated to be involved in driving monocytic differentiation, at the expense of the granulocyte compartment.

As demonstrated by the drastic reduction in the numbers of monocytes in knockout models, the development of monocytes depends on the growth factor receptor Csf-1R (CD115) [178]. CD115 is expressed by all the cells of the mononuclear phagocyte system (MPS), which comprises monocytes, macrophages, DC and their precursors [179]. The two known ligand of CD115, Csf-1 and IL-34, are both important for MPS development. PU.1 trans-activates CD115 promoter [180], as confirmed by CD115 lack of expression in PU.1 deficient myeloid progenitors [181]. However, CD115 expression is not sufficient by itself to drive macrophage differentiation [181].

Monocytes and macrophages highly express MafB and c-Maf transcription factors [182], which selectively drive monocyte fate in myeloid progenitors. Expression levels of PU.1 are fundamental to drive the differentiation of monocytes into macrophages or DC. Monocyte-derived macrophages express MafB and moderate levels of PU.1, whereas DC show high levels of PU.1 and no MafB expression [182].

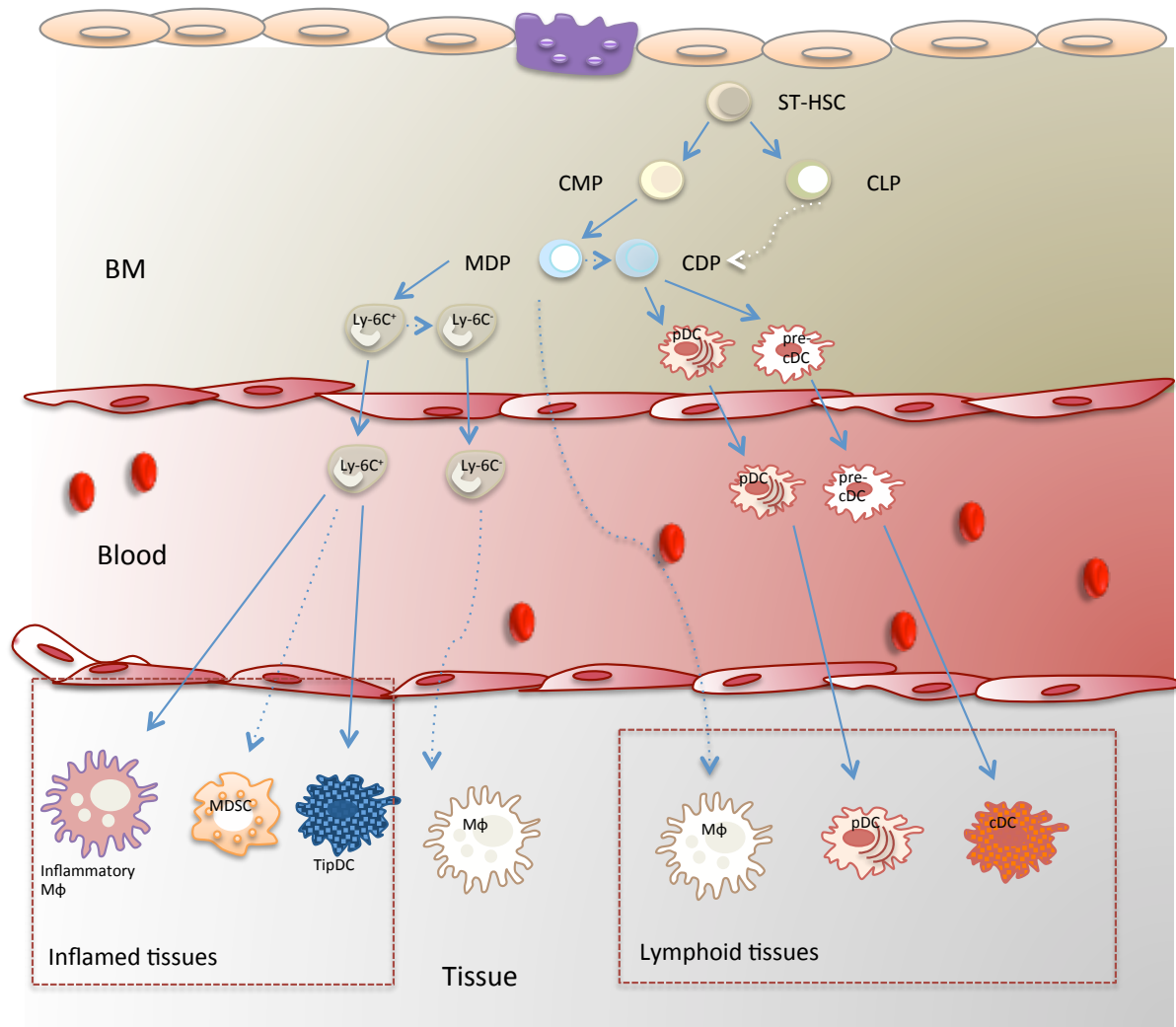


FIGURE 1.4. Development of myeloid lineages in mouse. In the BM, HSC produce CMP and CLP precursors. CMP give rise to MDP precursor. MDP give rise to monocytes, some populations of macrophages (dotted arrow). CDP (whose existence is still controversial (dotted arrow)) give rise to pre-cDC and pDC; pre-cDC and pDC circulate in blood and enter lymphoid tissue. Under homeostatic conditions, two monocyte subsets, Ly- 6C⁺ and Ly-6C⁻, leave the bone marrow to enter the blood, and transmigrate to nonlymphoid tissues, where they may give rise to tissue resident MΦ. During inflammation, Ly-6C⁺ monocytes give rise TipDC, inflammatory macrophages, and may contribute to MDSC associated with tumors (dotted arrow) It is unclear whether CLP contribute to CDP (dashed arrow). CDP, common DC precursor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; MDP, macrophage/DC precursor; MDSC, myeloid derived suppressor cells; MΦ, macrophage; pDC, plasmacytoid DC; pre-cDC, preclassical DC; ST-HSC, short-term haematopoietic stem cell; TipDC, TNF-α iNOS producing DC. (Modified from Geismann, Science)

1.2.2 The mononuclear phagocyte system

1.2.2.1 *The monocyte system*

Monocytes in mice represent a morphologically distinct population distinguished by their irregular shape, presence of cytoplasmic vesicles and high cytoplasm to nucleus ratio. A number of markers have been used to better characterise different subsets according to their origin and functional activity.

Mouse monocytes are identified in blood by the expression of the integrin CD11b (macrophage-1 antigen, Mac-1), CD115, F4/80 (member of EGF-TM7 family of leukocyte plasma molecules), Dectin-1 (β -glucan receptor), the chemokine receptor CX3CR1, and a variable expression of the Gr-1/lymphocyte antigen 6C (Ly6C) and 7/4 antigen [183, 184]. Anti-Gr-1 monoclonal antibody (RB6-8C5) binds with different specificity two molecules, Ly6C and Ly6G. The epitope Ly6C is not only expressed by monocytes, but also by NK cells, granulocytes and pDC, whereas the epitope Ly6G is expressed only by granulocytes [185].

Two main subsets of blood monocytes have been identified in mice, the Gr-1⁺ Ly6C^{high} ‘inflammatory’ subset, and the Gr-1⁻ Ly6C^{low} ‘resident’ subset [184]. The developmental relationship between these two subsets remains incompletely defined. Many investigators have proposed that Gr-1⁺ Ly6C^{high} shuttle between the blood and the BM and lose Ly6C expression, thus becoming Ly6C⁻ monocytes [165, 186, 187]. However, genetic defects impairing the presence of Gr-1⁺ Ly6C^{high} monocytes, such as cyclin-dependent kinase inhibitor p21 [188] and the transcription factor KLF4 [189], do not impair the presence of Gr-1⁻ Ly6C^{low} monocytes. In an attempt to define their relationship, additional subsets have recently been proposed, such as the Gr-1⁺ Ly6C^{int} subset [190].

1.2.2.1.1 Inflammatory $Gr-1^+$ $Ly6C^{high}$ monocytes

The main subset of blood monocytes is represented by the inflammatory subset, expressing CD115, $Ly6C$, $CCR2$ and $CD62L$ (adhesion molecule L-selectin), but low $CX3CR1$. These cells egress from the BM in $CCR2$ - $CCL7/CCL2$ dependent fashion and are recruited to inflamed tissues and lymph nodes *in vivo* [184, 191-193].

Monocytes differentiate into $TNF-\alpha$ iNOS producing DC (Tip-DC) via a $MyD88$ dependent mechanism [194] associated with the production of high levels of $TNF-\alpha$ and NO, and the up-regulation of $CD86$, $CD80$, $CD11c$ and MHC class II [195-197]. $CCR2$ appears to be a key molecule because $CCR2$ -deficient mice have a severe reduction in the number of Tip-DC, associated with a reduced control and clearance of bacterial infection [195].

Inflammatory monocytes have also been shown to replenish the macrophage and DC populations in skin [198], digestive tract [165] and lung during inflammation [199, 200].

Cells sharing the same phenotype of inflammatory monocytes have been found increased in spleen of tumour-bearing hosts [201]. These cells have the peculiar capability of inducing the recruitment of tumour-induced T regulatory cells (T_{REGS}) and inducing T cell anergy, and they are referred to as ‘myeloid derived suppressor cells’ (MDSC) [202-204]. It is still under investigation the hypothesis that $Gr-1^+$ $Ly6C^{high}$ circulating monocytes could give rise to these cells. It could also be suggested that inflammatory monocytes can give rise to MDSC in the tumour and Tip-DC in the inflammatory environment, but this remains to be confirmed.

1.2.2.1.2 Resident $Gr-1^-$ $Ly6C^{low}$ monocytes

The second subset of circulating monocytes is smaller in size and defined by the $Gr1^-$ $Ly6C^{low}$ $CD115^+$ phenotype. Characteristically, it exhibits high expression of the chemokine receptor

CX3CR1, lymphocyte function associated antigen 1 (LFA-1) and CD43, and lacks Gr-1, CCR2 and L-selectin (CD62L) [184]. This subset has been termed ‘resident’ because of its longer half-life and its presence in both resting and inflamed tissues.

During homeostasis, Gr-1⁻ Ly6C⁻ monocytes patrol blood vessels as demonstrated by intravital microscopy, and the process is mediated by the LFA-1/ICAM-1 interaction and CX3CR1 [205]. During tissue inflammation such as *Lysteria monocytogenes* infection, instead, these ‘resident’ monocytes extravasate and start to develop a peculiar transcriptional program with the up-regulation of cytokines, lysozyme, defensins, complement, and chemokines [205]. Interestingly, this type of monocytes is participating only in a transient response, as they are replaced by inflammatory monocytes within few hours after the infection [205]. After myocardial infarction, it has been observed that Gr-1⁻ Ly6C^{low} cells are recruited to the damaged tissue via CX3CR1 and, after its digestion, they express vascular endothelial growth factor (VEGF) and promote healing through myofibroblast accumulation, angiogenesis and deposition of collagen [191].

Studies performed on extravasated Ly6C^{low} resident monocytes demonstrate that the transcriptional program initiated by these monocytes resembles the one of M2-like (alternatively activated) macrophages [206]. Conversely, extravasated inflammatory monocytes start a transcriptional program similar to Tip-DC or M1 (classically activated) macrophages [205]. This concept of fate-defined subsets of monocytes is in apparent contradiction with the accepted view of monocytes as plastic cells [207]. Combinations of cytokines and growth factors can determine *in vitro* the differentiation of monocytes and macrophages. For example, monocytes can be induced to become DC by IL-4 and GM-CSF [185]; macrophages, in presence of macrophage colony-stimulating factor (M-CSF) [208]; M1 macrophages, with M-CSF and IFN- γ , or M2 macrophages, with M-CSF and IL-4 [209, 210]. It is therefore accepted that both the microenvironment and the transcriptional programs play a role in determining the fate of monocytes.

1.2.2.2 The macrophage system

Macrophages are scattered in almost every lymphoid and non-lymphoid tissue, where they exert fundamental effects in tissue homeostasis. They are responsible for clearing 200 billion erythrocytes per day, removing cell debris deriving from apoptotic and senescent cells, and remodelling the tissue after inflammation. Most of their actions are mediated independently of other immune responses. When stimulated by danger signals, such as heat shock proteins, DNA, histone proteins, macrophages alter their physiology, surface protein expression and production of cytokines, stimulating in such way an immune response.

1.2.2.2.1 Tissue distribution of macrophages

Macrophages are divided into subpopulations on the basis of their anatomical location and functional phenotype. Several specialized macrophages reside in tissues throughout the body, and these include osteoclasts in the bone, alveolar macrophages in the lungs, Langerhans cells in the epidermis, Kupffer cells in the liver, microglia in the brain. The gut and secondary lymphoid organs are also populated by distinct populations of macrophages with different phenotype and function. For instance, macrophages found in the thymus and in the white pulp of the spleen (called ‘tingible-body’ macrophages) are important for clearance of apoptotic lymphocytes generated during an immune response. High phagocytic and bactericidal activity is instead exhibited by lamina propria macrophages [211]. These tissue-specific macrophage subpopulations ingest foreign materials and recruit additional macrophages from circulation during an infection or following injury.

1.2.2.2.2 Phenotype of murine macrophage subsets

The phenotype of macrophages in mouse is hard to define. Although several molecules are in use to characterise macrophages (**Table 1.1**), their distinction from DC is still imprecise. CD11b and CD18 (Mac-1), F4/80, macrosialin (CD68), mannose receptor (MR, CD206) are some of the molecules used

to distinguish macrophages and DC. However, macrophages also express low amounts of CD11c, a marker that is used to identify DC, and, in turn, some DC but not all the macrophages express F4/80. Also, the nomenclature defying some subsets of DC or macrophages is misleading. As an example, the subset of Tip-DC is more similar to an inflammatory macrophage than an antigen-presenting cell (APC), whereas the subset of CD169⁺ macrophages has APC functions [212]. However, few combinations of markers can distinguish DC from macrophages, probably because they exist in a continuum and they arise from a common precursor.

Functionally, macrophages have been schematically classified in several subsets, which present a great overlap in surface marker expression. **Table 1.2** represent the markers used to distinguish the different subsets through gene expression. M1, or ‘classically activated’ macrophages, mediate defence of the host from bacteria, parasites and viruses, and have roles in antitumor immunity. M2, or ‘alternatively activated’ macrophages [213], have anti-inflammatory functions and regulate tissue remodelling. ‘Regulatory’ macrophages produce high levels of the anti-inflammatory cytokine IL-10 in response to TLR and immune complexes [214]. Further subsets are the tumour-associated macrophages (TAM), which suppress antitumour immunity and are closely linked to myeloid derived suppressor cells (MDSC). Although these subsets are distinguished functionally and phenotypically, it is generally believed that macrophages consist in a spectrum of activated phenotypes rather than discrete stable subpopulations.

Marker	Gene	Comments
CD115	Csf1r	Expressed on all monocytic cells, including macrophages and osteoclasts
CD11b	Itgam	Expressed on all myeloid lineage cells, including neutrophils
CD11c	Itgax	Expressed on many monocytic-derived cells, including macrophages. Enriched in certain populations of dendritic cells
CD169	Siglec1	Expressed on DC and on macrophages with APC functions; recently identified in a subset of BM macrophages regulating niche activity
CD206	Mrc1	Expressed on all tissue-specific macrophages but also on mesangial cells and hepatic endothelium; not clear if expressed by DC in vivo
CD62L	Sell	Expressed by neutrophils, monocytes, and subsets of T, B, and NK cells, mediating the rolling of blood cells
CD68	Cd68	Expressed on all macrophages
F4/80	Emr1	Expressed on most tissue macrophages in the mouse.
IL-4Ra	Il4ra	Expressed on most macrophages, but also on lymphocytes and other cell types that are responsive to IL-4 and IL-13
Ly-6C	Ly6c1	Enriched on monocytic myeloid lineages. A useful marker system when used together with LY6G to determine relative amounts of granulocytes and monocytes or macrophages.
Ly-6G	Ly6g	Enriched on granulocytes. A useful marker system when used together with LY6C to determine relative amounts of granulocytes and monocytes or macrophages
MAC2	Lgals3	Distributed in epithelia of many organs and various inflammatory cells, including macrophages, as well as dendritic cells and Kupffer cells

TABLE 1.1. Cell surface markers of murine macrophages. *Csf1r*, colony stimulating factor 1 receptor; *Emr1*, EGF-like module containing, mucin-like, hormone receptor-like sequence 1; *Il4ra*, interleukin-4 receptor, alpha; *Itgam*, integrin alpha-M; *Itgax*, integrin alpha-X; *Lgals3*, lectin, galactose binding, soluble 3; *Ly6*, lymphocyte antigen 6; *Mrc1*, mannose receptor, C type 1; *Sell*, selectin, lymphocyte; *Siglec*, sialic acid binding Ig-like lectin 1, sialoadhesin. Modified from [1].

Marker type	Gene	Comments
M1 markers	Cox2 (Ptgs2)	Highly induced in M1 activation
	Ido1	Useful marker of exposure to type 1 and 2 interferons
	Il12b	Highly induced in M1 activation
	Il23a (Il23p19)	Highly induced in M1 activation
	Marco	Calmodulin-associated. Also found in other activation scenarios
	Nos2	Highly upregulated in M1 and mediator of immunosuppressive properties
	Socs3	Induced by IL-10 and IL-6
M2 markers	Arg1	Highly upregulated in M2 and mediator of immunosuppressive properties
	Ccl12	Chemokine linked to TH2 cell responses
	Ccl24	Chemokine linked to TH2 cell responses
	Chi3l1 (Gp39, Ykl40)	Highly induced by IL-4 and IL-13. Not macrophage-specific
	Chi3l3 (Ym1)	Highly induced by IL-4 and IL-13
	Chia (Amcase)	Highly induced by IL-4 and IL-13. Not macrophage-specific
	Cxcl13	Chemokine linked to TH2 cell responses
	Fizz1 (Relma, Retnla)	Highly induced by IL-4 and IL-13
	Irf4	Highly induced by IL-4 and IL-13. Not macrophage-specific
	Klf4	Transcription factor induced by IL-4
	Socs2	Highly induced by IL-4 and IL-13. Not macrophage-specific

TABLE 1.2. M1-M2 markers for activated macrophages. *Arg1*, arginase 1; *Ccl*, CC-chemokine ligand; *Chi3l*, chitinase 3-like; *Chia*, chitinase, acidic; *Cxcl13*, CXC-chemokine ligand 13; *Ido1*, indoleamine 2,3-dioxygenase 1; *Il*, interleukin; *Irf4*, interferon regulatory factor 4; *Klf4*, Krüppel-like factor 4; *Marco*, macrophage receptor with collagenous structure; *Mrc1*, mannose receptor, C type 1; *Nos2*, nitric oxide synthase 2, inducible; *Ptgs2*, prostaglandin-endoperoxide synthase 2; *Relma*, resistin-like molecule alpha; *Socs*, suppressor of cytokine signalling. Modified from [1].

1.2.2.2.3 Classically activated macrophages

The term ‘classically activated’ refers to the effectors that are produced during cell-mediated immune responses (**Figure 1.5**). M1 cells have an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype, and they produce other inflammatory cytokines, such as IL-1 β , TNF- α and IL-6. They participate as inducers and effectors of Th1 immune responses, and mediate resistance against bacteria, protozoa, viruses and tumours. M1 macrophages are activated by IFN- γ , either alone or in combination with other cytokines, such as TNF- α and GM-CSF, or TLR stimulation by microbial products [215]. IFN- γ can be produced either by other cells of the innate immune system, such as NK cells, or by cells of the adaptive response, like Th1 cells. Once macrophages sense IFN- γ , they secrete pro-inflammatory cytokines, produce increased amounts of superoxide anions, oxygen and nitrogen radicals to increase their microbicidal activity [216]. However, if not tightly controlled, the amount of inflammatory cytokines produced by these cells can lead to host-tissue damage. M1 macrophages have been involved in several autoimmune diseases, such as rheumatoid arthritis [217] and inflammatory bowel disease [218].

1.2.2.2.4 Alternatively activated macrophages

‘Alternatively activated’ macrophages refer to various forms of macrophages sharing an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype, but with variable production of inflammatory cytokines (**Figure 1.5**). They are induced by IL-4 and IL-13, immune complexes, IL-10, glucocorticoids and secosteroid hormones [213], but also activin-a (member of TGF- β family) [219] and IL-21 [220]. They generally express high levels of scavenger, mannose and galactose receptors, and they up-regulate the enzyme arginase-1 (Arg-1) for the production of ornithine and polyamines. M2 macrophages differ from their M1 counterpart also in the regulation of components of the IL-1 system, with low levels of IL-1 β and caspase-1, and high levels of IL-1RA and IL-1RII receptor. They participate in parasite encapsulation [221], tissue remodelling and angiogenesis [222], and they promote tumour progression. Such actions are promoted through the production of growth factors, such as TGF- β 1 and PDGF [223], but also

matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP), which regulate extracellular matrix turnover, and immunoregulatory proteins, such as IL-10, Arg-1 [224] and resistin like α (Retnla, Fizz1) [225]. Although M2 wound healing and fibrosis-promoting activities contrast M1 responses, they can originate from M1 converted macrophages [226].

Based on their wide variety of physiological statuses, several classifications of M2 macrophages have been proposed. Mantovani *et al.* [227] have suggested the existence of three types of M2 macrophages, based on the stimuli needed to activate them. M2a are induced by IL-4 and IL-13; M2b by immune complexes and agonists of TLRs or IL-1R; M2c by IL-10 and glucocorticoid hormones. Other have proposed that ‘wound healing’ macrophages, activated by IL-4, are the one responsible for tissue remodelling, producing minimal amounts of pro-inflammatory cytokines and inducing regulatory effect on lymphocytes, whereas ‘regulatory’ macrophages, induced by glucocorticoids, TGF- β , IL-10, prostaglandins, and immune complexes, exert immunoregulatory functions by up-regulating IL-10 [228].

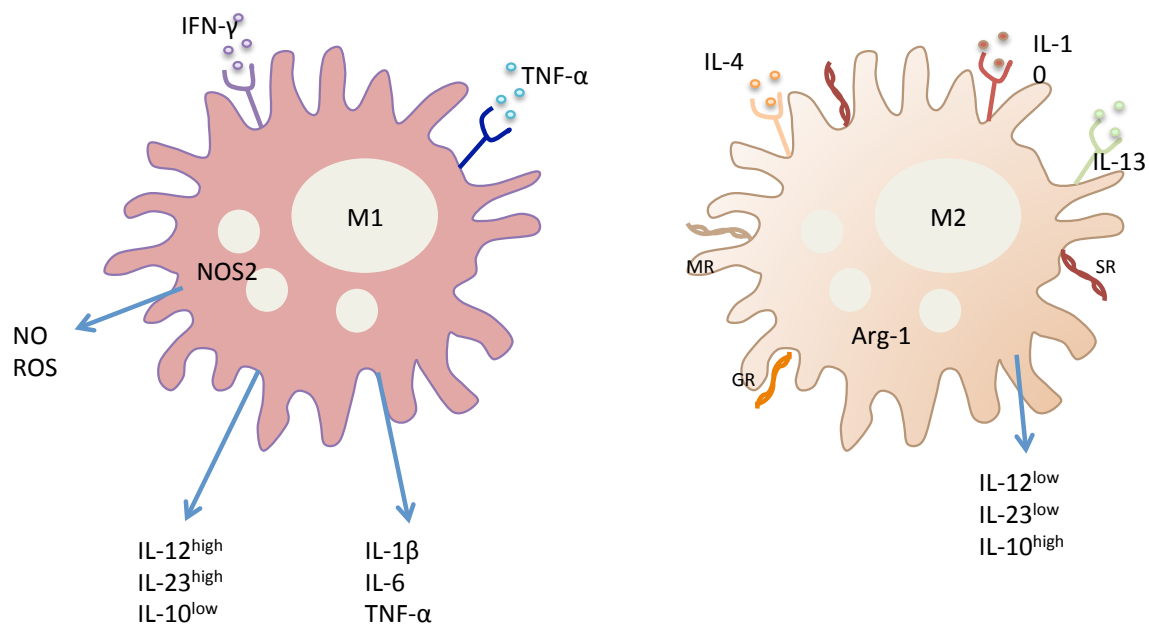


FIGURE 1.5. Macrophage subsets. M1 macrophages have a pro-inflammatory phenotype characterised by the expression of IL-12 and IL-23, and the up-regulation of NOS2, and they are induced by several pro-inflammatory stimuli. M2 macrophages have an anti-inflammatory phenotype, with high expression of IL-10, and characterised by the up-regulation of the enzyme Arg-1. They are induced by IL-4, IL-10 and other anti-inflammatory stimuli, and they are characterised by the up-regulation of scavenger (SR), mannose (MR) and galactose receptors (GR) on their surface.

1.2.2.2.5 Intrinsic and environmental regulation of macrophages

A complex interplay of intrinsic differentiation factors and environmental signals determines the existence of many different phenotypes and functions of macrophages. So far, there is not a comprehensive knowledge of the complex relation between transcriptional regulation and external cues.

PU.1 is a fundamental transcription factor for induction and maintenance of macrophage differentiation. A genome-wide role has been suggested for PU.1, which controls the formation and access of macrophage specific regulatory genomic regions and promotes the binding of other macrophage-specific transcription factors by acting through the methylation pattern of histone proteins [229]. Nevertheless, the interaction between PU.1 and macrophage-specific transcription factors needs to be further investigated.

Distinct signalling pathways determining M1 and M2 fates have been identified. IFN- γ , known to induce M1 macrophages, acts through the Janus kinase signal transducer and activator of transcription (JAK-STAT). Subsequently, STAT1 binds to ' γ -activated sequences' in the promoters of genes encoding NOS2, MHC class II transactivator (CIITA), and IL-12 [230]. STAT-1 and STAT-2 are also involved in TLR4 mediated signalling. LPS induces in fact the autocrine production of IFN- β , which acts through the induction of a heterodimer of STAT-1 and STAT-2 on interferon stimulated response elements (ISRE) that are found in several M1 genes [231].

STAT6, which can be induced by IL-4 [232], is instead regulator of M2 signature genes such as Arg-1, Fizz1, CD206, and chitinase-3 like 3 (Chi3l3).

Another important factor for the induction of M2 macrophages is the nuclear receptor peroxysome proliferator activated receptor- γ (PPAR- γ). Macrophages of adipose tissue constitutively express PPAR- γ , where it acts as an important regulator of macrophage-mediated lipid metabolism [233].

Nos2 expression is inhibited by PPAR- γ partly by antagonizing the activities of several transcription factors, including STAT. The activity of PPAR- γ can be augmented by IL-4 signalling through an interaction between PPAR- γ and STAT6 on promoters of PPAR- γ target genes [234].

C/EBP- β is another important transcription factor for M2 macrophage induction, and in particular it can induce Arg-1 expression in response to TLR signalling [235]. The induction of Arg-1 is not made redundant by C/EBP- β and STAT-6, as these two transcription factors are activated by different stimuli, and act on different cis-elements of Arg-1 promoter.

1.2.2.2.6 Macrophages and tissue injury

During inflammation, the number of resident macrophages is insufficient to mediate microbial control and tissue repair, and circulating monocytes are recalled from the blood (**Figure 1.6**). Tissue stress, acute or chronic inflammation, and sterile inflammation can induce ‘emergency myelopoiesis’, dependent on cytokines such as granulocyte colony stimulating factor (G-CSF), and chemokines like CCL5 and MCP-1 [192].

Inflammatory monocytes are recruited at the sites of inflammation in several types of acute infections. Rapid migration to the inflammatory tissue is induced by chemokines, such as MCP-1, produced by local inflamed tissues [236] or by BM MSC that sense low circulating amounts of TLR [102]. Once recruited at the site of inflammation, monocytes differentiate into macrophages or DC, and their activity is likely to be switched off by IL-10 [236].

It has not yet been elucidated if and how monocytes differentiate into macrophages. On the other hand, it has been recently demonstrated that macrophages are able to proliferate in situ in response to IL-4 [237]. In a Th2 type of inflammatory process, induced with infection from nematode *Litomosoides sigmodontis*, macrophage proliferation in situ was induced also after depleting circulating monocytes by treatment with clodronate liposomes.

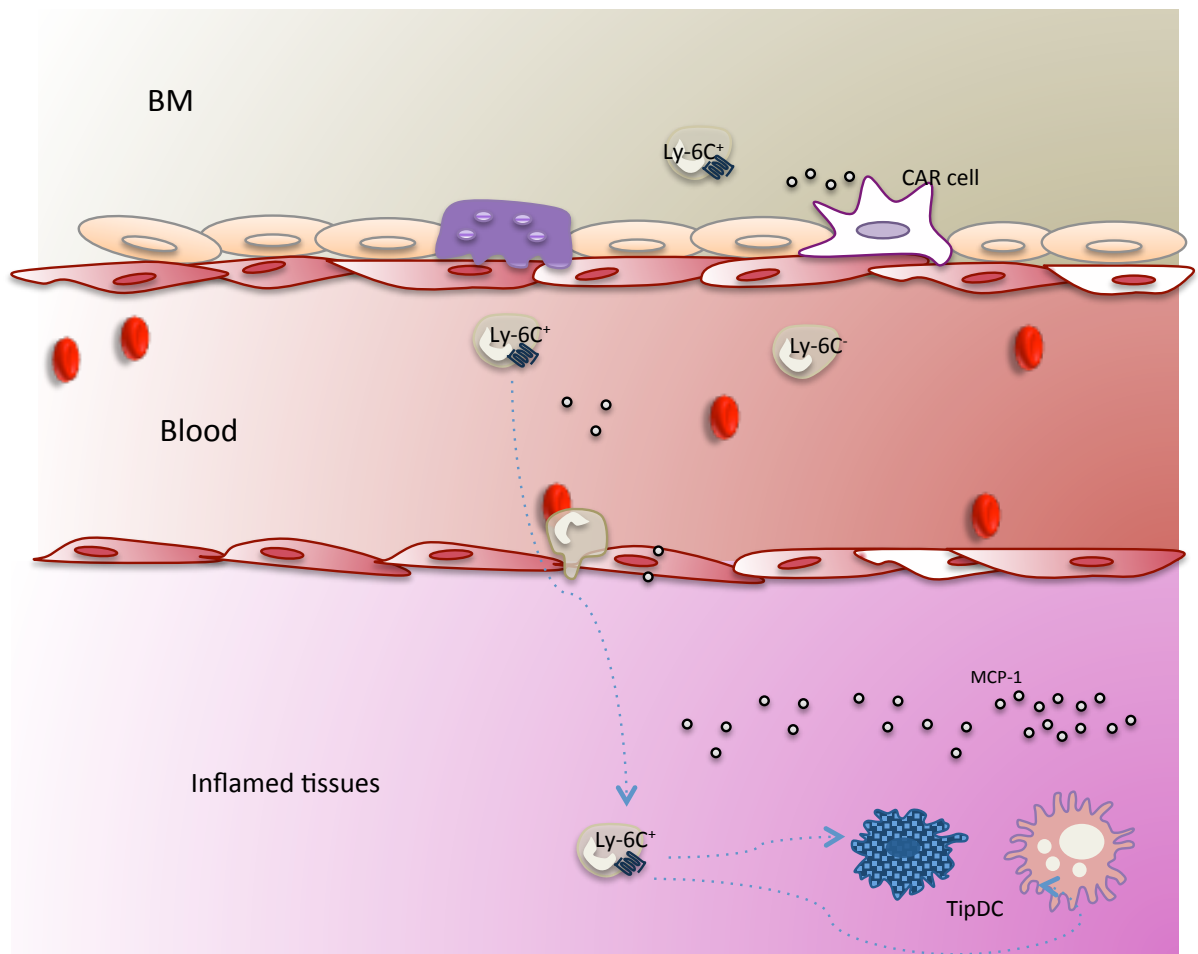


FIGURE 1.6. Macrophages are recruited during tissue injury. Ly6C⁺ monocytes egress from the BM and extravasate into the blood, in response to the chemokine MCP-1 produced by CAR cells and by inflamed tissues. Once in the tissue, monocytes differentiate either in Tip-DC or in M1 macrophages in order to help resolution of inflammation and tissue repair.

1.2.2.2.7 Macrophages and cancer

Macrophages play a key role in cancer (**Figure 1.7**) [238]. TAM are major components of the tumour leukocyte infiltrate. They are recruited by M-CSF, CCL2 and VEGF [238].

TAM express many genes typical of the M2 phenotype, and have therefore been described as ‘M2-skewed’. However, recent findings suggest that their phenotype varies with the stage of tumour development. M1-like cells have been found at the tumour site, whilst M2-like cells predominate during the stage of invasion, associated with vasculogenesis [239].

Accordingly, TAM promote angiogenesis [240], facilitate tumour invasion and metastasis [241], and protect tumour cells from chemotherapy-induced apoptosis [242]. They are also able to inhibit M1 tumouricidal responses, impair T cell activation, and produce IL-10 to stimulate Th2 and CCL22 to attract T_{REGS} [243]. T cell apoptosis is mediated through the expression of PD1 ligand 1 (PDL1) binding on programmed-death 1 receptor (PD1) on T cell surface [244] and by depleting L-arginine through up-regulation of Arg-1 [245].

1.2.2.2.8 Myeloid derived suppressor cells

Tumour cells also recruit a highly heterogenous myeloid population from BM and spleen with important functions for the promotion of neovascularization and metastasis of the tumour. This population, defined as myeloid derived suppressor cells (MDSC), [246] consists of progenitor and immature myeloid cells that exhibit immunoregulatory properties. They are present also in healthy individuals, where they differentiate into mature granulocytes, macrophages or DC without causing immunosuppression.

In the normal mouse BM, MDSC represent 20-30% of the total population, and 2-4% of the spleen, whereas they cannot be found in draining lymph nodes. During pathological conditions, such as

cancer, infectious diseases, trauma, sepsis, BM transplantation or autoimmune diseases, a block in the differentiation of immature myeloid cells causes their expansion [247].

Two main subsets of MDSC have been identified, both in mice and humans: monocytic MDSC and polymorphonuclear or granulocytic MDSC [248]. Polymorphonuclear MDSC, whose phenotype is defined as $CD11b^+ Gr-1^{high} Ly6C^{low} Ly6G^+ CD49d^-$, are the most represented subset in tumour-bearing mice, and also the major subset of circulating MDSC. They inhibit $CD8^+$ T cell responses by activation of reactive oxygen species (ROS), and they are less immunosuppressive than their monocytic counterpart [249, 250]. Monocytic MDSC are defined as $CD11b^+ Gr-1^{mid} Ly6C^{high} Ly6G^- CD49d^+$, and they have variable expression of F4/80, CD115, 7/4 and CCR2. They inhibit CD8 T cells through the induction of NOS2 and Arg-1, and the production of ROS [249, 250].

Corroborating the evidence that MDSC are a population of immature cells, some similarities with other myeloid cells can be found and MDSC have been shown to differentiate into mature myeloid cells, both *in vitro* and *in vivo*. Polymorphonuclear MDSC and neutrophils have a similar morphology [251], whereas monocytic MDSC are similar to inflammatory monocytes for their phenotype and morphology. Not only are MDSC distinguished for their immunosuppressive activity, but also for the coordinated up-regulation of enzymes such as NOS2 and Arg-1 [252]. Furthermore, MDSC includes progenitors of DC, macrophages and granulocytes. Several *in vitro* experiments have shown that MDSC can differentiate into mature cells. For instance, polymorphonuclear MDSC can differentiate into neutrophils [251], and culture of tumour-derived MDSC can give rise to mature macrophages and DC in absence of tumour soluble factors [253]. Finally, it has been shown that MDSC in the hypoxic microenvironment become TAM [254].

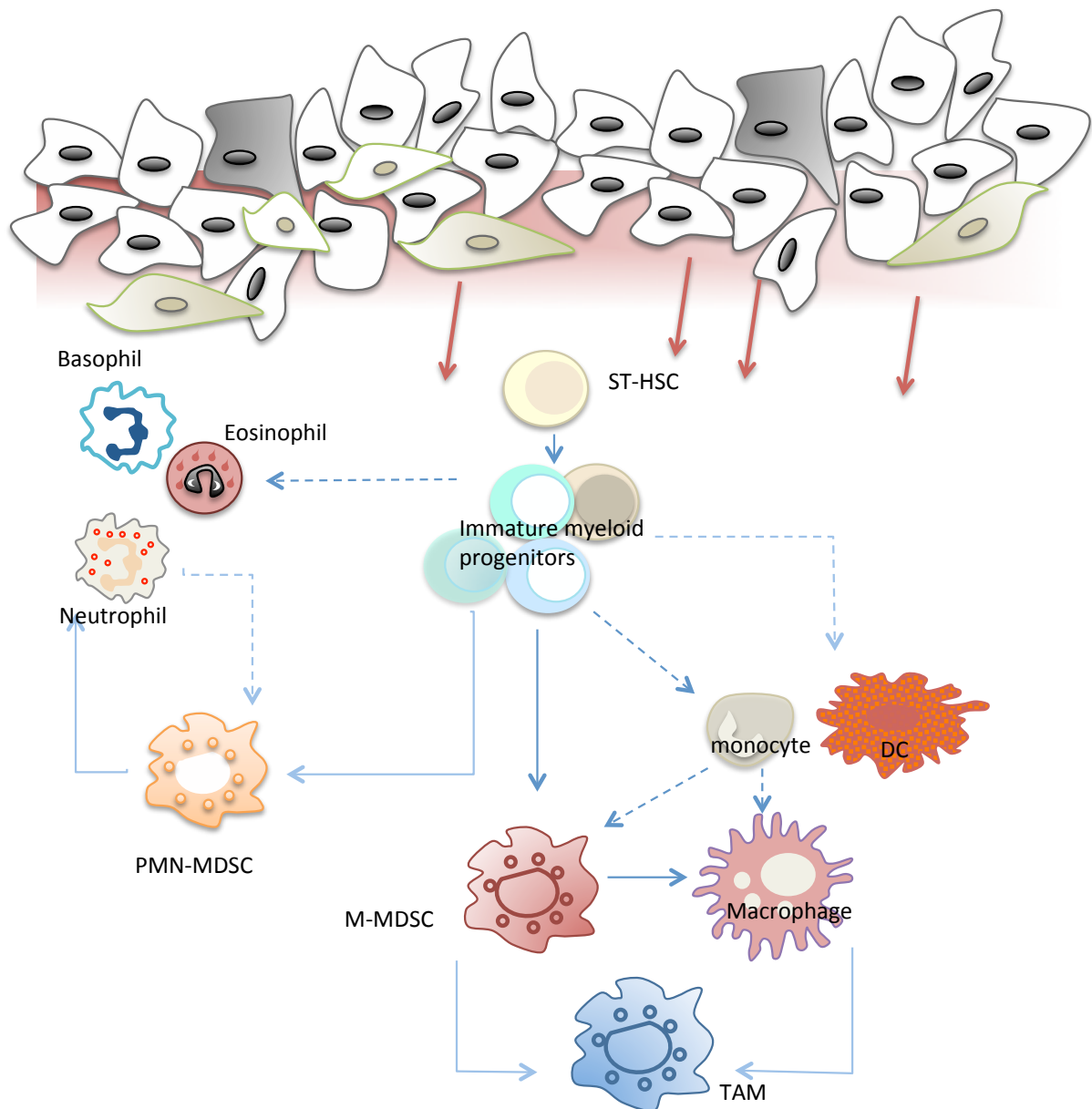


FIGURE 1.7. Myeloid cells in cancer. Factors produced in the tumour microenvironment by tumour cells and stromal cells promote the aberrant differentiation of myeloid cells. Immature myeloid cells normally differentiate into DC, monocytes, macrophages and granulocytes (dotted arrows). During cancer, aberrant differentiation gives rise to various immunosuppressive populations, such as monocytic MDSC (M-MDSC), polymorphonuclear MDSC (PMN-MDSC), and TAM. It is still unclear whether monocytes and neutrophils can give rise to M-MDSC and PMN-MDSC respectively. MDSC, myeloid derived suppressor cells; TAM, tumour-associated macrophages. (Modified from [286])

The mechanisms of modulation of innate and adaptive immune responses by MDSC are several and include depletion of nutrients [245], oxidative stress [255], inhibition of lymphocyte trafficking, induction of T cell apoptosis [256], and activation and expansion of T_{REGS} [257].

Stimulation of myelopoiesis and the subsequent inhibition of differentiation of mature myeloid cells cause MDSC expansion. The mediators are myelopoietic growth factors (GM-CSF, G-CSF, M-CSF, stem cell factor - SCF, and VEGF) [247], and pro-inflammatory cytokines (IL-6, IL-1 β , and IFN- γ , IL-4, IL-10 and IL-13) [258]. Most of these factors involve STAT3 signalling, which, amongst other functions, induces C/EBP β , fundamental for the differentiation of MDSC [259] and myelopoiesis in general. However, it is not clear whether the first wave of immature cells recruited to tumours is immunosuppressive. Continuous stimulation of myelopoiesis and activation of immature cells from soluble factors could therefore be fundamental to drive the accumulation and differentiation of MDSC.

1.2.2.2.9 Macrophages in the haematopoietic niche

BM contains at least two populations of macrophages, namely osteoclasts and tissue resident macrophages (**Figure 1.8**). Osteoclasts differentiate from precursors found in the GMP population, and their development depends on the presence of M-CSF. Tissue resident macrophages as well as osteoclasts are fundamental components of the HSC niche.

The function of BM macrophages has recently been investigated. Rare activated BM monocytes and macrophages with high expression of α -smooth muscle actin (α -SMA) and COX2 have been identified adjacent to haematopoietic stem/progenitor cells (HSPC). This myeloid population is thought to prevent HSPC exhaustion by limiting the production of ROS through the expression of PGE₂ [260]. Macrophages can also act as positive regulators of MSC and osteoblasts to retain HSC in the BM. *In vivo* depletion of endosteal macrophages, either in macrophage Fas-induced apoptosis (Mafia) transgenic mice in which a suicide fusion protein (made of the cytoplasmic domain of Fas and

the FK506-binding protein) is expressed under CD115 promoter, or with clodronate administration, recapitulated effects similar to administration of G-CSF: depletion of osteoblasts, suppressed endosteal bone formation, decreased expression of factors required for HSC retention and self-renewal, and HSC mobilization into the blood [261]. A seminal study showed that the selective depletion of a specific subset of Gr-1⁻ CD115^{int} F4/80⁺ CD169⁺ macrophages was sufficient to induce HSPC egress. Interestingly, this subset localises in the vicinity of Nestin⁺ MSC niche cells, and its depletion induces a selective down-regulation of HSC-retention genes in niche cells [262]. There is still controversy regarding the mechanisms underlying such activity of macrophages in the niche. G-CSF receptor signalling has been suggested to induce HSPC mobilization and osteoblast suppression either by monocytes [263], or through the expansion of neutrophils [264].

The role of osteoclasts in the niche seems more direct and essential for its constitution as demonstrated by the increased proportion of MSC progenitors in osteopetrotic mice [265] and the reduction of HSC after bisphosphonate treatment [266]. Also osteoclast activation, resulting into the secretion of proteolytic enzymes, can mediate progenitor mobilization in the peripheral blood. Stimulation of osteoclasts with RANK-L recruits mainly immature progenitors to the circulation, and this is mediated by cathepsin K mediated degradation of niche molecules such as CXCL12, OPN and SCF. On the other hand, inhibition of osteoclasts with calcitonin reduced progenitor egress in homeostatic conditions, G-CSF mobilization and stress [267].

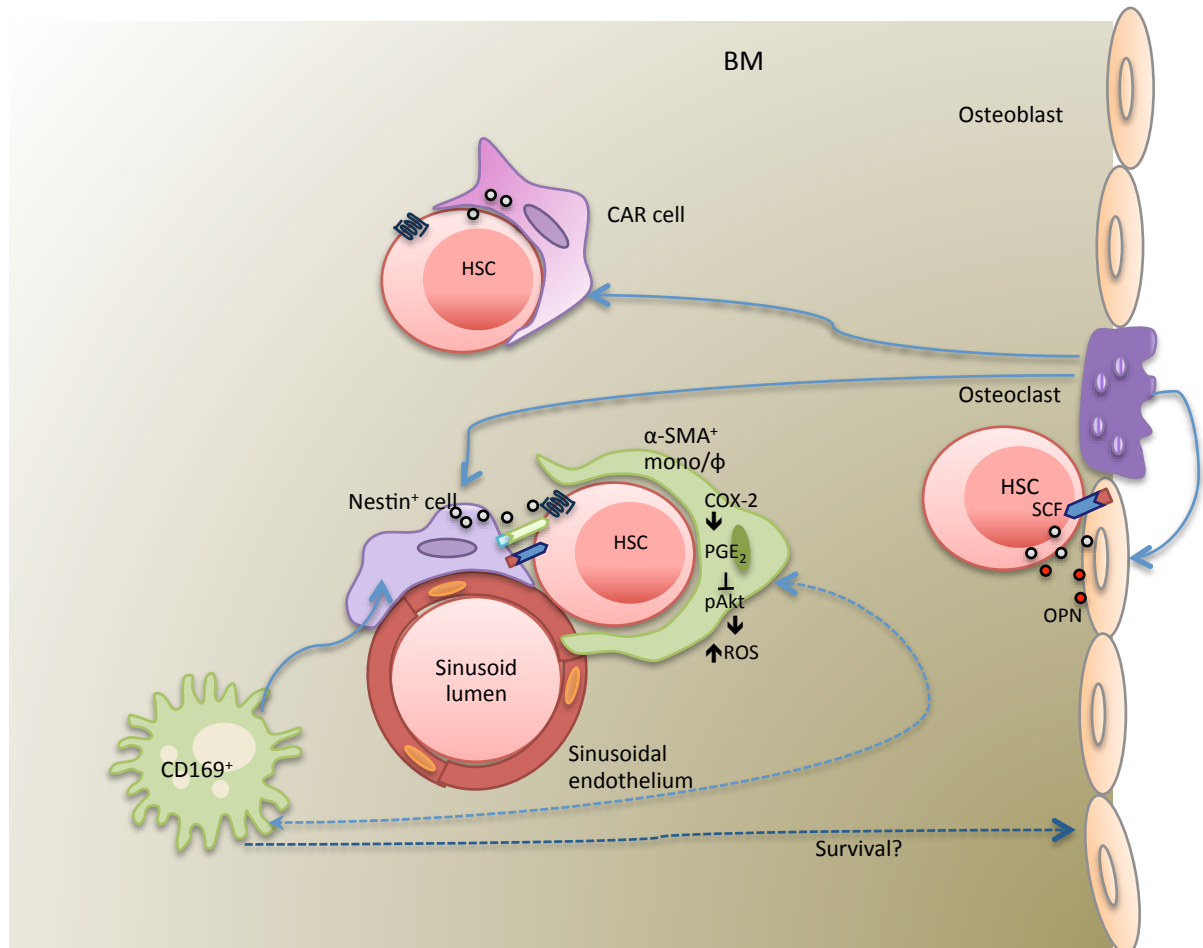


FIGURE 1.8. Macrophage role in the haematopoietic niche. Osteoclast activity is fundamental for osteoblast differentiation and MSC precursors capacity to retain HSC. BM monocytes and macrophages with high expression of α -SMA and COX2 (α -SMA⁺ mono/ ϕ) maintain the primitive phenotype of HSC by regulating Akt induction of ROS through PGE₂. In addition, PGE₂ results in higher CXCR4 expression on HSPC and higher CXCL12 expression in the nearby nestin⁺ MSC. A population of CD169⁺ macrophages is also fundamental to maintain nestin⁺ HSPC retention genes, and possibly to promote osteoblast survival (dotted arrow). The relationship between α -SMA⁺ cells and CD169⁺ macrophages has not yet been investigated (dotted arrow). CAR, CXCL12 abundant reticular cell; COX2, cyclooxygenase 2; OPN, osteopontin; PGE₂, prostaglandin E₂; ROS, reactive oxygen species; SCF, stem cell factor; α -SMA, α -smooth muscle actin.

1.3 THE ROLE OF NITRIC OXIDE IN IMMUNOSUPPRESSION

L-arginine is a conditionally essential amino acid in adult mammals, which must be supplied in the diet during some pathological conditions in which L-arginine biosynthesis cannot compensate for depletion or inadequate supply. It is mainly metabolized by arginase and nitric oxide synthase (NOS) enzymes to produce urea and L-ornithine, and nitric oxide (NO) and L-citrulline, respectively. Not only myelomonocytic cells, but also MSC exploit the metabolism of the amino acid L-arginine to restrain T lymphocyte proliferation [122] [268].

1.3.1 Nitric oxide synthase regulation

NOS is a family of enzymes present in three isoforms with different intracellular localization, regulation and catalytic properties: neuronal (nNOS, NOS1), inducible (iNOS, NOS2), endothelial (eNOS, NOS3). NOS1 and NOS3 are constitutively expressed in a variety of cell types at low levels, whereas NOS2 is expressed in various cells of the immune system, such as macrophages, eosinophils, neutrophils, DC and NK cells.

NOS enzymes are dimers that require the presence of calmodulin and the cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin (BH₄), iron–protoporphyrin IX (haem), flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) for full activity. Each isoform consists of an amino-terminal oxygenase domain and a carboxy-terminal reductase domain. Electron carriers FAD and FMN transfer the electron resulting from the conversion of NADPH to NADP to the oxygenase domain of NOS. The oxygenase domain then uses the cofactors haem and BH₄ to catalyse the reaction between oxygen and L-arginine, which generates L-citrulline and NO [269].

NOS2 expression is regulated by environmental cues. Th1-cytokines, such as IFN- γ , either alone or in combination with LPS, TNF- α , IFN- α , IFN- β and IL-1 up-regulate NOS2. In contrast, NOS2 transcription is down-regulated by steroids [270], anti-inflammatory cytokines (e.g., transforming growth factor- β [TGF- β], IL-10) [271], p53 [272], and NO itself [273]. IFN- α , β and γ mediate NOS2 induction by activating IRF-1, whereas LPS can induce NOS2 expression through stimulation of the mitogen-activated protein kinase (MAPK) pathway [274]. Hypoxia is another relevant factor in induction of NOS2 transcription due to the presence of hypoxia-responsive enhancer (HRE) on *NOS2* promoter [275]. Several transcription factors have been shown to be required for NOS2 gene expression, such as nuclear factor NF- κ B, Jak3/Stat1, and c-Jun NH2-terminal kinase (JNK) [276]. The promoter sequence of *NOS2* also contains a number of binding sites for transcription factors regulating cell proliferation, apoptosis and cell differentiation, such as C/EBP and hypoxia-inducible factor (HIF) [277]. The bioavailability of L-arginine, which is in turn influenced by the activity of arginase, is another important modulator of its function.

1.3.2 The immunoregulatory role of NO

NO is a diatomic free radical mediating a host of physiological events such as smooth muscle relaxation, vasodilatation, neurotransmission, inhibition of platelet aggregation, and immunomodulation [278-280]. Furthermore, at the cellular level, NO regulates cell growth, survival, apoptosis, proliferation, and differentiation [280]. As a result, NO is involved in the pathophysiology of several diseases [279].

Its downstream effects are mediated through either cyclic guanosine monophosphate (cGMP)-dependent or independent mechanisms. cGMP-independent effects of NO are mediated through its interactions with metal complexes, oxygen, and superoxide, DNA and proteins [281]. The reaction with other oxidants generates reactive nitrogen species (RNS), which can directly cause apoptosis or mutagenesis by damaging DNA and proteins [282]. Therefore, NO has a critical role in host-defence

against pathogens, but also in several immunological responses by regulating cytokine production, leukocyte chemotactic responses, cell survival, and thymic selection [269, 283, 284]. NO can also be involved in the pathogenesis of infectious diseases, chronic degenerative disorders and tumour growth [282, 285, 286].

The immunoregulatory activities mediated by NO on T cells [283, 287] are effected through the induction of apoptosis and/or by impairing cell activation. T lymphocyte apoptosis has been implicated in the regulation of T cell ontogeny both during thymic maturation and peripheral expansion [288].

The NO effects on the signalling cascade downstream of IL-2 are responsible for the impairment of T cell activation [289-291]. Phosphorylation and activation of signalling intermediates in the IL-2R pathway (JAK1, JAK3, STAT5, Erk, and Akt) are inhibited [255, 291].

NO exerts its effects also on other cells of the immune system. It impairs immunoglobulin expression on B cells [269, 292] and is required for NK IFN- γ mediated cytotoxic activity [293]. Th1 lymphocytes expansion is suppressed by high concentrations of NO, whereas low levels of NO induce their proliferation [294, 295].

During acute bacterial or parasitic infections, NO is described as a resistance factor elaborated by the host to fight pathogens. Persistent infection with parasites, such as *Trypanosoma*, *Chlamydia*, *Schistosoma*, or *Leishmania*, are instead commonly characterised by an increase in arginase expression concomitantly with the prevalent production of IL-4, IL-10, and TGF- β [296]. Recent results obtained in models of *Candida albicans* and *Trypanosoma cruzi* infections demonstrated the existence of an immunosuppressive mechanism depending on IFN- γ -induced secretion of NO by myeloid cells that colonize the spleen during the acute phase of infection [297-299].

1.3.3 NO and cancer

Tumour growth is often associated with an altered metabolism of the amino acid L-arginine, due to the activity of both NOS and arginase enzymes. The consequences of such activities by tumour-recruited myeloid cells and cancerous cells consist in L-arginine deprivation and/or release of NO, supporting tumour growth and inhibition of anti-tumour T cell responses.

Neoplastic transformation is a key step in cancer initiation. NOS2 mediates neoplastic transformation in oncogene- and chemical-induced tumourigenesis models, although conflicting results are reported in literature.

Oxidative and nitrosative stress are known for inducing DNA damage and inhibition of DNA repair enzymes through direct or indirect mechanisms. p53 tumour suppressor mutations, or S-nitrosylation of caspases, can induce apoptosis-resistance and facilitate the accumulation of further mutations. NO can also initiate and promote tumourigenesis. For instance, fewer polyps were found in the small and large intestines of mouse with genetic mutations in adenomatous polyposis coli (*Apc*) alone compared with mice carrying mutations in both *Apc* and *NOS2* [300]. In addition, *NOS2*^{-/-} mice showed decreased incidence of gastric carcinogenesis that is induced by *Helicobacter pylori* [301].

Another important effect of NO on tumour progression is determined by expression of angiogenic factors [302]. By up-regulating VEGF expression, several studies have shown that NOS2 induction in tumour cells promotes angiogenesis and consequently tumour progression. NO activates the transcription factor HIF1 α , and in turn the hypoxic microenvironment in growing tumours lesions induces both NOS2 and arginase enzymes through the stabilization of HIF1 α [303, 304], in particular in the populations of macrophages and immature myeloid cells associated with the tumour.

In contrast to these findings, NO has also been shown to inhibit tumour progression. DNA damage induces the accumulation of p53 and results in death of transformed cells, and relatively low levels of

NO up-regulate DNA-dependent protein kinase (DNA-PK), which maintains the integrity of the genome. Furthermore, in contrast with previous studies, increased intestinal adenomas in *Apc* and *NOS2* double-mutant mice were shown as compared to *Apc*-mutant mice [305].

A controversial effect of NO is also found in host stromal-cell derived NO, which inhibits the growth of NO-sensitive tumours and promotes the growth of NO-resistant tumours. For example, Wei *et al.* showed that *NOS2*-deficient tumour cells in a *NOS2*^{-/-} host grew faster and produced more lung metastases than those injected in wild type mice [306]. However, similar studies produced instead conflicting results. For example, lower VEGF expression and slower growth of B16 melanoma were shown in *NOS2*^{-/-} mice [307].

1.4 MESENCHYMAL AND HAEMATOPOIETIC STROMA INTERACTION

1.4.1 Interaction in peripheral tissues

The stroma of every lymphoid and non-lymphoid tissue is constituted of MSC, endothelial cells and resident macrophages, along with tissue-specific extracellular matrix (ECM) components. Tissue MSC, also referred to as tissue-resident fibroblasts, synthesize and remodel the ECM, but also regulate self-tolerance, organ development, wound healing, inflammation and fibrosis [308-311]. Tissue-resident macrophages have a broad role in the maintenance of tissue homeostasis, through clearance of apoptotic cells and resolution of inflammation.

Recent investigations delineated three main groups of stromal cells on the basis of their origin: MSC from mesenchyme, monocyte-derived fibroblasts, also referred to as ‘fibrocytes’, and fibroblasts arising from epithelial-mesenchymal transition (EMT).

As previously discussed, several studies have shown that ‘conventional fibroblasts’ share phenotype and immunosuppressive functions with BM MSC [13, 14], although further investigation is required to understand their developmental relationship.

Fibrocytes [312] are defined as a circulating blood leukocyte population with *in vitro* adherent properties, expressing markers of haematopoietic cells (CD34), leukocytes (CD11b, CD13 and CD45) as well as fibroblast products (collagens I, III, and fibronectin). Their identity is poorly defined regarding their relationship to cells of mesenchymal and haematopoietic origin but it is likely they represent a rare subset of macrophages with the ability to recirculate following injury [313, 314].

Although recent studies have indicated that macrophages and MSC interact to maintain HSC niche functions [262], few studies have addressed whether such interaction occurs also in peripheral organs. However, it is arguable that an MSC-macrophage interaction exists in other stem cell niches (**Figure**

1.9). In fact, the stem cell niche concept does not solely apply to the haematopoietic niche, but also to other adult tissues, such as liver, kidney, gut, skin and gonads.

During severe liver injury a cellular niche composed of myofibroblasts, macrophages and endothelial cells always surrounds the hepatic progenitor cells [315]. In particular, the interaction between Kupffer cells and hepatic stellate cells (which resemble MSC) has been recently investigated for its role in the process of hepatic injury and fibrotic progression. Stellate cells differentiate towards a pro-fibrotic myofibroblast phenotype, which undergoes apoptosis during recovery from fibrosis. *In vitro* and *in vivo* experiments have suggested that macrophages are able to regulate proliferation and apoptosis of stellate cells [316, 317]. Using a transgenic mouse model of liver fibrosis, which allowed discrete depletion of CD11b⁺ cells under the influence of diphtheria toxin, Duffield *et al.* were able to demonstrate that depletion of macrophages during the early phases of recovery resulted in sustained accumulation of scarring matrix. However, when macrophages were depleted during the progression phase of fibrosis, decreased scarring and fewer myofibroblasts were detected [317].

Similar results have been obtained in a model of crescentic glomerulonephritis. Depletion of macrophages reduced proliferation and apoptosis of tubular cells, and this was associated with a reduced number of glomerular crescents and improved renal function. The presence of macrophages, instead, supported a population of interstitial myofibroblasts with increased levels of proliferation and apoptosis [318].

The colonic epithelial niche also harbors myofibroblasts and macrophages. Subepithelial myofibroblasts continuously migrate upward from the crypt base, and it has been suggested that they assume a strategic position to establish and maintain instructive communications with stem cells. Like the myofibroblasts, macrophages have been shown to support and promote the proliferation of colonic epithelial progenitors in a model of dextran sodium sulphate (DSS) mediated epithelial injury [319].

Suggestions of interaction between macrophages and MSC are found also in the epidermal niche, in which CXCL14 and epidermal tissue have been shown as attractant and niche of differentiation for Langerhans cells respectively in a human tissue model [320]. In particular, human epidermal equivalents were able to affect the supra-basal positioning, survival and differentiation into Langerhans cell-like cells of CD14⁺ DC precursors.

Although both MSC and macrophages have been extensively studied for their role in tumour initiation and progression, few studies have addressed their interaction. In a murine model of mammary cancer, knock-out of specific hypoxia regulatory genes, such as HIF-1 α , in tumour stromal fibroblasts, led to accelerated tumour growth and reduction of F4/80⁺ cells infiltration [321]. Furthermore, myeloid progenitor cells recruited in a lung tumour have been shown to promote EMT of metastatic tumour cells through the expression of versican, an extracellular matrix proteoglycan [322]. Recently, several *in vitro* studies have shown that MSC isolated from tumours support survival and proliferation of tumour cells through the recruitment and differentiation of myeloid cells. For instance, MSC obtained from follicular lymphoma patients up-regulate CCL2 and exhibit increased ability to recruit monocytes compared to MSC from healthy donors [323].

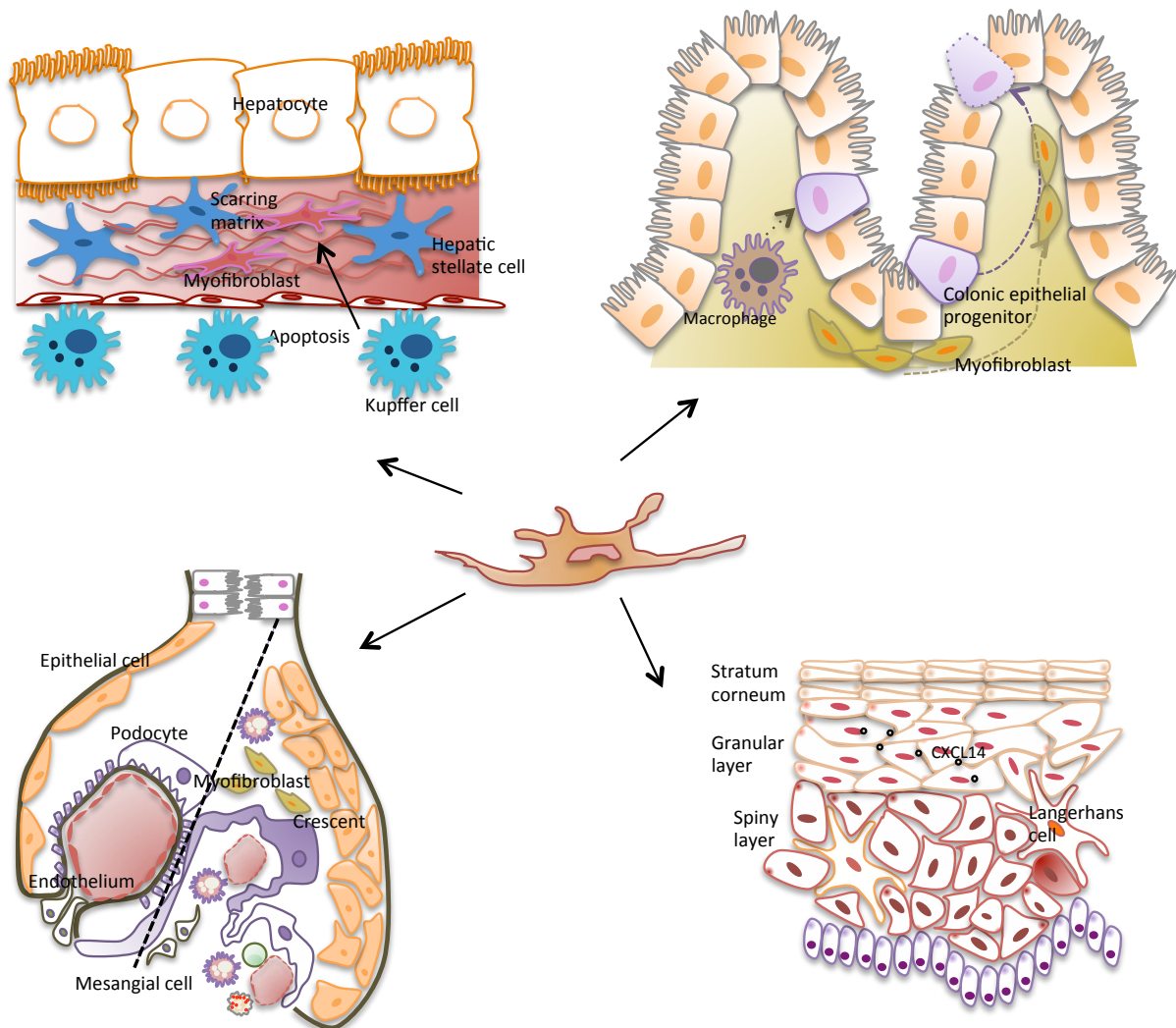


FIGURE 1.9. Evidence for a mesenchymal and haematopoietic stroma interaction in peripheral tissues. Kupffer cells in the liver regulate the apoptosis and proliferation of stellate cells, which differentiate into myofibroblasts during hepatic injury. Macrophages and myofibroblast support and promote the proliferation of colonic epithelial progenitor cells under inflammatory conditions. In the epidermal niche, the presence of stromal cells is important to promote the differentiation of Langerhans cells. In a model of crescentic glomerulonephritis, macrophages support the proliferation of myofibroblasts.

1.4.2 Interaction in the haematopoietic niche

It is still unclear how the cellular components of the BM niche interact together to regulate HSC functions. Nevertheless, recent investigations are highlighting the role of several soluble factors, such as cytokines, chemokine and growth factors, bioactive lipids, cell adhesion molecules in regulating HSPC egress from the BM. The further sections will discuss the involvement of these factors in HSPC mobilization and their possible role as candidate molecules involved in MSC and macrophages interaction in the niche.

1.4.2.1 *The molecular profile of the BM niche*

The modalities by which the BM niche regulates HSC activities are currently a hot topic, and several new investigations have highlighted the role of cellular components of the niche and their interactions, as well as the central nervous system, as potential players.

Osteoblasts and HSC are known to interact through Notch-Jagged-1 signalling, which prevents HSC differentiation in favour of self-renewal, and this, in turn, leads to HSC expansion [49]. Another important interaction is represented by osteoblast angiopoietin-1 and -2 (Ang-1 and Ang-2) with Tie2 on HSC [324]. This interaction determines the activation of phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway, and in turn activates cell survival pathways and cell cycle regulators, which may prevent HSC from metabolic exhaustion [324].

One of the first studies demonstrating a role of the sympathetic nervous system (SNS) in the HSC niche came from Katayama *et al.* [325], who demonstrated that G-CSF mobilization was reduced in mice in which the SNS was impaired. Osteoblasts and nestin⁺ MSC are innervated by the SNS [60], suggesting that SNS acts on several niche components. In particular, SNS inhibits cell cycle and differentiation of nestin⁺ cells, as demonstrated by chemical sympathectomy [60]. Furthermore, β 2-

adrenergic and dopamine receptors on HSC are up-regulated by G-CSF [326], and neurotransmitters can attract HSC.

Fatty acids have also been explored for a role in modulating HSC niches activities. Mice deficient in galactocerebrosidase (GALC), an enzyme that synthesizes the lipid components of myelin sheaths, have a defective niche in which HSC fail to engraft [327]. HSPC express the receptor for sphingosine-1-phosphate (S1P), which is part of the GALC metabolic pathway. It has recently been demonstrated that S1P induces CXCL12 secretion from BM MSC via ROS signalling, thus modulating steady state egress of HSPC [328]. Eicosanoids are also able to regulate haematopoiesis. PGE₂ regulates the expression of CXCR4 on HSPC and facilitates their attraction to CXCL12 and homing [329]. Furthermore, a population α -SMA⁺ COX2 expressing monocytes and macrophages preserves the primitive stem cell phenotype of the progenitors in the BM by controlling their ROS content and inducing a higher CXCL12 expression in nestin⁺ MSC [260].

Extracellular matrix proteins (ECM) are structural and functional organizers of the niche, augmenting progenitor cell localization within the haematopoietic microenvironment [330], and mediating adhesion of HSC to stromal cells [331]. Agrin, an extracellular matrix protein belonging to the family of heparan sulphate proteoglycans (HSPG), has recently been demonstrated as a non-redundant signal for HSC survival and proliferation mediated by niche MSC [332]. Agrin is a critical regulator of neuromuscular synapses where it binds to skeletal muscle Lrp4, leading to activation of MuSK, a receptor tyrosine kinase essential for initiating clustering of acetylcholine receptors under the nerve terminal [333]. The function of non-neuronal isoforms of agrin, expressed in numerous cell types, is poorly understood, although its receptor at the immunologic synapse has been identified as α -dystroglycan (α -DG) [334], a broadly expressed cell surface receptor critical for the early development stages [335]. Recent investigations on the role of agrin in post-natal haematopoiesis have shown that the absolute number of total leukocytes in all hematopoietic and peripheral compartments is reduced in agrin-deficient mice compared with control mice [332]. In particular, agrin-deficient mice show a significantly reduced frequency of monocytic cells in BM, spleen, and

peripheral blood, but not in lymphocytes or granulocytes, indicating that the absence of agrin specifically affects monocytes and macrophages [336]. The lack of agrin inhibits the differentiation of promonocytes into mature monocytes, and monocytes into macrophages, whereas it does not affect the differentiation of GMP into neutrophils [336]. LSK cells pre-treated with inhibitor of α -DG co-injected with control cells into lethally irradiated recipients displayed a reduced reconstitution ability, whereas agrin^{-/-} LSK were able to differentiate normally in an agrin-sufficient recipient [332], demonstrating that agrin expression is fundamental in the stromal but not in the haematopoietic compartment.

1.4.2.2 Role of pro-inflammatory cytokines

During inflammation, immune cell are highly consumed, and are replenished by proliferating haematopoietic precursors from the BM [337]. Mature blood cells are produced at a rate of more than one million cells per second in humans, whereas most of the HSC from which they derive are quiescent [338].

In recent times, it has been demonstrated that inflammatory cytokines can also contribute to shape the HSC compartment. *In vivo* injection of bacterial component LPS increases the proliferation and self-renewal of LSK through TLR4 binding on the surface of HSC [339, 340]. The role of pro-inflammatory cytokines, such as TNF- α and IFN- γ , is still controversial. It has been shown that TNF- α and IFN- γ can induce HSPC to proliferate [341] and determine impairment in self-renewal of these cells [342, 343]. In line with this finding, BM cells from TNF- α receptor knockout (TNFR^{-/-}) mice have a competitive advantage over wild type (WT) BM cells for their ability to reconstitute a myeloablated recipient, indicating that TNF- α may act as a potent suppressor of HSC activity *in vivo* [344]. Similarly, BM of mice deficient for IFN- γ show increased engraftment after transplantation when compared to WT mice [345]. In contrast with these data, mice lacking TNFR1 have increased

numbers of hematopoietic progenitors at baseline and develop increased marrow cellularity with age [346, 347].

Although it is not clear whether TNF- α and IFN- γ can indirectly act on HSC function, other pro-inflammatory molecules have been instead shown to modify the cellular components of the niche. Exposure to TLR ligands induces a block in the maturation of osteoclasts [348, 349], which have been shown to be fundamental for a normal formation of the HSC niche [265]. G-CSF inhibits CXCL12 production by osteoblasts and leads to increased mobilization of haematopoietic progenitors, in a process mediated by G-CSF receptor (G-CSFR) mediated signals in BM monocytes [263]. In a similar vein, a recent study provides evidence that the expansion of neutrophils in the BM in response to G-CSF, induces MSC progenitors and osteoblasts apoptosis through increased ROS production and reduces their expression of factors crucial for HSC retention in the BM [264]. Interestingly, CXCL12 is not only expressed by osteoblasts but also by CAR cells, affiliated to MSC, which express MCP-1 in response to circulating TLR ligands or bacterial infection, and induce an egress of monocytes from BM into the bloodstream [102].

The expression of HSC retaining genes, such as CXCL12, angiopoietin 1, SCF and VCAM1 by niche MSC is regulated by CD169⁺ BM macrophages. Accordingly, macrophage depletion promotes the egress of HSC in the niche [262]. The macrophage-derived factors mediating these effects are still unknown, although pro-inflammatory molecules, such as insulin-like growth factor 1, IL-1, IL-10 and TNF- α , have been proposed.

1.4.2.3 Role of complement

During inflammation, the human complement cascade represents a first line defence, not only eliminating foreign bodies, but also acting as a surveillance system capable of discriminating between host cells and pathogens or cell debris [350].

Several lines of evidence suggest that complement is also involved in the BM niche. C5a and G-CSF can activate granulocytes and monocytes to release proteolytic enzymes that cause detachment of HSPC from their niches [351, 352]. C3 fragments inhibit mobilization of HSPC, whereas C5 cleavage fragments promote it, as demonstrated by knockout mouse models [351, 353].

Even more directly, complement factors are involved in HSPC homing and engraftment. C3a can potentiate the homing gradient of CXCL12 and increase the responsiveness of HSPC to this gradient by including CXCR4 in lipid rafts. C3b instead has been shown to tether HSPC to the stroma [354]. C3- and C5-deficient mice show a delayed haematopoietic recovery after transplantation [354]. Transplantation of WT HSPC into C3^{-/-} mice resulted in a delay in platelet and leukocyte recovery and in a reduced number of BM GMP, whereas HSPC from C3^{-/-} engrafted normally into WT mice, suggesting a defect of the niche environment of C3^{-/-} [354]. Furthermore, an accumulation of soluble C5b–C9 membrane attack complex (MAC) has been shown after conditioning for haematopoietic transplantation by lethal irradiation. This complex has a role in homing and engraftment, as it activates signalling in HSPC, enhances adhesion of HSPC to BM MSC through the HSPC-expressed CR3 (CD11b/CD18) receptor and increases secretion of CXCL12 from BM stroma [355].

These data have provided the rationale to tested ex vivo priming of HSPC with C3a before transplantation to improve engraftments (Charlottesville, VA; and Minneapolis, MN) [356].

1.4.2.4 Role of hypoxia and NOS

Several pieces of evidence suggest that stem cell niches are prevalently hypoxic [357, 358], a factor that is believed to enhance greater self-renewing ability in HSC [261]. Analysis of HSC proteome has shown a profile compatible with an anaerobic environment, having increased expression of antioxidant enzymes [359]. HIF-1 α up-regulates erythropoietin production [360], proliferation and survival genes, the angiogenic VEGF [361], and other genes. G-CSF, mediator of HSPC mobilization, determines an up-regulation of HIF-1 α expression [362], increasing in turn VEGF and CXCL12 production [363], and CXCR4 expression [364]. Interestingly, a population of MSC precursors [17], VSEL, was reported to mobilize as a result of hypoxia, coincident with an increased plasma level of CXCL12 [365].

Hypoxia is also a typical feature of the cancer stem cell niche. It is believed that, like normal stem cells, also cancer stem cells self-renew and differentiate, and hypoxia might contribute to sustain these properties [366]. HIF-1 α can induce the up-regulation of NOS in solid tumours [304], and in neural progenitor cells [367], thus suggesting a role for NOS in the tumour niche.

NO has recently been investigated as a putative regulator of HSPC mobilization in the BM niche. NO production by murine BM cells was shown to inhibit the colony-forming ability of isolated Lin⁻ cells *in vitro* [368]. Inhibition of NOS activity *in vivo* results in an increase in the number of stem and early progenitor cells in the BM, followed by an increase in the neutrophil content in the blood [369]. In particular, BM cells isolated from mice treated with NOS inhibitors were more efficient in repopulating lethally irradiated mice than control cells in a competitive repopulation assay [369].

Interestingly, stromal cell derived neuronal NOS (nNOS) produces NO that acts as a paracrine regulator of HSC [370]. Further studies conducted in eNOS knockout mice demonstrate that eNOS expressed by BM MSC influences recruitment of stem and progenitor cells in a hind limb ischemia model [371]. Moreover, eNOS knockout mice exhibit markedly reduced capacity to produce

endothelial progenitor cells from hematopoietic stem cells [372]. Another confirmation to the ability of NO to regulate stem cells divisions come from studies in neurons, where it has been shown that infusion of NOS inhibitors impairs the proliferation of neural stem cells [373].

1.5 AIMS

We hypothesized that MSC isolated from BM or peripheral tissues can modulate the recruitment, expansion and differentiation of myeloid cells. To test this hypothesis we investigated:

1. The effect of MSC on expansion and differentiation of haematopoietic cells;
2. The phenotype, and function of BM mononuclear cells exposed to MSC;
3. The role of candidate molecules in MSC-driven haematopoietic differentiation;
4. The cellular players involved in mesenchymal-haematopoietic stroma interaction.

CHAPTER 2.

MATERIALS AND METHODS

2.1 ANIMALS

C57BL/6 and BALB/c mice were purchased from Olac, Bicester UK. Nos2^{-/-} mice in C57BL/6 background were kindly provided by Prof. Vincenzo Bronte (Verona University Hospital and Department of Pathology, Immunology Section, Verona). Tnfr1/r2^{-/-} mice in C57BL/6 background were kindly provided by Dr. Jorge Caamano (Medical Research Council (MRC) Centre for Immune Regulation, Institute for Biomedical Research, Birmingham Medical School, Birmingham). C3^{-/-} mice in BALB/c background were kindly provided by Prof. Marina Botto (Centre for Complement and Inflammation Research, Imperial College, London). Musk-L;Agrn^{-/-} and Musk-L;Agrn^{+/-} mice in C57BL/6 background were kindly provided by Prof. Antonella Viola. Musk-L;Agrn^{-/-} mice have been described elsewhere [374]. Briefly, mice lacking all isoforms of agrin were crossed with mice overexpressing *Musk* transgene (Musk-L), increasing *Musk* expression by 3-fold and achieving an adequate level of Musk activation in muscle that prevents neonatal lethality of agrin mutant mice [374].

BM and spleen cells were obtained from 8-12 week old mice. All procedures were conducted in accordance with the Home Office Animals Act of 1986.

2.2 CELLS AND CELL CULTURE

All cell culture procedures were performed under sterile conditions in a flow cabinet. Cell cultures were incubated at 37°C and 5% CO₂ in a humidified incubator. MSC were cultured in Mesencult medium with MSC stimulatory supplement (StemCell Technologies, Canada). MDSC were cultured

in RPMI 1640 medium supplemented with 10% heat inactivated FBS (Superior, Biochrom), 100U/ml penicillin G, 100U/ml streptomycin sulphate and 100U/ml glutamine (Invitrogen). Skin MSC and mesangial cells were cultured in RPMI 1640+GlutaMAXTM medium supplemented with 20% heat inactivated foetal bovine serum (FBS) (Biosera, South America) 100U/ml penicillin G, 100U/ml streptomycin sulphate (Invitrogen). Mesangial cells isolated from C57Bl/6 mice were a gift from Dr. Jacques Behmoaras (Centre for Complement and Inflammation Research, Imperial College London, London). Briefly, glomeruli from C57Bl/6 mice were isolated by sieving, and digested with collagenase type 1 for 20 min. Partially digested glomeruli were cultured in 25-cm² tissue culture flasks at 600 glomeruli/ml in RPMI 1640 medium (Invitrogen) that contained 20% FBS (F-539), penicillin (100 U/ml; Invitrogen), streptomycin (100 µg/ml; Invitrogen), and l-glutamine (2 mM, Invitrogen) and was supplemented with insulin-transferrin-selenite (Sigma). The cultures were maintained at 37°C with 5% CO₂ for 6 days, allowing glomerular mesangial cells to grow out.

BM and spleen cells were cultured in RPMI 1640+GlutaMAXTM medium supplemented with 10% heat inactivated FBS (Biosera, South America) 100U/ml penicillin G and 100U/ml streptomycin sulphate (Invitrogen). In co-culture experiments with MSC the same medium was used.

2.2.1 MSC

MSC were generated from 8 to 12 weeks old mice. Femurs and tibias of C57Bl/6, Nos2^{-/-} and Tnfr1/r2^{-/-} mice were directly crushed and plated in Mesencult. After 72 hours non-adherent cells were removed and fresh medium was added to the cultures. The medium was changed every 3 to 4 days for 8 to 10 weeks and a homogeneous cell population was obtained. The identity of MSC was confirmed by immunophenotypic criteria based on the expression of PDGFR α and Sca-1, and the absence of haematopoietic marker CD45. BM derived MSC isolated from Musk-L;Agrn^{+/-} and Musk-L;Agrn^{-/-} mice [332] were kindly provided by Prof. Antonella Viola (Istituto Clinico Humanitas IRCCS and Department of Translational Medicine, University of Milan, Milan). BM derived MSC

isolated from C3^{-/-} (BALB/c background) and BALB/c mice were kindly provided by Prof. Marina Botto.

2.2.2 MDSC

Tibias and femurs from 8 to 12 weeks old C57BL/6 mice were removed using sterile techniques and BM was flushed. Red blood cells (RBCs) were lysed with ammonium chloride (Lonza). To obtain BM-derived MDSC, 2.5×10^6 cells were plated into dishes with 100 mm diameter (Falcon, BD, NJ, USA) in 10 ml of medium supplemented with a combination of GM-CSF (40 ng/ml) and IL-6 (40 ng/ml) cytokines. Cells were maintained at 37°C in 5% CO₂-humidified atmosphere for 4 days.

2.2.3 Skin MSC

In order to obtain skin MSC, 5 mm skin biopsies were obtained from C57BL/6 mice, cut into pieces and plated in RPMI 1640+GlutaMAXTM medium supplemented with 20% heat inactivated FBS (Biosera, South America), and 100U/ml penicillin G, 100U/ml streptomycin sulphate (Invitrogen). After 72 hours non-adherent cells were removed and fresh medium was added in the cultures. The medium was changed every 3 to 4 days for 2 weeks and a homogeneous cell population was obtained.

2.2.4 Spleen and BM cells

Spleen and limbs were removed from freshly euthanized C57BL/6 mice and placed in complete RPMI. Spleens were mashed through a cell strainer (70µm, BD Bioscience, UK), washed with complete RPMI, and centrifuged at 1500 rpm for 6 minutes. BM cells were obtained by flushing the tibia and femur of freshly euthanized mice. For the lysis of RBCs spleen and BM cells were treated with ammonium chloride (Lonza) for 5 minutes at room temperature and washed again.

2.3 Co-cultures

For co-cultures experiments with BM cells, MSC were plated at 2×10^5 cells/well in 24 well plates (Costar), and kept at 37°C, 5% CO₂ and 95% humidity in a cell incubator. When the MSC were attached to the plastic, 1×10^6 BM cells were added, either directly or in a 0.4µm transwell system (Corning, Costar). After 4 days, cells were collected by adding PBS containing 2mM ethylenediamine-tetraacetic acid (EDTA). Where stated, cytokines or antibodies were added to the co-cultures: murine TNF-α (Peprotech), murine IFN-γ (Peprotech), human complement C3 (Calbiochem), murine IL-6 (Peprotech).

2.4 CD11b⁺ ISOLATION

For CD11b⁺ separation, co-cultures were collected by adding PBS containing 2mM EDTA and separated with CD11b⁺ Microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Purity of isolation was assessed by FACS.

2.5 *IN VIVO* EXPERIMENTS

For adoptive transfer of WT or Nos2^{-/-} MSC, lethally irradiated (800cGy) WT C57Bl/6 recipients were transferred (by tail vein injection) with 2×10^6 BM cells and 0.2×10^6 WT or Nos2^{-/-} MSC 4 hours after the second dose of irradiation.

For the transplantation experiment, lethally irradiated (800cGy) WT or Nos2^{-/-} recipients received liposome-encapsulated clodronate (Dr. Nico Van Rooijen) at day -1 by intraperitoneal injection, and were transferred (by tail vein injection) with 2×10^6 BM cells 4 hours after the second dose of irradiation.

Spleen and BM from transplanted mice were recovered at day 10 (adoptive transfer of MSC) or day

14 (transplantation experiment) and analysed by FACS.

2.6 FACS STAINING AND SORTING

Cells were blocked with PBS supplemented with 1% FBS, and mouse serum IgG before incubation with the respective monoclonal antibody (mAb) at 4°C for 20 minutes. After incubation the cells were washed with PBS and analysed by flow cytometry.

For intracellular cytokine staining, 0.3×10^6 cells obtained from the co-cultures were plated in 96 well U-bottom plates and either non stimulated or stimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (250 ng/ml) for 4 hours. Golgi stop (BD Pharmingen) was added for the subsequent 12 hours. Cells were collected, by adding PBS 2mM EDTA, fixed and permeabilized following manufacturer instructions (BD Cytofix/Cytoperm™), and stained for intracellular markers. A list of antibody used is represented in Table 2.1.

Samples were acquired with a BD FACSCalibur or with a BD LSRII (BD Biosciences) and data was subsequently analysed using FlowJo software (Oregon, USA).

For sorting primitive HSC and myeloid progenitors, BM cells were stained for Lineage antibody Cocktail (CD3e, CD11b, CD45R/B220, TER-119, and Ly-6G and Ly-6C), c-Kit (CD117), IL-7R α , CD34, CD16/32 (Fc γ R), Sca-1 and Flk2 antibodies. Samples were acquired with a BD FACSARIA (BD Biosciences).

For sorting CD11b⁺ cells and CD11b⁺ Gr-1^{high}, CD11b⁺ Gr-1^{int-low} and CD11b⁺ Gr-1⁻, BM cells were stained for CD11b and Gr-1 antibodies. Samples were acquired with a BD FACSARIA (BD Biosciences).

Antibody	Clone	Manufacturer
CCR2	475301	R&D Systems
CD115 (c-fms)	AFS 98	eBioscience
CD117 (c-Kit)	2B8	BD Biosciences
CD11b	M1/70	Biolegend
CD124 (IL4R α)	mIL4R-M1	BD Biosciences
CD127 (IL-7R α)	A7R34	eBioscience
CD135 (Flk2)	A2F10.1	BD Biosciences
CD140a (PDGFR α)	APA5	eBioscience
CD16/32 (Fc γ R)	93	eBioscience
CD169 (Sialoadhesin)	3D6.112	AbDSerotec
CD206 (mannose receptor C type 1)	MR5D3	AbDSerotec
CD34	RAM34	eBioscience
CD45	30-F11	eBioscience
CD45.1	A20	eBioscience
CD45.2	104	eBioscience
CD62L (L-selectin)	MEL-14	BD Biosciences
CD68	FA-11	Biolegend
F4/80	CI:A31	AbDSerotec
IFN γ	XMG1.2	BD Biosciences
IL-10	JES5-16E3	BD Biosciences
IL-12 (p40/p70)	C15.6	BD Biosciences
IL-4	11B11	BD Biosciences
Lineage antibody cocktail		BD Pharmingen
Ly-6C	AL-21	BD Biosciences
Ly-6G	1A8	BD Biosciences
Ly-6G/Ly-6C (Gr-1)	RB6-8C5	Biolegend
Sca-1 (Ly-6A/E)	D7	eBioscience
TNF α	MP6-XT22	BD Biosciences

TABLE 2.1. List of monoclonal antibodies for FACS analysis and sorting.

2.7 RT-PCR

Total RNA was extracted from CD11b⁺ cells from the co-cultures, or from MSC, using the RNeasy[®] plus kit (Qiagen, UK) according to manufacturers instruction. RNA concentration was measured using a NanoDrop 1000 spectrometer (Thermo Fisher Scientific, USA). 25 ng of sample were analysed with TaqMan[®] RNA-to-C_T[™] *I-Step* Kit, according to manufacturers instruction. RNA was reverse transcribed for 15 minutes at 48°C, and the AmpliTaq Gold[®] DNA Polymerase (Applied Biosystems) was activated for 10 minutes at 95°C. mRNA was quantitated with TaqMan[®] Gene Expression Assay. Samples were run in triplicate and data was analysed using the delta delta CT ($\Delta\Delta CT$) relative quantification method, with each sample normalized to HPRT to correct for differences in RNA quantity.

2.8 IMMUNOSUPPRESSIVE ASSAY

For immunosuppressive assays of CD11b⁺ cells isolated from the co-cultures, CD11b⁺ cells were plated at different percentages of the total number of cells in the cultures (12%, 6%, 3% and 1.5%) in a 96 well flat bottom plate (Costar), and either 5x10⁵ Concanavalin-A (ConA) stimulated or non-stimulated splenocytes were added. For immunosuppressive assays of skin stromal cells or MSC, stromal cells were plated at 5x10⁴ cells/well in a 96 well flat bottom plate (Costar), and either 5x10⁵ Con-A stimulated or non-stimulated splenocytes were added. Cell proliferation was measured by incorporation of tritiated thymidine (³H-TdR). Cell cultures were pulsed with 0.5μCi/well of ³H-TdR (Amersham, UK) and incubated for 18 hours. Cells were then harvested onto filters (Wallac Perkin Elmer, USA) using a 96-well cell harvester. Scintillation fluid (Wallac) was added to the filter, which contains fluorophores that absorb radioactive energy and converts it into light that is then detected by the beta counter (Wallac). The results of the incorporated thymidine were expressed in counts per minute (cpm) and were a mean of triplicate.

2.9 DIFFERENTIATION ASSAY

MSC were tested for their ability to differentiate into adipocytes and osteoblasts. MSC were plated in 6-well plates at a density of 1×10^5 cells per well in 2 ml of medium. Adipocytic differentiation was induced by $1 \mu\text{M}$ dexamethasone (SIGMA), 0.5 mM methyl-isobutylxanthine (IBMX) (SIGMA), and $10 \mu\text{g/ml}$ insulin (SIGMA), whereas 100 nM dexamethasone, 10 mM β -glycerol phosphate (SIGMA), and $50 \mu\text{M}$ ascorbic were used for osteoblastic differentiation. Oil red O and Alizarin red S dyes were used to identify adipocytes and osteoblasts respectively after 21 days.

2.10 CYTOSPIN PREPARATIONS

After 4 days, BM cultured alone, with cytokines GM-CSF and IL-6 or with MSC were harvested and CD11b^+ cells were separated. Cytospin preparations of CD11b^+ cells were fixed and stained with May-Grünwald-Giemsa staining.

2.11 CONFOCAL

BM cultured alone grown on coverslips or cytopins of CD11b^+ cells sorted from co-cultures of BM cells and MSC were rinsed with PBS, fixed in 4% paraformaldehyde (PFA) overnight at 4°C , and air-dried. The coverslips were incubated with $5 \mu\text{g/ml}$ of wheat germ agglutinin (WGA, AlexaFluor 488,) for 10 minutes, and blocked with PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 hour. After removing the medium, the coverslips were incubated overnight with a solution containing monoclonal antibody anti CD11b ($0.5 \mu\text{g/ml}$, Abcam) and anti-iNOS (1:100, Abcam) or anti-Arginase-1 (1:100, Abcam) in PBS containing 1% BSA. The coverslips were washed three times with PBS, and then incubated with a solution containing Dylight 594-conjugated anti-Ig directed to the native species of the primary antibody (1:200) in PBS containing 1% BSA. This secondary incubation was done for 1 hour at 25°C . The coverslips were washed three times in PBS

and mounted to the slides in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA). The slides were then sealed with nail polish and stored at 4°C. Confocal images were obtained using a Leica TCS-SP laser-scanning confocal microscope (Leica, Heidelberg, Germany).

2.12 STATISTICAL ANALYSIS

Data were analysed using unpaired two-tailed Student's t test with confidence interval of 95%, and expressed as mean $\pm$ standard deviation (SD). For analysis of *in vivo* experiments, Mann-Whitney U test was used.

CHAPTER 3.

MSC DRIVE MYELOID CELL DIFFERENTIATION

3.1 INTRODUCTION

MSC are a heterogeneous population derived from the BM that comprises a large proportion of progenitors, capable of differentiating into osteoblasts, adipocytes and chondrocytes [2, 3] and of forming a haematopoietic niche when transferred *in vivo* [32]. MSC precursors have been shown to play a fundamental role in the regulation of self-renewal and differentiation of HSC [25, 58, 60]. However, cells of myeloid origin play a key role in modulating MSC functions. BM macrophages modulate HSPC retention genes in MSC precursors, thus regulating HSPC mobilization from the niche [262]. G-CSF mediated HSPC egress correlates with an expansion of neutrophils population in the BM, and this in turns reduces MSC expression of factors crucial for HSC retention in the BM [264]. Finally, MSC progenitors modulate HSC entry into the bloodstream during infections [102]. Therefore, the interaction between myeloid populations and MSC is a crucial regulator of the haematopoietic niche.

An interaction between MSC and macrophages has also been shown in regards to MSC mediated immunosuppressive functions. Indeed, MSC can exert potent immunosuppressive activities under particular circumstances, which can be potentiated by the presence of monocytes [99, 375].

On the basis of these notions, we wanted to investigate the ability of MSC to induce the expansion and terminal differentiation of myeloid cells from haematopoietic cells of BM origin.

3.2 RESULTS

3.2.1 Characterization of isolated MSC

The first aim was to establish a reference MSC preparation in order to make the results more comparable throughout the project.

The preparation of BM derived MSC was quantitated at serial intervals during its expansion for the expression of PDGFR α , Sca-1 [32], c-kit, and the stromal marker CD90. Any contaminating haematopoietic cells were evaluated by the use of CD45. The results of a FACS analysis of a representative MSC preparation are reported in **Figure 3.1A**. MSC were cultured for 8 weeks and underwent 8 passages. The preparation was confirmed as CD45⁻, and the proportion of PDGFR α ⁺/Sca-1⁺ cells within this population was 97.6%. No expression of the marker c-Kit was detected in BM derived MSC, whereas 95.6% cells were CD90⁺.

In order to assess the multipotent activity of the isolated MSC, we investigated their ability to differentiate into adipocytes and osteoblasts in the presence of specific media. After 3 weeks of culture, differentiation of our MSC preparation was confirmed by using Oil Red O to stain lipid droplets (**Figure 3.1C**, left panel) or Alizarin Red S (**Figure 3.1C**, right panel) to stain bone nodules.

Since BM MSC have been demonstrated to be immunosuppressive [79], we tested the ability of our MSC preparations to inhibit T-cell proliferation by culturing escalating concentrations of MSC with ConA stimulated splenocytes (**Figure 3.1B**). T-cell proliferation was evaluated by ³H-thymidine uptake. The suppressive activity of our MSC preparation was dose dependent (**Figure 3.1B**). The proliferation of activated splenocytes was completely abolished when MSC were present up to a 1:80 ratio, whilst the percentage of inhibition was 50% at a 1:160 ratio.

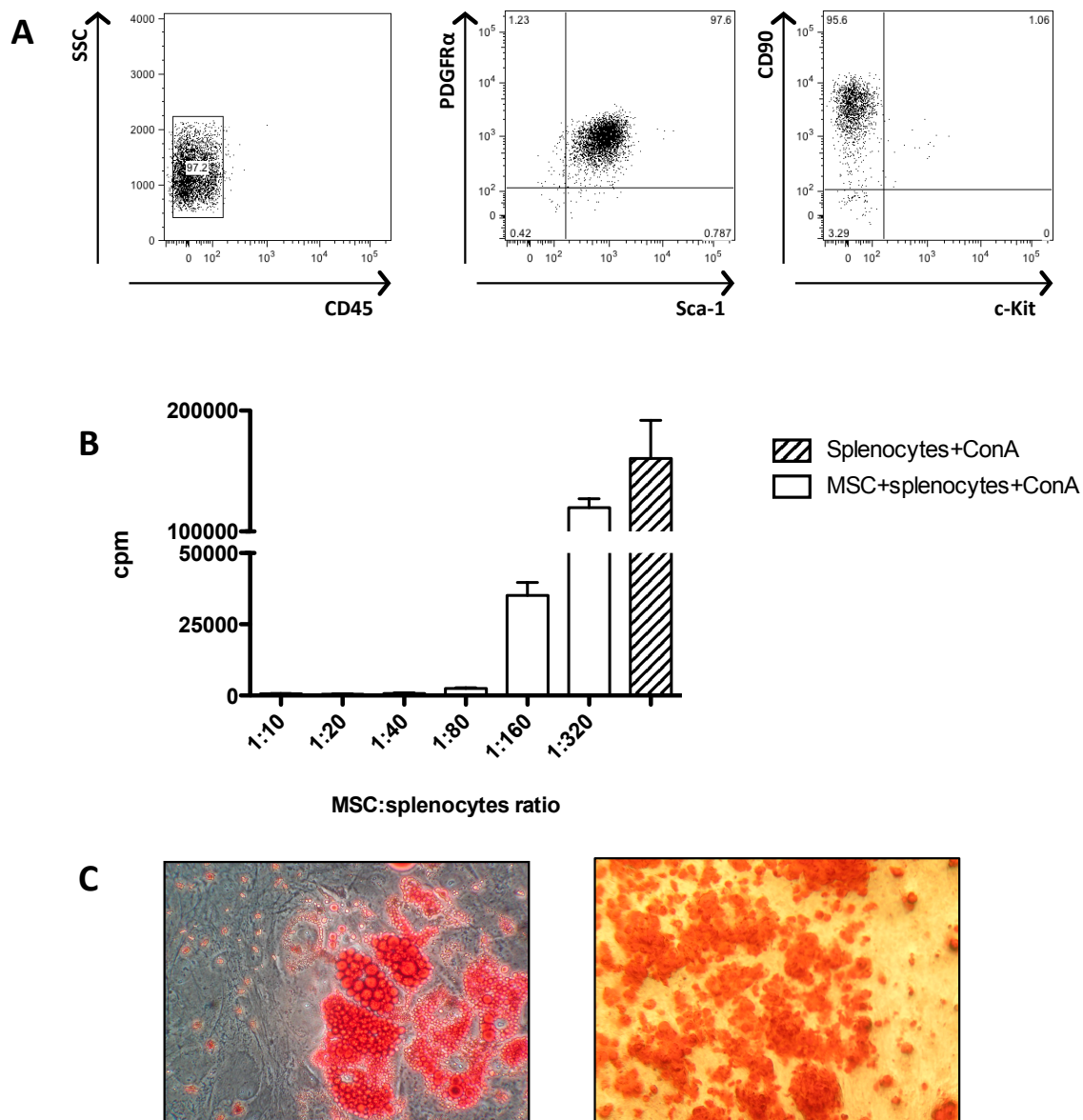


FIGURE 3.1. Characterization of BM derived MSC preparation. **A.** Proportion of CD45⁻ cells is represented in the live gate. Proportions of PDGFR α ⁺ Sca-1⁺ cells and CD90⁺ c-Kit⁺ cells are represented in the CD45⁻ gate in dot plot. A representative example out of 3 independently derived preparations of MSC is shown. **B.** MSC were plated at 5×10^4 cells/well, with 1:2 serial dilutions, and splenocytes were added at 5×10^5 cells/well and stimulated with 3 μ g/ml of ConA for 3 days. ^{3} H-thymidine was added for the last 18h. A representative experiment out of 3 independent experiments is shown. Each column represents the mean $\pm$ SD of triplicates of cultures. (cpm: count per minute). Results are expressed as the value obtained by subtracting the (MSC+Splenocytes) value from the (MSC+Splenocytes ConA) value. **C.** Adipogenic and osteogenic differentiation of BM derived MSC. Adipose droplets and bone nodule mineralization are visualized by oil red O and Alizarin Red S staining, respectively. Representative images of 3 independent adipogenesis and osteogenesis differentiation experiments.

3.2.2 MSC expand and differentiate haematopoietic cells

In order to investigate the effect of MSC on terminal myeloid differentiation of haematopoietic cells, short-term co-cultures of BM derived MSC and unfractionated BM were set up.

MSC were plated overnight and unfractionated BM cells were added at a 5:1 ratio to the MSC cultures and incubated for 4 days. BM cells treated with GM-CSF and IL-6 were used as control cultures to differentiate myeloid cells as previously reported [259]. A further control consisted of BM cells cultured alone.

After 4 days the BM cells present in the cell suspension were harvested and assessed for the expression of CD11b and Gr-1 to define myeloid cell populations, and F4/80 for macrophages [376]. The vast majority of BM cells cultivated in the presence of MSC were CD11b⁺, thus indicating a prominent myeloid differentiation. The analysis of Gr-1 expression within the CD11b⁺ gated population identified three main subsets of CD11b⁺ cells: CD11b⁺ Gr-1^{high}, CD11b⁺ Gr-1^{int-low} and CD11b⁺ Gr-1⁻ (**Figure 3.2A**, left panel). Cytokine-induced differentiation typically consisted of a higher percentage of CD11b⁺ Gr-1^{int-low} cells (33.8% ± 9.3% versus 24.6% ± 8.6% in BM alone, p=0.0346) and a higher percentage of CD11b⁺ Gr-1⁻ cells (19.7% ± 5.3% versus 4.9% ± 2% in BM alone, p<0.0001) as compared to BM cultured alone. The proportion of CD11b⁺ Gr-1^{high} cells was instead decreased (46.9% ± 9.8% versus 70.6% ± 9.1% of BM alone, p<0.0001) (**Figure 3.2A** and **Figure 3.2B**). Within the CD11b⁺ population, the presence of MSC induced a decrease in the CD11b⁺ Gr-1^{high} (32.2% ± 10.4%) and in the CD11b⁺ Gr-1^{int-low} component (17.3% ± 6.7%) as compared to BM alone or treated with cytokines, respectively (CD11b⁺ Gr-1^{high}: p<0.0001 versus BM alone, and p=0.0044 versus BM+GM-CSF+IL-6; CD11b⁺ Gr-1^{int-low}: p=0.0465 versus BM alone, and p=0.0003 versus BM+GM-CSF+IL-6) (**Figure 3.2B**). The CD11b⁺ Gr-1⁻ component was highly increased (50.6% ± 12.5%) in presence of MSC as compared to BM alone and BM treated with cytokines (p<0.0001 versus BM alone and BM+GM-CSF+IL-6) (**Figure 3.2B**).

In line with a marked increase in the proportion of CD11b⁺ Gr-1⁻, the expression of F4/80/CD11b⁺ was higher in MSC-induced myeloid cells (39.1% ± 12.3%) than in BM cell cultures (6.9% ± 4.3%) or BM with cytokines (14.9% ± 4.9%) (p<0.0001 and p=0.0003 versus BM alone and BM+GM-CSF+IL-6 respectively, **Figure 3.2C**). Cytokine-induced differentiation of myeloid cells was also confirmed by an increase in the percentage of CD11b⁺ F4/80⁺ cells as compared to BM cultured alone (p=0.0044). Representative contour plots for the expression of F4/80 versus Gr-1 are shown in **Figure 3.2A**.

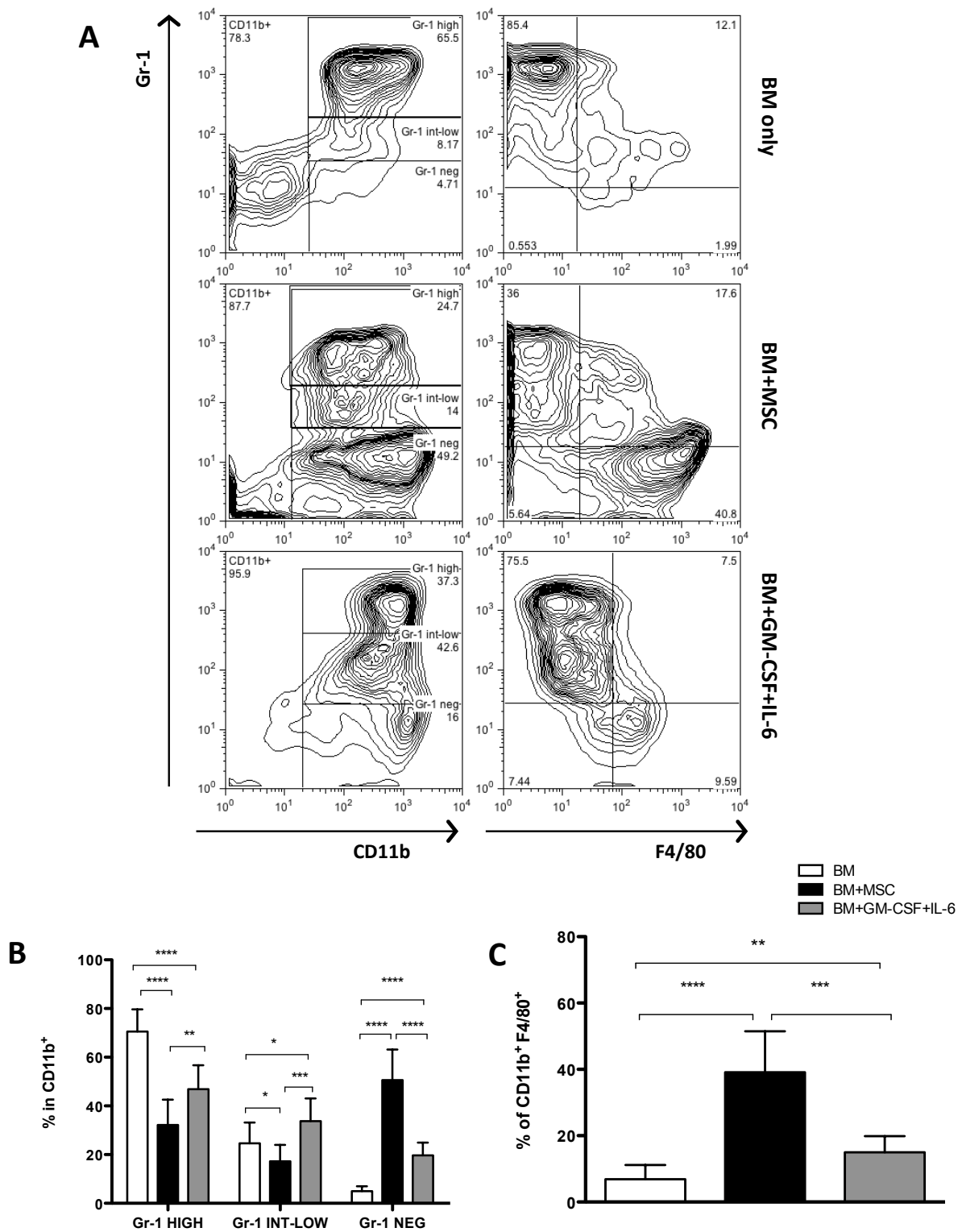


FIGURE 3.2. MSC induce the differentiation of myeloid cells. BM was cultured alone, with MSC (ratio 5:1) or with GM-CSF and IL-6 (40ng/ml) for 4 days. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the expression of F4/80 versus Gr-1 is represented in the CD11b⁺ gate. A representative example out of 10 independent experiments is shown. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ cells. Mean of 10 independent experiments, $\pm$ SD * p <0.05 ** p <0.005 *** p <0.001 **** p <0.0001 Unpaired t test. **C.** Percentage of CD11b⁺ F4/80⁺ cells, within the CD11b⁺ cells population. Mean of 10 independent experiments, $\pm$ SD ** p <0.005 *** p <0.001 **** p <0.0001 Unpaired t test.

In order to understand whether MSC could drive not only the differentiation but also an expansion of the myeloid population, the absolute number of CD11b⁺ cells recovered at day 4 was analysed (**Figure 3.3**). The figures were obtained by multiplying the number of cells recovered at the end of the cultures by the percentage of CD11b⁺ cells. The selection of CD11b⁺ excluded possible contaminations by MSC. MSC or the cytokine combination, induced in BM cells an expansion in the absolute number of CD11b⁺ cells ($5.7 \times 10^6 \pm 1.8$ and $10.3 \times 10^6 \pm 3.4$), as compared to the amount of CD11b⁺ recovered from BM cultured alone ($1.7 \times 10^6 \pm 1.4$) ($p=0.0014$ and $p=0.0002$ versus BM alone, respectively).

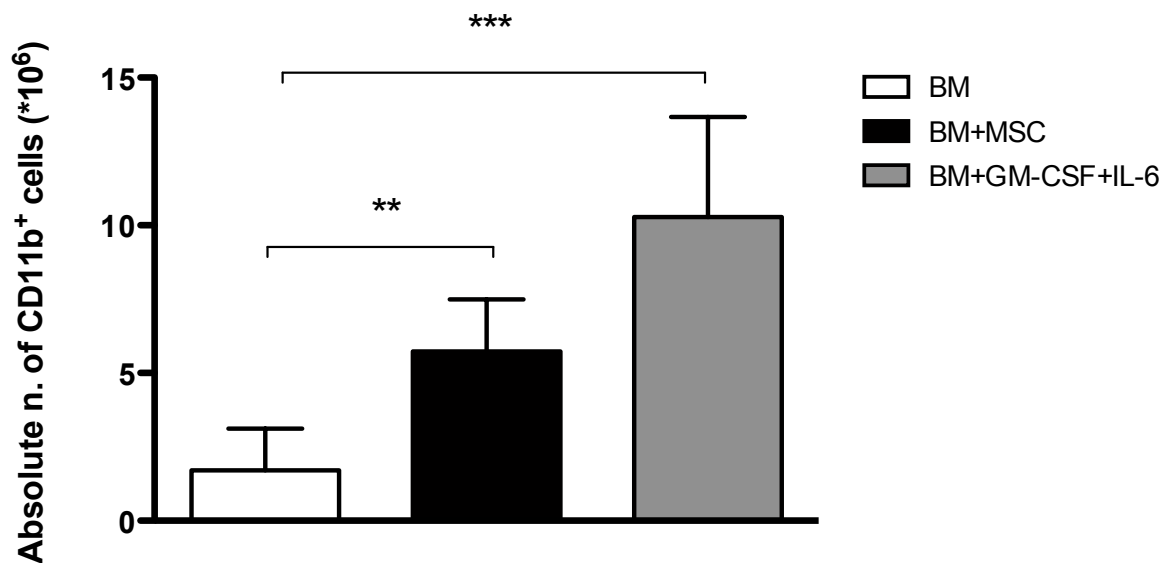


FIGURE 3.3. MSC induce the expansion of myeloid cells. Absolute number of CD11b⁺ cells recovered from initial seeding from BM cultured alone, with MSC or treated with GM-CSF and IL-6 for 4 days. Mean of 10 independent experiments, $\pm$ SD ** $p<0.005$ *** $p<0.001$ Unpaired t test.

To further characterise the profile of the CD11b⁺ cells obtained after the co-culture, several markers preferentially expressed by macrophages (F4/80, CD206, CD68 and CD169) and by monocytes (CD115, IL4R α and CD62L) were analysed in BM cells cultured alone, with MSC or with cytokines.

CD11b⁺ cells obtained from BM cultured alone showed low expression of F4/80 (6.9% $\pm$ 4.3%), CD115 (4.1% $\pm$ 2.3%), IL4R α (4.9% $\pm$ 4.1%), and CD206 (1.5% $\pm$ 1.5%) (**Figure 3.4A**). Very low levels of expression of CD169 (0.7% $\pm$ 0.3%), and CD68 (0.8% $\pm$ 0.2%) was detected on BM cultured alone. High expression of CD62L was instead documented on BM cultured alone (24.4% $\pm$ 4.2%). The presence of GM-CSF and IL-6 produced an increase in F4/80 (14.9% $\pm$ 4.9%), CD115 (33.5% $\pm$ 10.3%), IL4R α (22.9% $\pm$ 4.2%), CD206 (2.8% $\pm$ 0.3%), and CD68 (1.5% $\pm$ 0.4), and a decrease in the amount of CD62L (6.8% $\pm$ 2.1%) as compared to BM cultured alone (**Figure 3.4A**). MSC induced a remarkable 6-fold increase in F4/80 (36.5% $\pm$ 10.3%), a 3-fold increase in IL4R α (18.2% $\pm$ 7.5%), and a 2-fold increase in CD169 expression (2.3% $\pm$ 0.6%) as compared to BM cultured alone. Also, MSC were able to induce the expression of CD115 (48.6% $\pm$ 12.4%), CD206 (20.6% $\pm$ 2) and CD68 (16.5% $\pm$ 4.9%). Expression of CD62L was found reduced in BM cultured with MSC as compared to BM cultured alone (12.1% $\pm$ 2.2% versus 24.4% $\pm$ 4.2% of BM only).

The expression of each marker in CD11b⁺ cells obtained from BM and MSC co-cultures was evaluated within the different Gr-1 subset. F4/80, IL4R α , CD206, CD169 and CD68 were expressed only in the Gr-1⁻ subset (**Figure 3.4A**, right panel), whilst CD115 was detected both in the Gr-1^{high} and the CD11b⁺ Gr-1⁻ subsets.

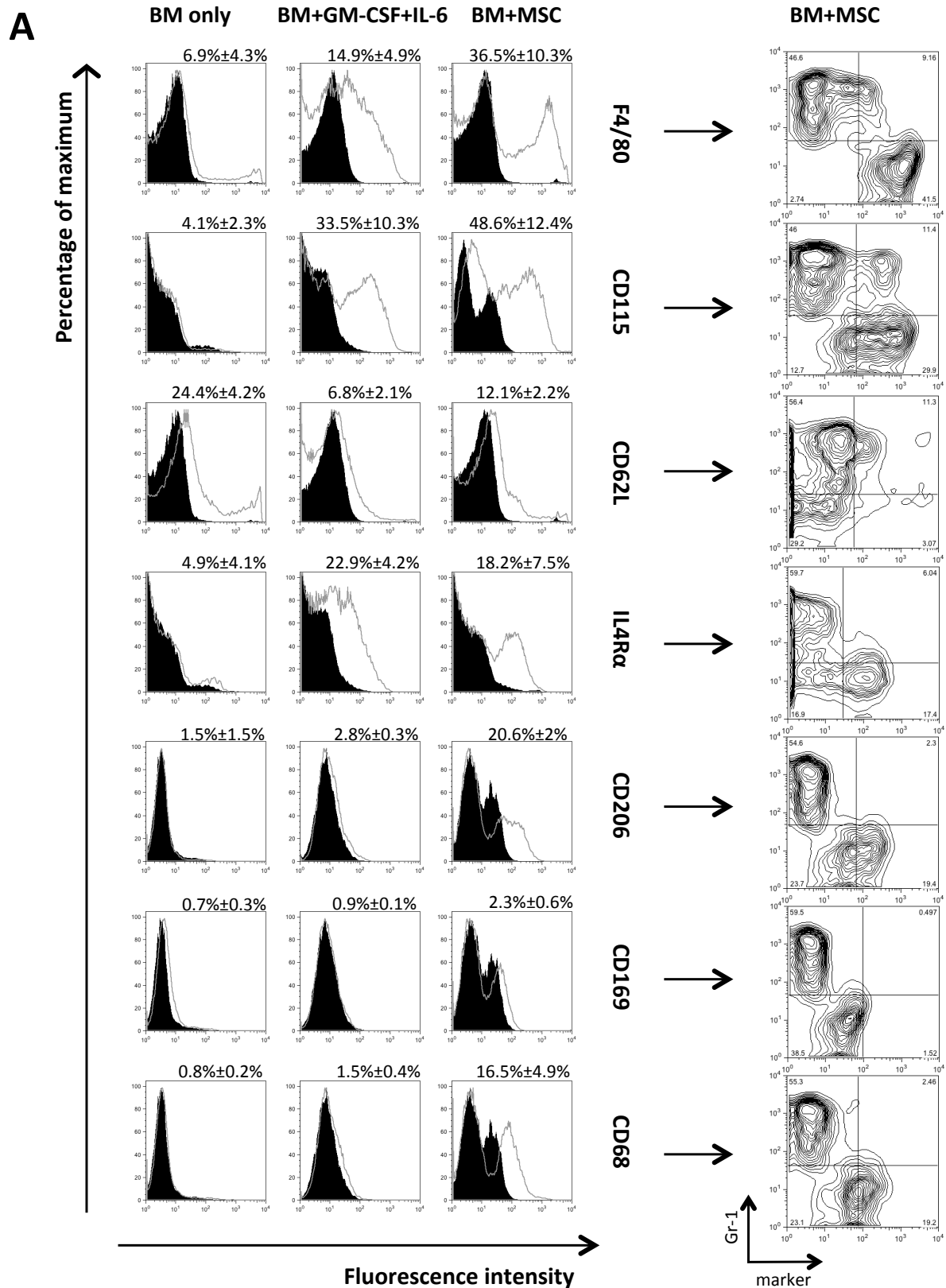


FIGURE 3.4. MSC induce the differentiation of macrophages. A. BM cultured alone, with GM-CSF and IL-6 for 4 days was evaluated for the expression of various macrophage surface markers within the CD11b⁺ gated population (open histograms) against their matched isotype controls (filled histograms). Contour plots within the CD11b⁺ gated population show the expression of the different surface markers versus Gr-1 expression in BM cultured with MSC. Percentage of fluorescence $\pm$ SD of 6 independent experiments is represented in histogram plots.

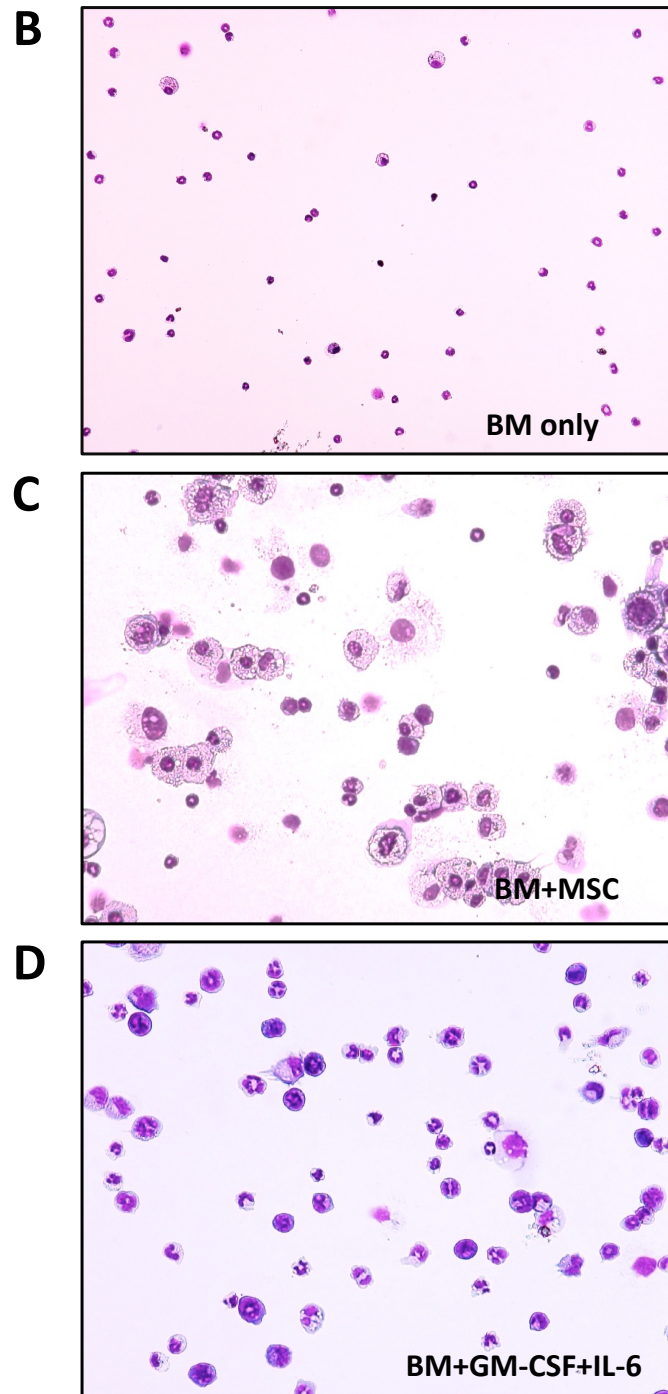


Figure 3.4. MSC induce the differentiation of macrophages. B. May-Grünwald Giemsa staining of cytopsin preparations of CD11b⁺ cells isolated from BM cultured alone, with MSC or GM-CSF and IL-6.

The morphology of CD11b⁺ cells isolated from BM cultured alone, with MSC or with cytokines was evaluated by May-Grünwald Giemsa staining. The presence of MSC in culture induced an accumulation of a fairly homogeneous cell population of large size with abundant pale vacuolated cytoplasm with some granules and reniform nucleus (**Figure 3.4B**). In contrast, cytokine-treated BM cells were characterised in majority by segmented neutrophils and myeloid cells mostly at terminal stages of their differentiation (pro-myelocytes and meta-myelocytes) (**Figure 3.4B**).

3.2.2.1 Kinetic of expansion and differentiation

In order to determine the kinetics of MSC-induced expansion and differentiation of BM cells, analysis of expression of CD11b and Gr-1 markers was carried out at day 1, 2, 3 and 4 on the co-cultures, using BM cultured alone or in the presence of cytokines GM-CSF and IL-6 as controls.

At day 1, BM cultured alone, with MSC or with cytokines showed similar proportions of the three populations of CD11b⁺ cells, with a majority of CD11b⁺ Gr-1^{high} cells (62.7%, 54.2% and 58.6%, respectively), and a very small percentage of CD11b⁺ Gr-1⁻ cells (3.07%, 7.66% and 6.29%, respectively) (**Figure 3.5A**). By day 2, changes in the proportions of CD11b⁺ cells were already detectable between the three different conditions of culture. A remarkable 2-fold increase in the CD11b⁺ Gr-1⁻ cells percentage (13.8% versus 7.66%) and in the CD11b⁺ Gr-1^{int-low} percentage (27.6% versus 15.5%) was detected in the BM cultured with MSC and with cytokines, respectively (**Figure 3.5A**). At day 3, a 2-fold decrease was detected in the percentage of CD11b⁺ Gr-1^{high} cells in BM cultured with MSC (21.1% versus 54.2%), with a commensurate increase in the percentage of CD11b⁺ Gr-1⁻ cells (25.1% versus 7.66%). BM cultured with cytokines, instead, induced a 3-fold increase in the percentage of CD11b⁺ Gr-1^{int-low} cells (45.2% versus 15.5%) as compared to day 1 (**Figure 3.5A**). At day 4, BM cultured alone still maintained the same proportions of CD11b⁺ cells. BM cultured with MSC, instead, showed a further expansion of CD11b⁺ Gr-1⁻ cells (34% versus 7.66%), and a related decrease in the percentage of CD11b⁺ Gr-1^{high} cells (16.3% versus 54.2%).

Notably, BM cells cultured with cytokines expanded the population of CD11b⁺ Gr-1⁻ cells only at day 4 (12% versus 6.29%), with a slight decrease in the percentage of CD11b⁺ Gr-1^{high} cells (38.7% versus 58.6%) (**Figure 3.5A**).

Analysis of the absolute number of CD11b⁺ Gr-1⁻ cells confirmed the expansion induced by the presence of MSC (**Figure 3.5B**). The figures were calculated by multiplying the number of cells recovered by the percentage of CD11b⁺ Gr-1^{high}, CD11b⁺ Gr-1^{int-low} or CD11b⁺ Gr-1⁻ cells.

Furthermore, we assessed the kinetics of MSC induced CD11b⁺ cell expansion in comparison to BM cells alone (**Figure 3.5C**). The absolute cell number was decreased throughout the days in both conditions. At day 1 no difference could be detected in the number of CD11b⁺ cells recovered from the cultures, whereas by day 2 and 3 the number of CD11b⁺ cells recovered from the co-cultures of BM and MSC had increased by 1.5-folds (5.7×10^6 versus 4.1×10^6 , and 4.8×10^6 versus 3.9×10^6). At day 4, a greater expansion of CD11b⁺ cells could be detected in BM cultured with MSC (4.1×10^6 versus 1.76×10^6) (**Figure 3.5C**).

In order to mimic a physiological condition in which MSC and myeloid cells interact, different ratios of BM and MSC were investigated in the short-term co-cultures. As shown in **Figure 3.6**, higher ratios (up to 40:1) between BM and MSC did not impair the MSC ability to induce the same pattern of differentiation as in the lowest ratio of 5:1 (9.28% versus 12.5% of CD11b⁺ Gr-1^{high}; 22.7% versus 15.4% of CD11b⁺ Gr-1^{int-low}; 37.4% versus 38.9% of CD11b⁺ Gr-1⁻). It seemed therefore that, as long as MSC could grow in a homogeneous layer making contact with BM cells, myeloid differentiation was induced.

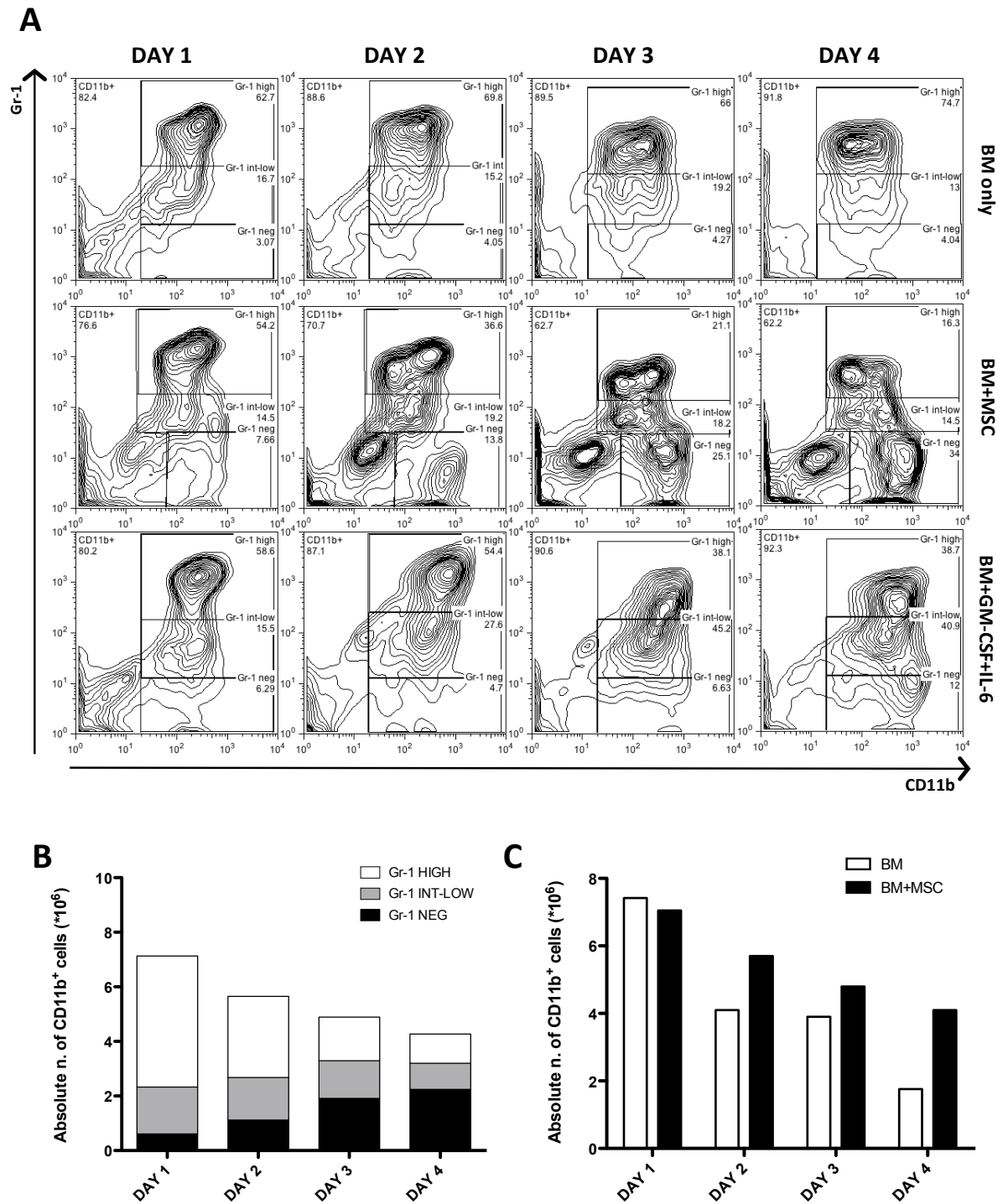


FIGURE 3.5. MSC mediate the differentiation of myeloid cells in 4 days, and maintain BM cells viability. **A.** Kinetic of differentiation of BM cultured alone, with MSC or treated with GM-CSF and IL-6 (40ng/ml) for 1, 2, 3 and 4 days. Contour plots represent the proportion of $CD11b^+ Gr-1^{high}$, $CD11b^+ Gr-1^{int-low}$ and $CD11b^+ Gr-1^{-}$ cells in the live gate. **B.** Absolute number of $CD11b^+ Gr-1^{high}$, $CD11b^+ Gr-1^{int-low}$ and $CD11b^+ Gr-1^{-}$ cells recovered from BM and MSC co-cultures at day 1, 2, 3 and 4. A representative result of 2 independently performed experiments is shown. **C.** Absolute number of $CD11b^+$ cells recovered from BM cultured alone or with MSC after 1, 2, 3 and 4 days. A representative result of 2 independently performed experiments is shown.

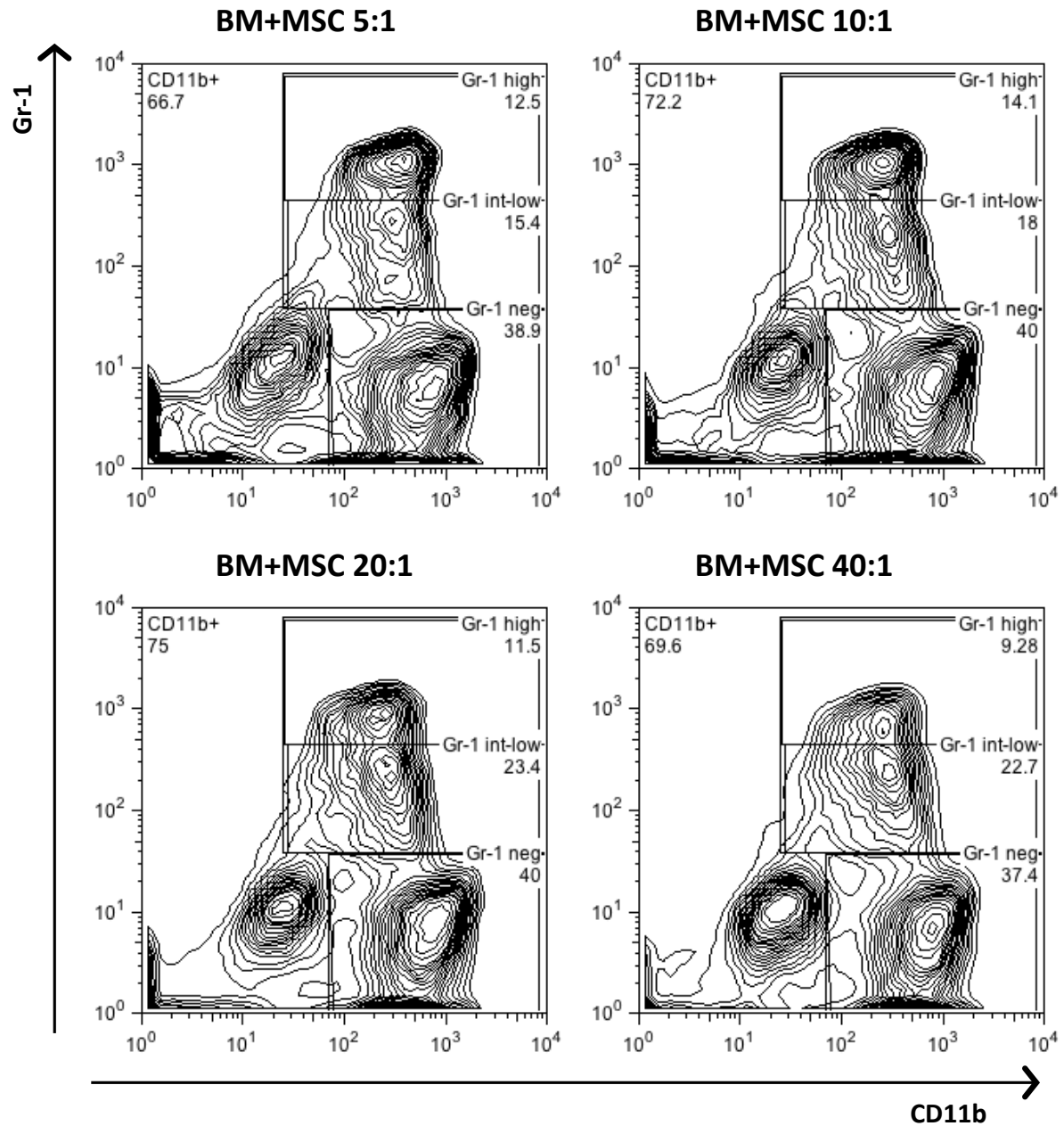


FIGURE 3.6. The differentiation of myeloid cells induced by MSC is not dose dependent. BM was cultured with MSC in different ratios (ratio 5:1, 10:1, 20:1, 40:1) for 4 days. Contour plots represent the proportion of CD11b and Gr-1 cells in the live gate. A representative example from 2 independent experiments is shown.

3.2.3 Functional characterization of MSC-induced myeloid cells

We further characterised the functional and enzymatic profile of CD11b⁺ cells obtained from culturing the BM alone, with MSC or with GM-CSF and IL-6.

3.2.3.1 Myeloid cells generated by MSC are immunosuppressive

Immunosuppression of activated T cells is a function that is shared by macrophages and myeloid cells generated with GM-CSF and IL-6 [259]. Therefore, we investigated whether CD11b⁺ cells obtained from the co-cultures were able to inhibit the proliferation of ConA activated splenocytes.

After 4 days of culture, CD11b⁺ cells isolated from BM cultured alone, with MSC or with cytokines were plated at different percentages of the total number of cells in the cultures (12%, 6%, 3% and 1.5%), and cultured with ConA activated splenocytes. CD11b⁺ cells from BM cultured with cytokines were used as positive control of immunosuppression. CD11b⁺ cells isolated from BM cultured with MSC were able to suppress the proliferation of activated splenocytes (50% of inhibition) even when representing only 1.5% of the total number of cells in culture. Furthermore, they were more immunosuppressive than CD11b⁺ cells isolated from BM cultured in presence of cytokines, as shown by the inhibitory effect when they represented 3% of the total cells in culture (90% versus 65% of inhibition in CD11b⁺ from BM+GM-CSF+IL-6) (**Figure 3.7**).

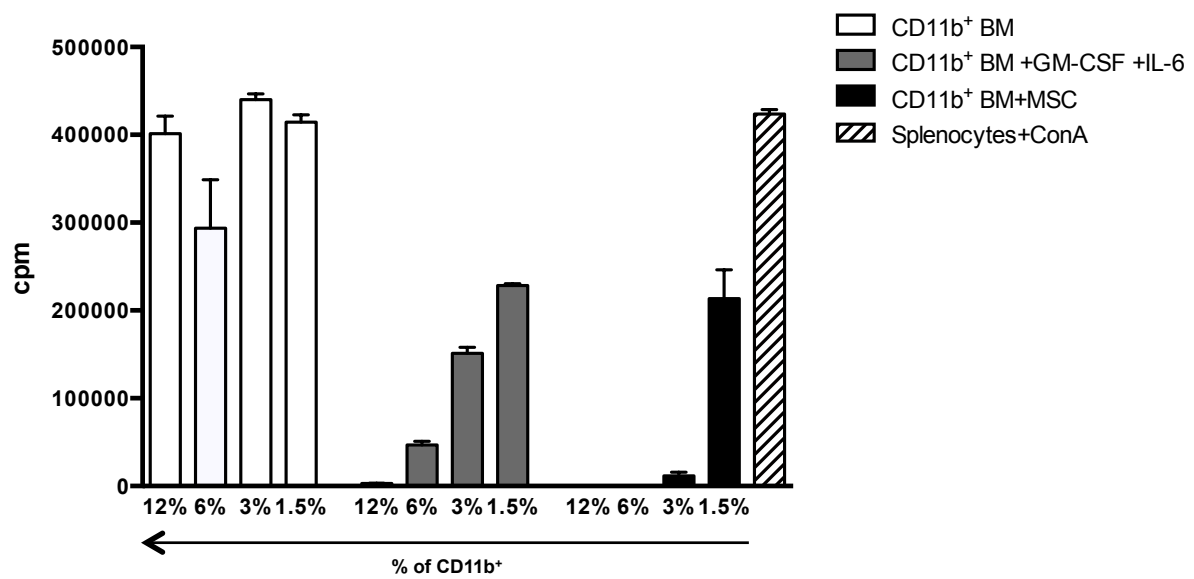


FIGURE 3.7. MSC-induced CD11b⁺ cells are immunosuppressive. BM was cultured alone, with MSC or with GM-CSF and IL-6 for 4 days. CD11b⁺ cells were sorted from the cultures, and plated into 96 well flat bottom plate; cells were present at different percentages of the total number of cells in the cultures (12%, 6%, 3% and 1.5%). ConA stimulated splenocytes were then added for 72 hours, and ³H-thymidine was added for the last 18 hours of co-culture. Representative experiment from 3 independent experiments; each column represents the mean±SD of triplicates. (cpm: count per minute). Results are expressed as the value obtained by subtracting the (CD11b⁺ cells+Splenocytes) value from the (CD11b⁺ cells+Splenocytes ConA) value.

3.2.3.2 M1-M2 profile of MSC-induced myeloid cells

Murine macrophages are generally classified in two subsets, ‘classically activated’ or M1 macrophages, and ‘alternatively activated’ or M2 macrophages, mirroring the Th1/Th2 nomenclature of pro- and anti-inflammatory T helper subsets. In order to further characterise the molecular profile of MSC-induced CD11b⁺ cells, we investigated the expression of several M1 and M2 markers by Real-Time PCR (RT-PCR).

CD11b⁺ cells from BM cultured alone, with MSC or with cytokines were isolated and RNA was extracted. CD11b⁺ cells isolated from BM cultured with cytokines were used as control for expression of *Nos2* and *Arg-1* genes [377]. RT-PCR data analysis documented a 30-fold and a 10-fold increase in *Nos2* and *Arg-1* expression after co-culture with MSC as compared to BM alone, respectively (p=0.003 and p=0.0023, respectively) (Figure 3.8). The presence of MSC induced a 2-fold downregulation of cyclooxygenase 2 (*Cox2*) (p=0.0004), a 100-fold downregulation of indoleamine 2,3-dioxygenase 1 (*Ido1*) (p<0.0001), a 3-fold downregulation of interleukin 12b (*Il12b*) (p<0.0001), a 25-fold downregulation of macrophage receptor with collagenous structure (*Marco*) (p<0.0001), and a 6-fold downregulation of M2 marker interferon factor 4 (*Irf4*) (p=0.0132), as compared to BM cultured alone. The marker Found in inflammatory zone 1 (*Fizz1*) was expressed only by CD11b⁺ cells isolated from BM cultured with cytokines (**Figure 3.8**).

As *Nos2* and *Arg-1* genes were upregulated in BM after culture with MSC, their protein expression was further confirmed with confocal staining. Cytospin preparations of CD11b⁺ cells isolated from BM in culture with MSC were stained for expression of proteins Nos2 or Arginase-1, nuclear DNA content (DAPI) and wheat germ agglutinin (WGA, for cytoplasm staining) (**Figure 3.9**). BM cultured alone for 4 days onto a cover glass was used as control. Arginase-1 and Nos2 expression in the cytoplasm of BM cells and CD11b⁺ cells isolated from co-cultures was confirmed.

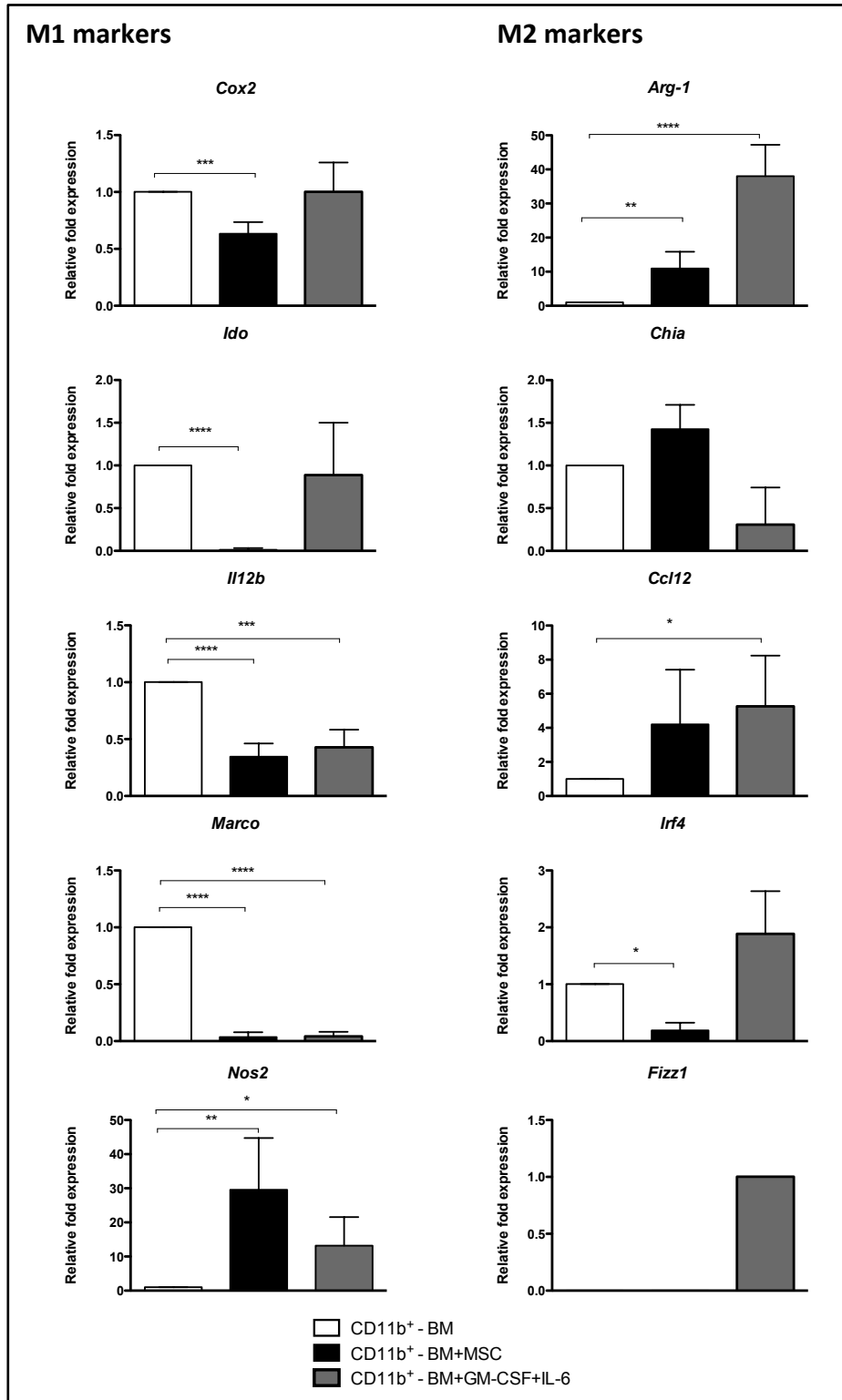


FIGURE 3.8. M1-M2 profile of CD11b⁺ cells from BM cultures. Quantitative RT-PCR data of CD11b⁺ cells isolated from 4 days cultures of BM alone, with MSC or with GM-CSF and IL-6. Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in CD11b⁺ from BM alone. Mean of 5 independent experiments, $\pm$ SD. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$ Unpaired t test. *Arg-1*, arginase 1; *Ccl12*, CC-chemokine ligand 12; *Chia*, chitinase, acidic; *Cox2*, cyclooxygenase 2; *Fizz1*, found in inflammatory zone 1 (*Relma*, resistin-like molecule alpha); *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; *Ido1*, indoleamine 2,3-dioxygenase 1; *Il12b*, interleukin 12b; *Irf4*, interferon factor 4; *Marco*, macrophage receptor with collagenous structure; *Nos2*, nitric oxide synthase 2.

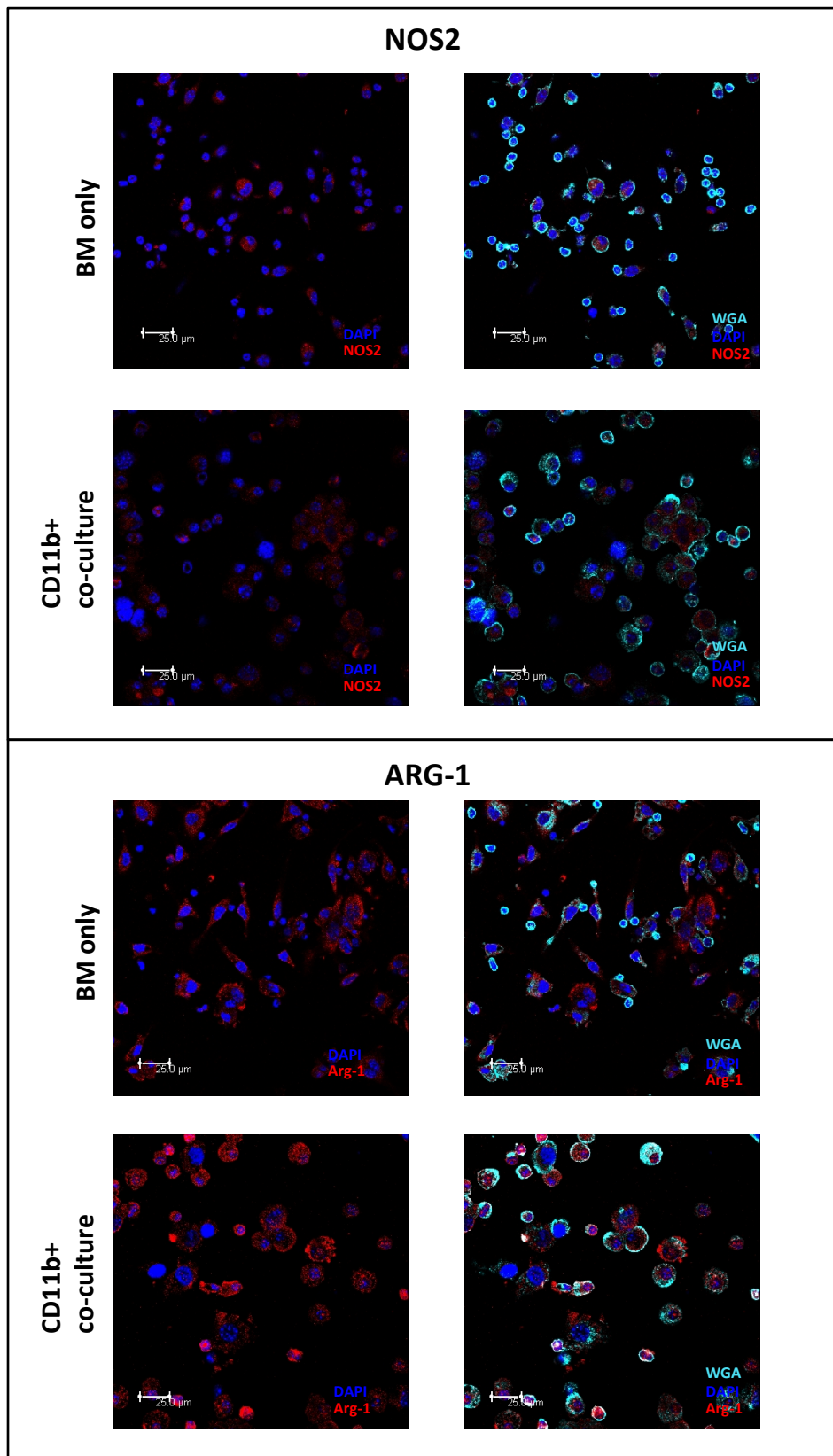


FIGURE 3.9. Confocal analysis of NOS2 and ARG-1 expression in BM cells cultured alone or with MSC. Confocal images of BM cells cultured alone (BM only) for 4 days and cytospin preparations of CD11b⁺ cells isolated from BM and MSC 4 days co-cultures stained for either NOS2 or ARG-1 (red), nuclear DNA content (DAPI, blue) and wheat germ agglutinin (WGA, light blue). Upper panel, cells stained for NOS2; lower panel, cells stained for ARG-1. Scale bar, 25 μm. ARG-1, Arginase-1; NOS2, nitric oxide synthase 2.

3.2.3.3 Expression of *Nos2* and *Arg-1* in *CD11b*⁺ cells subsets

We further asked which is the subset of *CD11b*⁺ cells that retains the highest expression of the two genes *Arg-1* and *Nos2*. For this purpose, BM cultured with MSC or treated with GM-CSF and IL-6 were sorted in 4 different populations: total *CD11b*⁺, *CD11b*⁺ Gr-1^{high}, *CD11b*⁺ Gr-1^{int-low} and *CD11b*⁺ Gr-1⁻. RNA was extracted from the cells obtained and RT-PCR was used to evaluate the expression of the two genes. *Nos2* was mainly expressed by the *CD11b*⁺ Gr-1^{high} subset in BM cultured with MSC (3.5±0.9, p=0.0458 versus *CD11b*⁺), whereas it was mainly expressed by the *CD11b*⁺ Gr-1^{high} and *CD11b*⁺ Gr-1^{int-low} subset in BM treated with cytokines (2.8±0.3, p=0.0144 and 3.1±0.6, p=0.0439 versus *CD11b*⁺, respectively). *Arg-1* was instead mainly expressed by the *CD11b*⁺ Gr-1⁻ subset (1.2±0.4 versus *CD11b*⁺) and the *CD11b*⁺ Gr-1^{int-low} subset (4.9±0.8, p=0.0224 versus *CD11b*⁺) in BM cultured with MSC or with cytokines, respectively (**Figure 3.10**).

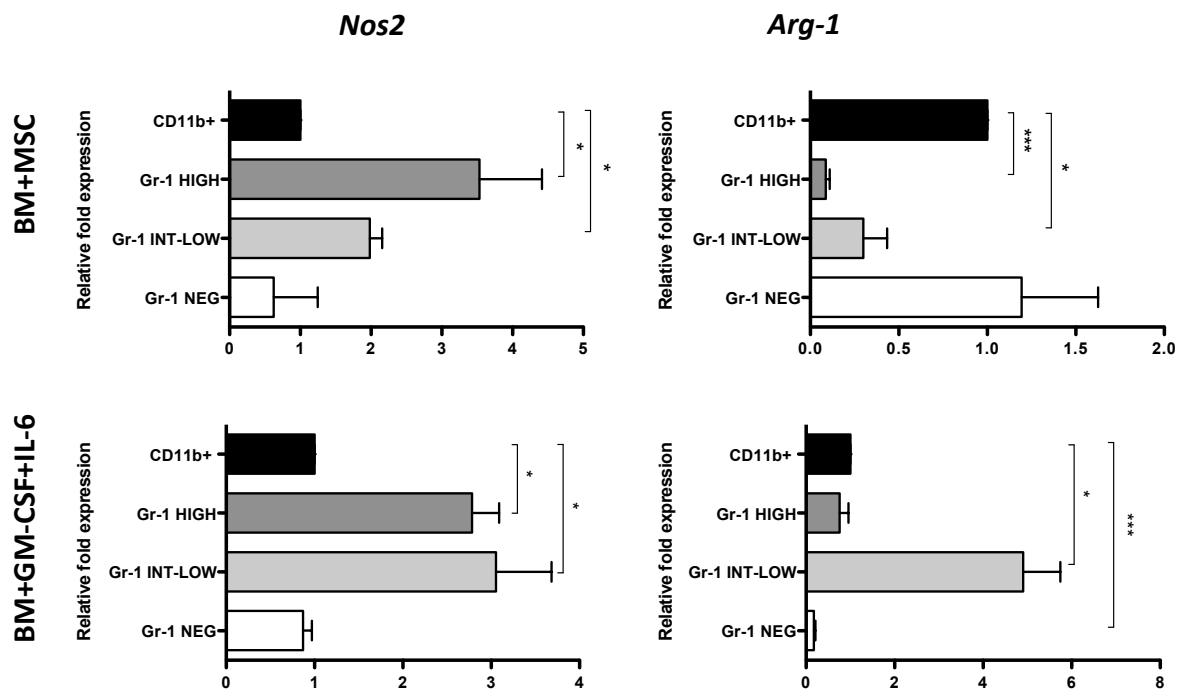


FIGURE 3.10. Expression of *Nos2* and *Arg-1* in CD11b⁺ subsets from BM cultures. Quantitative RT-PCR data of total CD11b⁺ cells and Gr-1^{high}, Gr-1^{int-low}, Gr-1⁻ subsets sorted from 4 days cultures of BM with MSC or with GM-CSF and IL-6. Data are expressed as normalized to HPRT1 housekeeping gene, and compared to abundance in the total CD11b⁺ population. Mean $\pm$ SD of 2 independently performed experiments. * $p < 0.05$, *** $p < 0.001$, Unpaired t test. *Arg1*, arginase 1; *Nos2*, nitric oxide synthase 2.

3.2.3.4 Essential aminoacid enzymes are expressed by MSC-induced myeloid cells

Enzymes sequestering essential aminoacid (EAA) have been recently demonstrated to be one of the mechanisms mediating T cell tolerance [378]. In particular, several of these enzymes have known roles in mediating MSC and macrophages immunosuppressive abilities. On the basis of these notions, we investigated the gene expression of enzymes histidine decarboxylase (*Hdc*), branched-chain aminoacid transaminase 1 (*Bcat1*), and interleukin 4 induced 1 (*Il4i1*) on CD11b⁺ cells isolated from BM cultured alone, with MSC or with cytokines GM-CSF and IL-6. As *Arg-1* and *Nos2* RNA were significantly upregulated in CD11b⁺ after co-culture with MSC, we also investigated the expression of other arginase and NOS isoforms, namely arginase-2 (*Arg-2*), neuronal NOS (*Nos1*) and endothelial NOS (*Nos3*).

A 25-fold downregulation was detected in *Arg-2* expression in BM cultured with MSC as compared to BM alone (p<0.0001) (**Figure 3.11**). In contrast, a 10- and 2-fold upregulation of *Nos1* and *Nos3* was induced in presence of MSC in comparison to the BM alone (non-significant and p=0.0113, respectively). *Nos1* and *Nos3* were not expressed by BM treated with cytokines. A 4-fold increase was documented in *Bcat1* expression in BM cultured with MSC (p=0.0035 versus BM alone), whereas 12- and 8-fold decreases were detected in *Hdc* and *Il4i1* expression as compared to BM alone (p<0.0001).

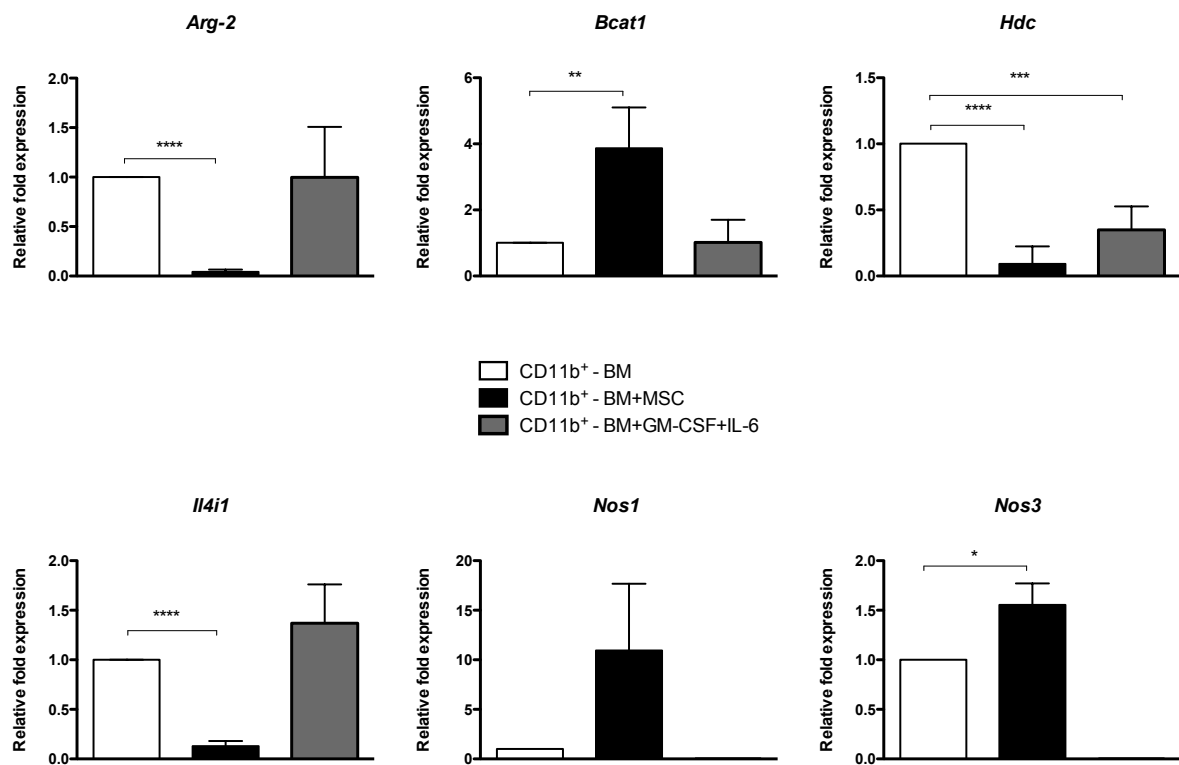


FIGURE 3.11. Enzymatic profile of CD11b⁺ cells from BM cultures. Quantitative RT-PCR data of CD11b⁺ cells sorted from 4 days cultures of BM alone, with MSC or with GM-CSF and IL-6. Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in CD11b⁺ from BM alone. Mean of 5 independent experiments, $\pm$ SD. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$ Unpaired t test. *Arg-2*, Arginase-2; *Bcat1*, branched chain aminotransferase 1; *Hdc*, histidine decarboxylase; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; *Il4i1*, Interleukin-4 induced 1; *Nos1*, nitric oxide synthase 1 (neuronal NOS); *Nos3*, nitric oxide synthase 3 (endothelial NOS).

3.3 DISCUSSION

In this chapter, we have shown that MSC drive the expansion (**Figure 3.3**) and differentiation (**Figure 3.2**) of BM mononuclear cells into three different subsets of CD11b⁺ cells, according to a differential expression of Gr-1: granulocytes (CD11b⁺ Gr-1^{high}), early myeloid cells (CD11b⁺ Gr-1^{int-low}) and macrophages (CD11b⁺ Gr-1⁻). Since CD11b is a marker expressed also by NK and B cells, we cannot exclude the presence of these two populations in the co-cultures. However, since the majority of Gr-1⁻ cells were F4/80⁺, we chose not to further characterise these populations.

The negligible proportion of MSC in unfractionated BM (estimated around 0.01-0.001% mononuclear cells) cannot account for the differentiation of BM cells alone. Therefore, further work is required to study the limiting dilution at which the phenomenon of macrophage differentiation can still be seen, as the ratio used in the experiments does not represent the physiological ratio between MSC and haematopoietic cells in the marrow. However, we argue that this culture system magnifies stromal and haematopoietic cell contact in the niche.

The main subset induced by the presence of MSC is represented by macrophages, as confirmed by phenotypical and morphological analysis (**Figure 3.4**). However, the phagocytic activity of these cells should be investigated to confirm their macrophage phenotype. In this population, M1 and M2 enzymes, such as *Arg-1* (**Figure 3.10**), *Ccl12*, *Marco*, *Il12b* and *Cox2* (data not shown), were highly up-regulated, suggesting a typical immature 'M0' profile.

Expression of *Nos2* was mainly detected in the CD11b⁺ Gr-1^{high} subset (**Figure 3.10**), suggesting the immaturity of MSC-induced granulocytes. Early myeloid cells (CD11b⁺ Gr-1^{int-low}) were not responsible for the up-regulation of any enzyme. These cells were not able to differentiate into Gr-1^{high} or Gr-1⁻ cells after a second round of co-culture with MSC (data not shown), suggesting that these cells either possess a primitive phenotype lacking of the required ligand/receptor for the differentiation induced by MSC or they present an irreversible differentiation stage. Further analysis

of expression of M1 and M2 enzymes at a protein level and investigation of the immunosuppressive properties of each of these populations is however needed to confirm their phenotype.

Characterization of the whole CD11b⁺ population revealed that MSC-induced myeloid cells were able to suppress the proliferation of mitogen-stimulated splenocytes (**Figure 3.7**). The up-regulation of *Nos2* and *Arg-1*, which are the two main enzymes exploiting the metabolism of L-arginine, together with a high expression of *Bcat1*, an enzyme that mediates T cell suppression through its catabolic activity on essential amino acids valine, leucine, and isoleucine, was consistent with the immunosuppressive properties of MSC-induced myeloid cells. However, the technical limitations of the assay make it impossible to know if MSC-induced myeloid cells are intrinsically immunosuppressive or whether this is the result of having been exposed to the inflammatory cytokines released in culture by activated splenocytes. A similar cytokine-dependent activation has been described for the immunomodulatory activities of MSC. Further assessment of the immunosuppressive properties of MSC-induced myeloid cells in a mixed-leukocyte reaction could provide a more physiological system, in order to minimise the confusing effect produced by inflammatory cytokines released upon mitogen stimulation of splenocytes. Another important effect to study is the profile of these cells after exposure to an inflammatory environment, both at mRNA and protein level.

The differentiation pattern mediated by MSC was similar to the one induced by GM-CSF and IL-6 on BM cells (**Figure 3.2**). The use of these cytokines has been shown to mediate the differentiation of MDSC [259]. This population has been characterised by the ability to mediate tumour immune-evasion through the suppression of T cell responses.

Neutrophils (CD11b⁺ Gr-1^{high}) and early myeloid cells (CD11b⁺ Gr-1^{int-low}) were mainly represented in MDSC, as confirmed by analysis of their phenotype and morphology (**Figure 3.7**). CD11b⁺ Gr-1^{int-low} cells were the main subset expressing M1 and M2 enzymes, and up-regulating IL-4R α , a marker that has been associated to the subset of MDSC in which the immunosuppressive ability resides [258].

Despite differences in the proportions of the three subsets of myeloid cells induced, both MSC and cytokines mediated the differentiation of immature cells, as confirmed by the low levels of *Hdc* expression. A recent study has shown that cancer cells down-regulate expression of *Hdc* expression and inhibit myeloid cell maturation [379]. Since the presence of GM-CSF and IL-6 induced a slightly higher expression of *Hdc* as compared to the presence of MSC, it could be inferred that MDSC are more mature than MSC-induced myeloid cells (**Figure 3.11**).

Nos2 and *Arg-1* up-regulation in the CD11b⁺ Gr-1^{int-low} subset, as well as the up-regulation of *Ido* and *Il4i1* in the total population (**Figure 3.10** and **3.11**), could probably account for MDSC immunosuppressive ability [380]. The marker profile that has been used was relatively limited and may benefit of further refinement to analyse the differences in the phenotype and function of these two populations.

Taken together, these data suggest that MSC can drive the expansion and differentiation of immature myeloid cells with a phenotype and function similar to heterogeneous myeloid populations that accumulate in tumour-bearing hosts. It is therefore plausible that stromal cells are one of the mechanisms by which macrophages are recruited and differentiated at the BM and in peripheral sites, thus opening a major line of investigation on the possible modulation of stromal cell activity in peripheral tissues under physiological and pathological conditions.

CHAPTER 4.

INVESTIGATING THE MOLECULAR BASIS OF HAEMATOPOIETIC AND MESENCHYMAL STROMA INTERACTION.

4.1 INTRODUCTION

In the previous chapter we have provided evidence that MSC induce *in vitro* the differentiation of three populations of myeloid cells according to the differential expression of Gr-1.

In particular, CD11b⁺ Gr-1⁻ cells exhibit a profile compatible with M0 type of macrophages, as defined by the up-regulation of both M1 and M2 markers and the ability to suppress the proliferation of T cells. Since NOS2 is one of the principal mediators of the immunosuppressive ability of mouse BM derived MSC [122] and macrophages, we investigated whether NOS2 could also regulate the ability of MSC to induce the differentiation of myeloid cells. Furthermore, NO, one of the metabolites produced by NOS activity, has recently been investigated also for its role as putative regulator of the HSC niche. Its inhibition causes an accumulation of HSPC progenitors in the BM, and an increase in the number of neutrophils in peripheral blood [369]. Accordingly, *in vitro* studies have demonstrated that NO can inhibit the differentiation of erythroid cells and induce instead myeloid differentiation in CD34⁺ cells [381].

Several factors are known to up-regulate the expression of NOS2, in particular hypoxia and pro-inflammatory cytokines, such as TNF- α and IFN- γ . Indeed, also the inflammatory environment has

emerged as potential regulator of HSPC niche activity, although it is not clear whether such regulation acts directly on HSC functions or on other cellular components of the HSC niche.

In fact, several pieces of evidence indicate that also other niche components, such as osteoclasts, osteoblasts, neutrophils, macrophages, MSC precursors and the extracellular matrix [332] can modulate self-renewal and differentiation of HSPC.

Taken altogether, our discovery that MSC are capable of differentiating myeloid cells, along with the notion that macrophages and MSC precursors interact in the HSC niche [262], led us to investigate several putative molecules involved in the regulation of haematopoietic differentiation as mediators of MSC ability to induce the differentiation of myeloid cells.

4.2 RESULTS

4.2.1 Contact is required for MSC induced differentiation of CD11b⁺ Gr-1⁻ cells

To determine whether cell contact or soluble factors mediate MSC induced myeloid differentiation, BM cells were cultured with MSC either in contact or separated by a transwell membrane for 4 days.

The percentage of CD11b⁺ Gr-1⁻ cells was significantly reduced when BM was separated from MSC by a transwell membrane (7.1% $\pm$ 1.3% versus 50.6% $\pm$ 12.5% in BM+MSC, $p=0.0003$, **Figure 4.1B**). However, the presence of MSC in the transwell cultures was still able to induce an increase in the percentage of CD11b⁺ Gr-1^{int-low} cells as compared to BM cultured alone (22.3% $\pm$ 2.5% versus 10.4% $\pm$ 1.6% in BM alone, $p=0.0008$, **Figure 4.1B**), thus suggesting that differentiation of this subset is mediated by a soluble factor.

Analysis of the expression of F4/80 and Gr-1 revealed that when BM was cultured separated from MSC, the percentage of CD11b⁺ Gr-1⁻ F4/80⁺ cells was reduced (2.19% versus 40.8% of BM+MSC), whereas the percentage of CD11b⁺ Gr-1^{high} and Gr-1^{int-low} was increased (33.8% versus 17.6% of BM+MSC) (**Figure 4.1**).

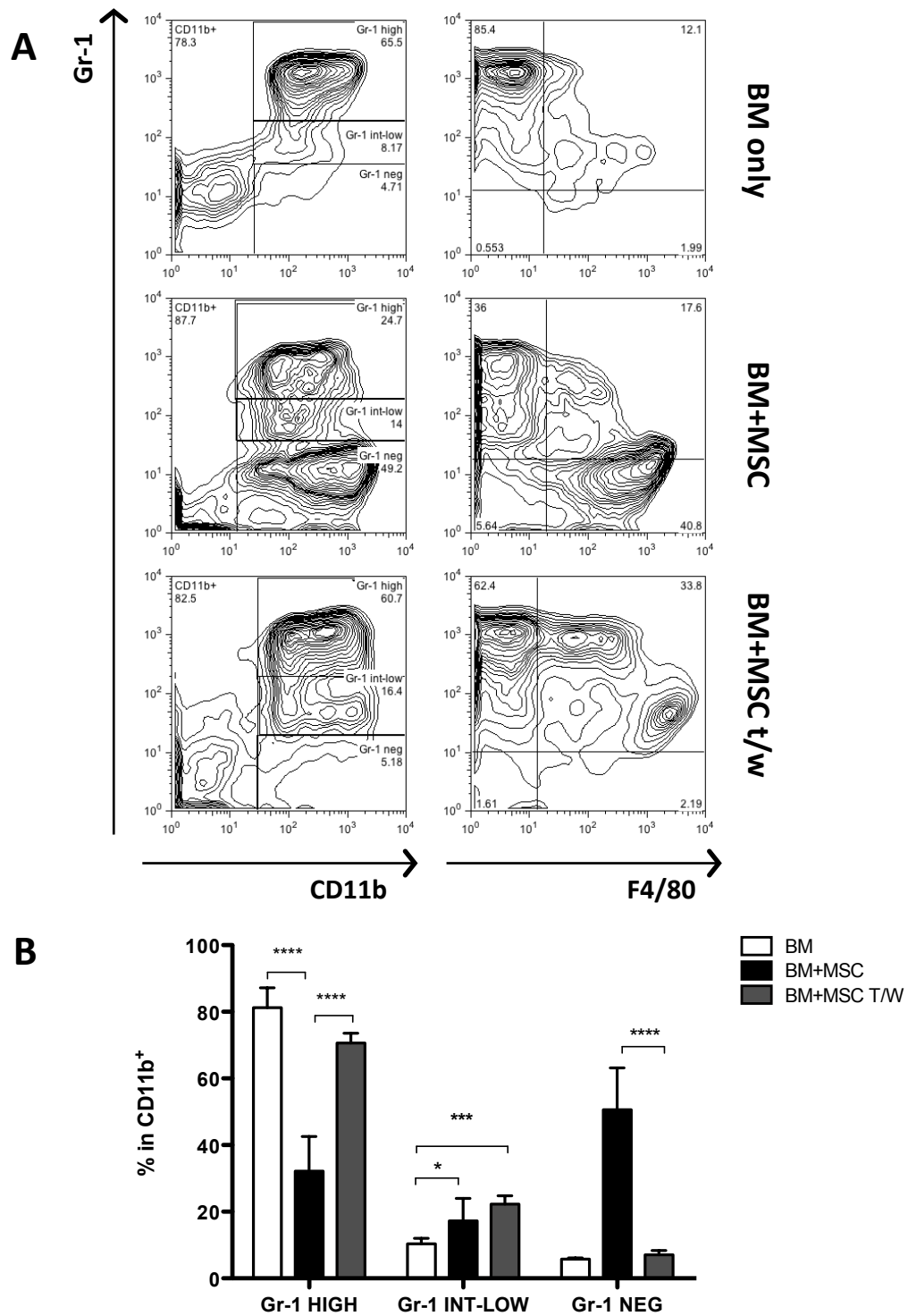


FIGURE 4.1. MSC induced differentiation of myeloid cells depends on cell contact. BM was cultured alone or with MSC (ratio 5:1) either in contact (BM+MSC) or separated by a transwell membrane (BM+MSC t/w) for 4 days. **A.** Contour plots represent the proportion of CD11b⁺ Gr-1^{high}, CD11b⁺ Gr-1^{int-low} and CD11b⁺ Gr-1^{neg} cells in the live gate, and expression of F4/80 versus Gr-1 in the CD11b⁺ gate. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1^{neg} in CD11b⁺ cells. Mean of 3 independent experiments, $\pm$ SD * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$ Unpaired t test.

4.2.2 MSC-induced differentiation of CD11b⁺ Gr-1⁻ cells requires NOS2 expression

In order to investigate the effect of NOS2 in MSC-mediated differentiation of myeloid cells, short-term co-culture experiments of BM cells and BM derived MSC isolated from WT or Nos2^{-/-} mice were performed.

BM derived MSC isolated from WT and Nos2^{-/-} mice were plated overnight in a 24 well plate, and unfractionated WT BM cells were added for 4 days. In order to assess myeloid differentiation, cells were collected from the co-cultures and analysed for the expression of markers CD11b, Gr-1, F4/80, CD115, CD206, CD169 and CD68 (**Figure 4.2**).

Nos2^{-/-} MSC exhibited a reduced ability to induce an increase in the percentage of CD11b⁺ Gr-1⁻ cells (16.6% ± 2.3% versus 30.8% ± 4.9% of BM WT+MSC WT, p=0.0020), but they induced an increased proportion of CD11b⁺ Gr-1^{high} cells (71.9% ± 3.6% versus 58.8% ± 2.3% of BM WT+MSC WT, p=0.0009) as compared to WT MSC in the CD11b⁺ gated population (**Figure 4.2B**). Representative contour plots of Gr-1 versus CD11b are shown in **Figure 4.2A**.

Analysis of macrophage markers F4/80, CD206, and CD68 confirmed a decrease in the proportion of macrophages obtained from BM cultured with Nos2^{-/-} MSC. In particular, the percentage of CD11b⁺ F4/80⁺ cells was decreased in the co-cultures of BM cells and Nos2^{-/-} MSC (27.8% ± 8.2% versus 41.5% ± 8.7% in BM WT+MSC WT, p=0.0183, **Figure 4.2C**), as compared to BM cultured with WT MSC. A significant decrease was also detected in the percentage of CD11b⁺ CD206⁺ cells from BM cultured with Nos2^{-/-} MSC (12.3% ± 3% versus 20.6% ± 2% in BM WT+MSC WT, p=0.0074 **Figure 4.2C**). No difference was detected in the percentage of CD11b⁺ CD115⁺ cells (42.7% ± 13.7% versus 45% ± 13.9% in BM WT+ MSC WT), CD169⁺ cells (1.5% ± 0.9% versus 2.3% ± 0.6% in BM WT+MSC WT) and CD68⁺ cells (11.8% ± 0.08% versus 16.8% ± 4.9% in BM WT+MSC WT)

between the two co-cultures. In particular, the expression of F4/80 and CD206 was decreased in the CD11b⁺ Gr-1⁻ subset. Reduced, albeit not significant, was also the expression of markers CD115 and CD68 in the CD11b⁺ Gr-1⁻ subset (**Figure 4.2A**).

In order to rule out whether the lack of Nos2 could impair also the expansion of CD11b⁺ cells, we evaluated the percentage of CD11b⁺ cells recovered after the co-cultures. A 2-fold decrease in the percentage of CD11b⁺ cells collected at day 4 was documented in BM cultured with Nos2^{-/-} MSC ($5.3 \times 10^6 \pm 0.4 \times 10^6$ versus $8.4 \times 10^6 \pm 0.5 \times 10^6$, $p=0.0016$ versus BM+MSC WT, **Figure 4.2D**).

A

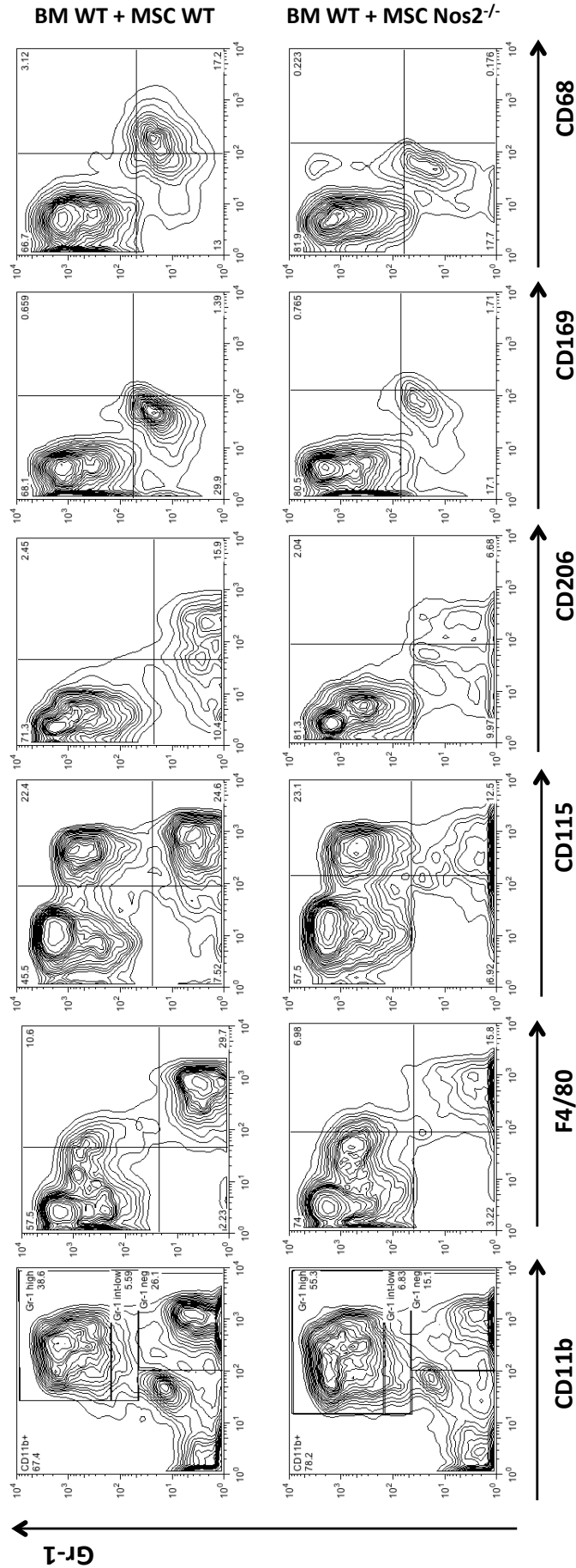


FIGURE 4.2. MSC induced differentiation of CD11b⁺ Gr-1⁻ cells requires NOS2 expression. BM cells were cultured with MSC isolated from WT or Nos2^{-/-} mice for 4 days. A. Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the expression of F4/80, CD115, CD206, CD169, and CD68 in the CD11b⁺ gated population.

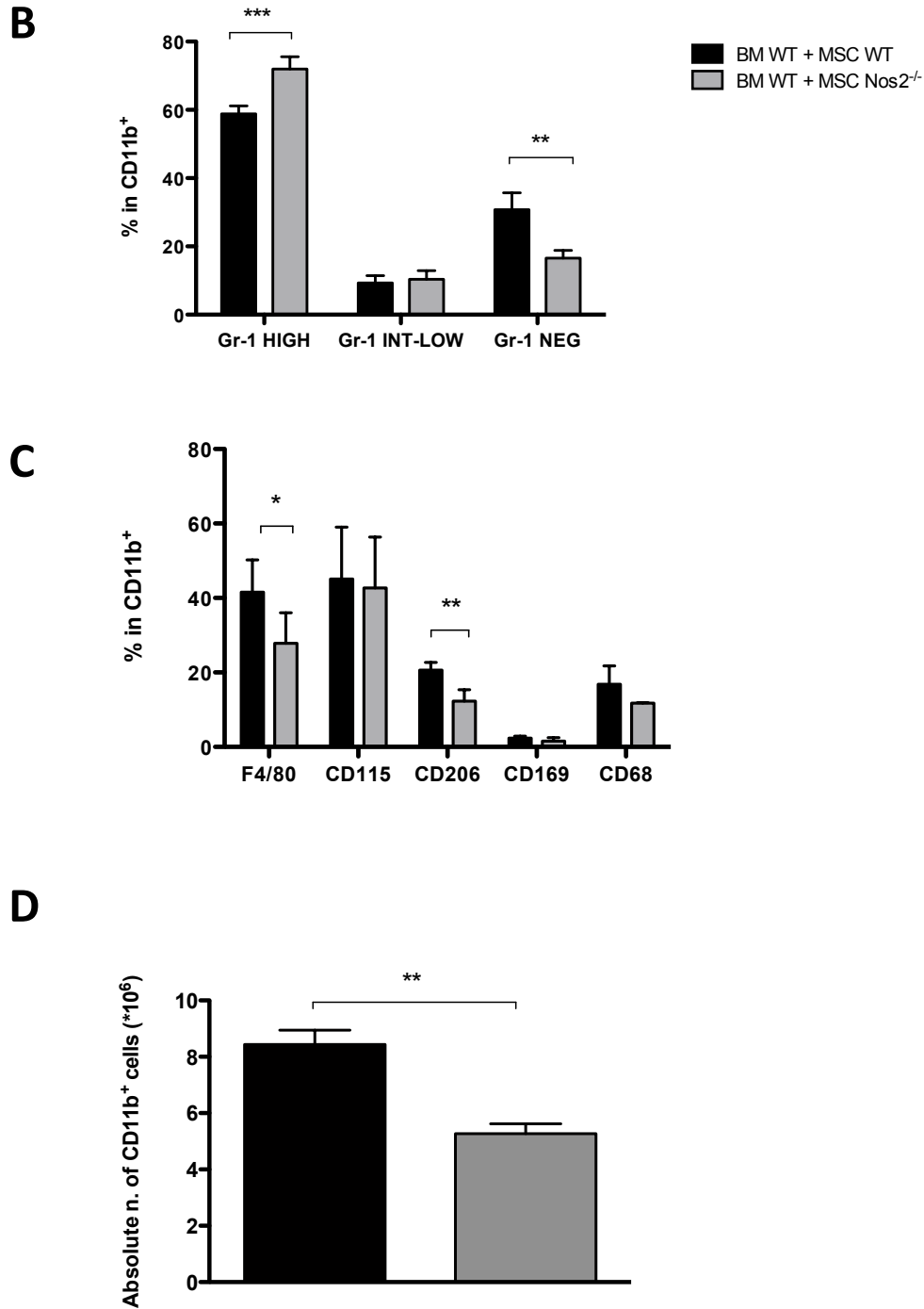


FIGURE 4.2. MSC induced differentiation of CD11b⁺ Gr-1⁻ cells requires NOS2 expression. BM cells were cultured with MSC isolated from WT or Nos2^{-/-} mice for 4 days. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ cells. Mean of 6 independent experiments, $\pm$ SD ** $p < 0.005$ *** $p < 0.001$ Unpaired t test. **C.** Percentage of CD11b⁺ F4/80⁺, CD115⁺, CD206⁺, CD169⁺, and CD68⁺ cells, within the CD11b⁺ cells population. Mean of 6 independent experiments, $\pm$ SD * $p < 0.05$ ** $p < 0.005$ Unpaired t test. **D.** Absolute number of CD11b⁺ cells recovered from BM cultured with MSC WT or Nos2^{-/-} for 4 days. Mean of 6 independent experiments, $\pm$ SD ** $p < 0.005$ Unpaired t test.

4.2.2.1 Nos2 is required on MSC but not on BM cells for myeloid differentiation

We further asked whether a lack of Nos2 in the haematopoietic cells could also determine an impairment of myeloid differentiation. BM isolated from Nos2^{-/-} or WT mice was co-cultured with MSC isolated from Nos2^{-/-} or WT mice for 4 days. Analysis of Gr-1 and CD11b expression showed that the lack of Nos2 in BM cells did not influence the differentiation of myeloid cells induced by MSC (**Figure 4.3**). Nevertheless, a small decrease in the percentage of CD11b⁺ Gr-1⁻ cells was detected in co-cultures of Nos2^{-/-} BM with WT MSC as compared to WT BM cultured with WT MSC (23.4% versus 29.2% of BM WT+MSC WT, **Figure 4.3A and B**). A similar decrease was documented in co-cultures of NOS2^{-/-} BM with Nos2^{-/-} MSC as compared to WT BM cultured with Nos2^{-/-} MSC (9.74% versus 12.2% of BM WT+MSC Nos2^{-/-}, **Figure 4.3A and B**).

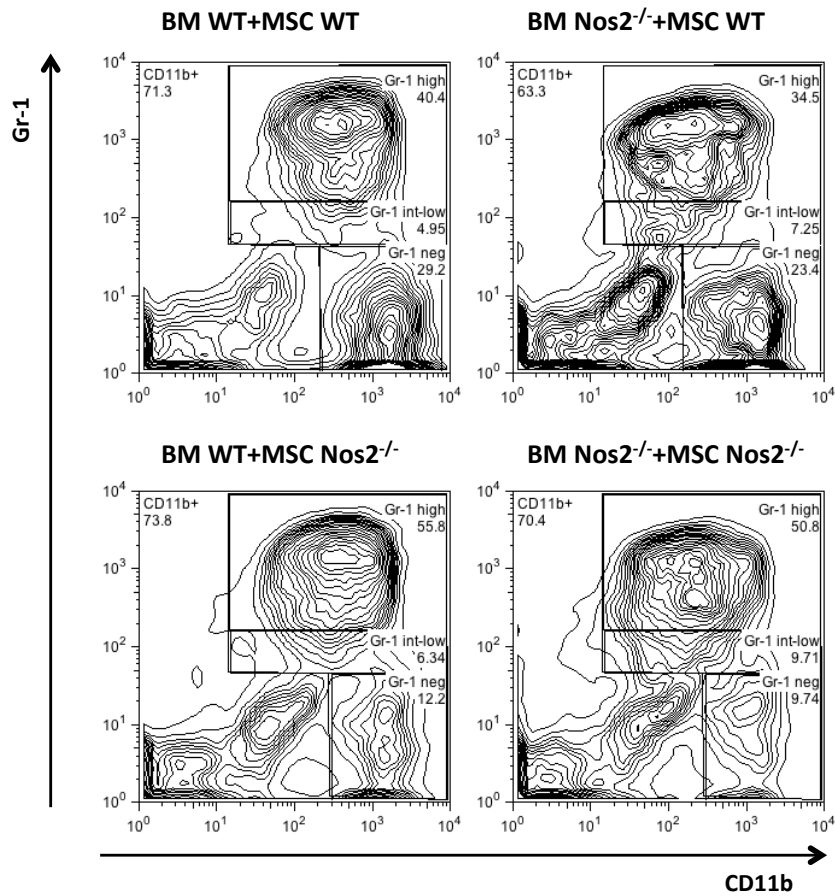
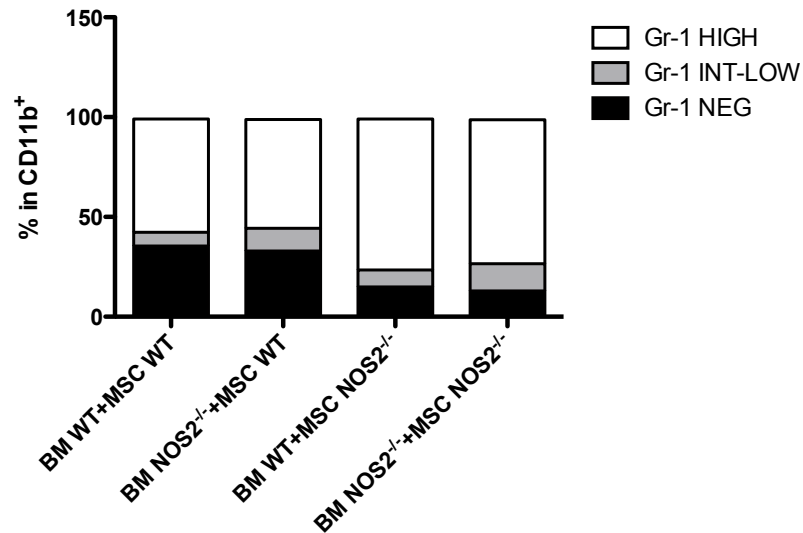
A**B**

FIGURE 4.3. BM cells do not require *Nos2* expression when differentiation is driven by MSC. BM cells isolated from WT or *Nos2*^{-/-} mice were cultured with MSC isolated from WT or *Nos2*^{-/-} mice for 4 days. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ gated population. A representative example of two independent experiments is shown.

4.2.2.2 Functional characterization of *Nos2*^{-/-} MSC-induced myeloid cells

On the basis of the impaired differentiation seen in the co-cultures of BM and *Nos2*^{-/-} MSC, we further characterised the enzymatic and functional profile of CD11b⁺ cells obtained from culturing the BM with *Nos2*^{-/-} MSC or WT MSC.

CD11b⁺ cells isolated from co-cultures of BM cells with MSC WT and *NOS2*^{-/-} were analysed for the expression of M1 and M2 macrophage functional markers by RT-PCR (**Figure 4.4**). A significant 2-fold decrease in the expression of *Cox2* and *Nos2* was found in BM cultured in presence of *Nos2*^{-/-} MSC (0.36 ± 0.3 , $p=0.0106$ and 0.37 ± 0.24 , $p=0.0218$ versus BM WT+MSC WT, respectively). A 2-fold increase was documented in *Marco* expression in BM cultured with *Nos2*^{-/-} MSC, but such variation was not significant (3.8 ± 2.6 versus BM WT+MSC WT). *Arg-1* and *Ccl12* were down-regulated in presence of *Nos2*^{-/-} MSC as compared to WT MSC (0.28 ± 0.16 , $p=0.0232$ and 0.42 ± 0.03 , $p=0.014$ versus BM WT+MSC WT respectively). No significant difference was found in the expression levels of *Il12b*, *Chi3l1* and *Irf4* in the two cultures (0.58 ± 0.5 , 0.63 ± 0.39 , and 0.52 ± 0.49 versus BM WT+MSC WT).

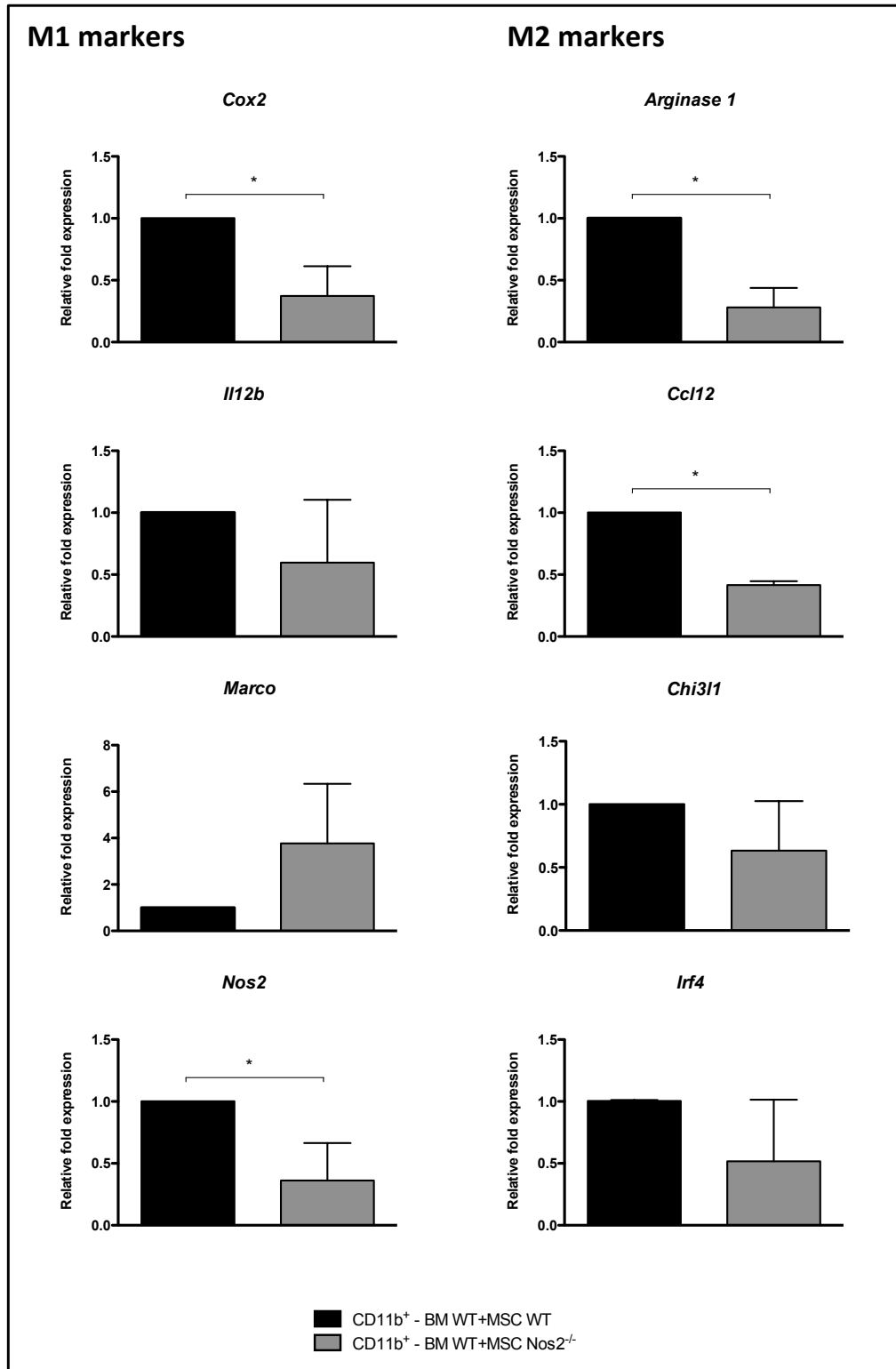


FIGURE 4.4. CD11b⁺ cells isolated from BM and WT or Nos2^{-/-} MSC co-cultures have a different M1-M2 profile. Quantitative RT-PCR data of CD11b⁺ cells isolated from 4 days cultures of BM with MSC WT or Nos2^{-/-}. Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in CD11b⁺ isolated from BM and MSC WT co-cultures. Mean of 3 independent experiments, $\pm$ SD. * $p < 0.05$, Unpaired t test. *Arg1*, arginase-1; *Ccl12*, CC-chemokine ligand 12; *Chi3l1*, chitinase 3-like 1; *Cox2*, cyclooxygenase 2; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; *Il12b*, interleukin 12b; *Irf4*, interferon factor 4; *Marco*, macrophage receptor with collagenous structure; *Nos2*, nitric oxide synthase 2.

CD11b⁺ cells isolated from BM and MSC cultures can suppress activated T cells. As *Nos2* and *Arginase-1*, mediators of M0 macrophages immunosuppressive ability, were remarkably down-regulated in CD11b⁺ cells obtained from BM cultured with *Nos2*^{-/-} MSC, we tested these cells for the suppression of T cell proliferation.

After 4 days, BM cultured with MSC isolated from WT or *Nos2*^{-/-} mice was collected and CD11b⁺ cells were isolated. The cells were plated at different percentages of the total number of cells in the cultures (12%, 6%, 3% and 1.5%), and let adhere for 4 hours. Concanavalin-A (ConA) activated splenocytes were added for 72 hours and proliferation was assessed by tritiated (³H) thymidine uptake. The proliferation of activated splenocytes was suppressed by CD11b⁺ cells isolated from BM cultured with *Nos2*^{-/-} MSC until they were present as 3% of the total amount of cells in culture, whereas CD11b⁺ cells obtained from BM cultured with WT MSC were immunosuppressive even when they represented only 1.5% of the total cells in culture (50% of inhibition) (**Figure 4.5**).

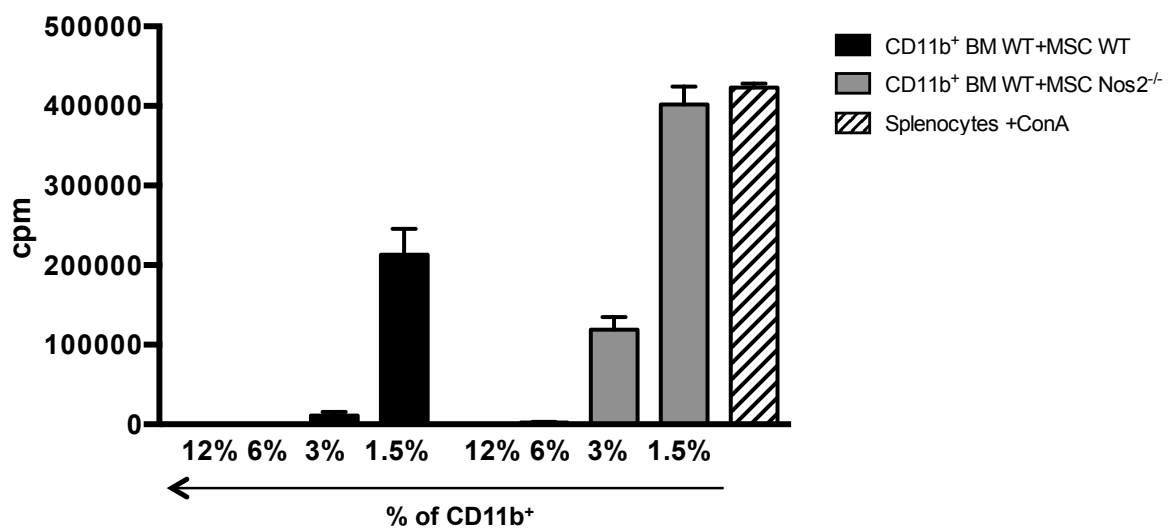


FIGURE 4.5. CD11b⁺ cells isolated from BM cultured with WT or Nos2^{-/-} MSC are immunosuppressive. BM cells were cultured with MSC isolated from WT or Nos2^{-/-} mice for 4 days. CD11b⁺ cells were sorted from the cultures, and plated into 96 well flat bottom plate; cells were present at different percentages of the total number of cells in the cultures (12%, 6%, 3% and 1.5%). ConA stimulated splenocytes were then added for 72h, and ³H-thymidine was added for the last 18 hours of co-culture. Representative experiment from 3 independent experiments; each column represents the mean±SD of triplicates. (cpm: count per minute). Results are expressed as the value obtained by subtracting the (CD11b⁺ cells+Splenocytes) value from the (CD11b⁺ cells+Splenocytes ConA) value.

4.2.2.3 *Nos2^{-/-} mice have a normal mononuclear phagocyte system*

In order to understand whether *Nos2^{-/-}* mice had an intrinsic impaired ability to differentiate macrophages in steady-state, we analysed the proportions of BM and spleen mononuclear phagocyte cells in *Nos2^{-/-}* and WT mice. Following the gating strategy proposed by the group of Frenette [262], neutrophils granulocytes are represented in the Gr-1⁺ CD115⁻ gate (**Figure 4.6A**, gate I), monocytes are represented in the Gr-1⁺ CD115⁺ (gate II) and Gr-1^{low} CD115⁺ gate (gate III), whereas the Gr-1⁻ CD115^{int} F4/80⁺ fraction consists of eosinophils (gate V) and macrophages (gate IV), which can be discriminated by forward and side scatter characteristics. No difference was seen in the percentage of granulocytes (WT=35.4%±1.97 versus *Nos2^{-/-}*=34.14%±5.2%), monocytes (Gr-1^{high}: WT=6.86%±1.43% versus *Nos2^{-/-}*=8.24%±1.07%; Gr-1^{low}: WT=2.17%±1.24% versus *Nos2^{-/-}*=1.86%±0.54%), eosinophils (WT=0.59%±0.1% versus *Nos2^{-/-}*=0.59±0.25%) and macrophages (WT=4.58%±1.1% versus *Nos2^{-/-}*=4.55%±0.86%) in BM of WT or *Nos2^{-/-}* mice (**Figure 4.6B**).

Following the definition of Geissmann group [163], CD11b⁺ cells (**Figure 4.7A**, gate A) were divided into Ly6G⁺ Ly6C^{int-low} (neutrophils, gate B), and Ly6G⁻ cells. Ly6G⁻ cells were subdivided into Ly6C⁻ CCR2⁻ CD115⁻ resident monocytes (gate C), Ly6C^{dim} CCR2⁻ CD115⁻ monocytes (gate D), and Ly6C⁺ CCR2⁺ CD115⁺ inflammatory monocytes (gate E). No difference was documented in the percentages of CD11b⁺ cells (WT=45.87%±3.9% versus *Nos2^{-/-}*=46.24%±6.3%), Ly6G⁺ Ly6C^{int-low} cells (WT=32.37%±3.06% versus *Nos2^{-/-}*=31.92%±5.87%), Ly6C⁻ CCR2⁻ CD115⁻ cells (WT=0.9%±0.02% versus *Nos2^{-/-}*=1.07%±0.10%), Ly6C^{dim} CCR2⁻ CD115⁻ cells (WT=7.22%±0.34% versus *Nos2^{-/-}*=7.91%±0.73%) and Ly6C⁺ CCR2⁺ CD115⁺ cells (WT=4.11%±0.36% versus *Nos2^{-/-}*=4.06%±0.80%) in WT or *Nos2^{-/-}* mice (**Figure 4.7B**).

Analysis of these populations in spleen of WT or *Nos2^{-/-}* recipients showed no differences (data not shown).

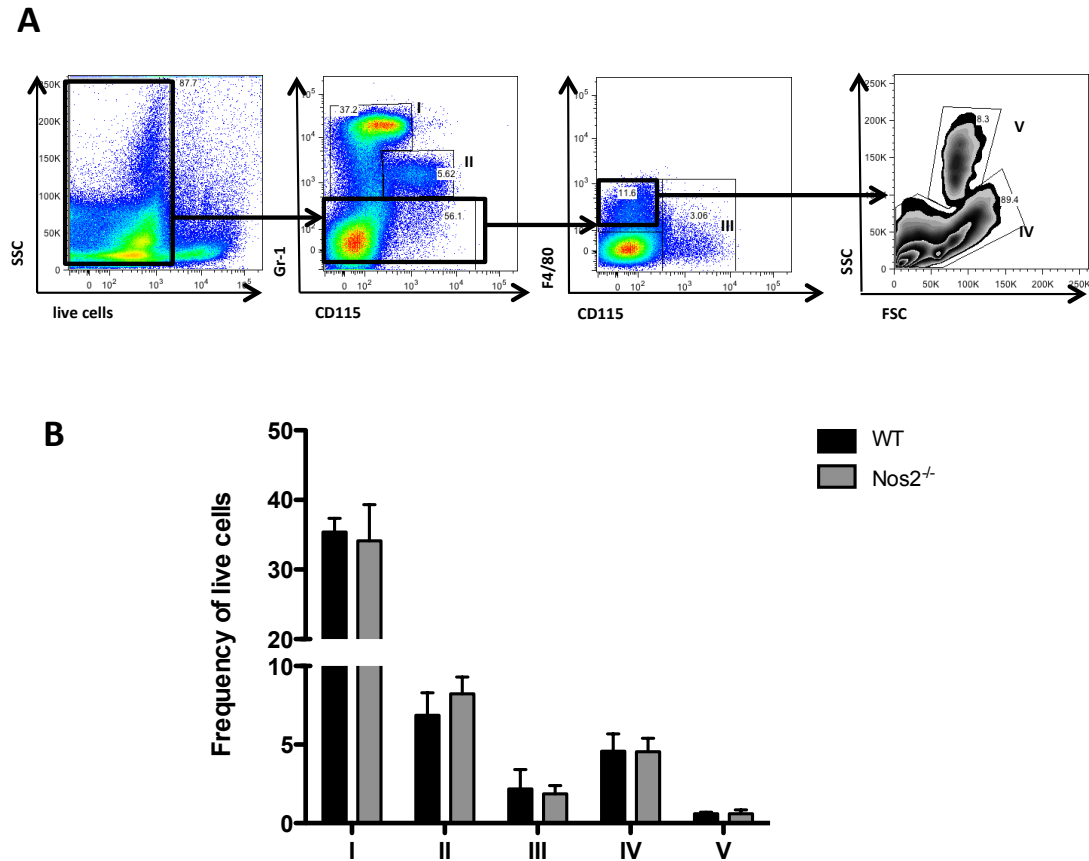


FIGURE 4.6. Proportions of BM mononuclear phagocyte populations in naïve WT and *Nos2*^{-/-} mice are similar. BM cells of naïve WT and *Nos2*^{-/-} mice were analysed by FACS. **A.** Gating strategy. Alive Gr-1⁺ populations were divided into a CD115⁻ fraction comprised of neutrophils (I) and CD115⁺ fraction of Gr-1^{high} monocytes (II). The Gr-1^{low} fraction was further subdivided into two populations: CD115⁺ Gr-1^{low} monocytes (III) and an F4/80⁺CD115⁻ population, which can be subdivided into SSC^{high} eosinophils (V) and SSC^{int/low} macrophages (IV). **B.** Percentage of BM mononuclear phagocyte populations within the live gate (n=5 per group).

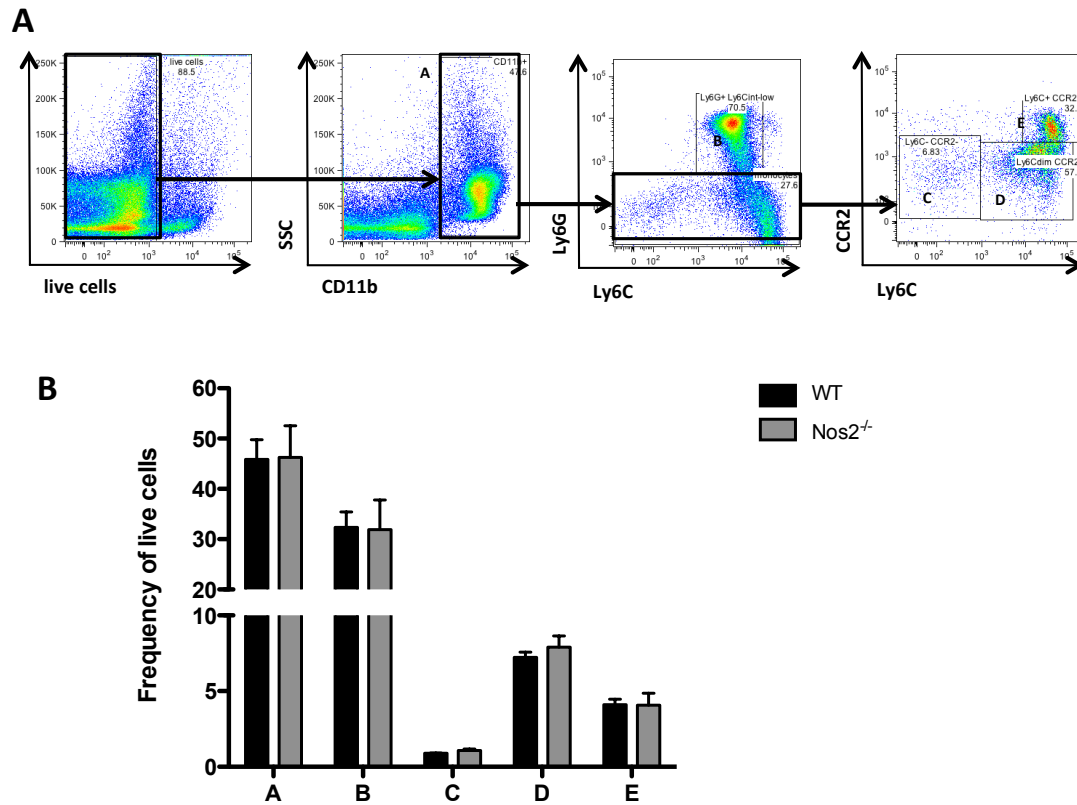


FIGURE 4.7. Proportions of BM mononuclear phagocyte populations in naïve WT and *Nos2*^{-/-} mice are similar. BM cells of naïve WT and *Nos2*^{-/-} mice were analysed by FACS. **A.** Gating strategy. Alive CD11b⁺ cells (A) were divided into Ly6G⁺ Ly6C^{int-low} (neutrophils, B), and Ly6G⁻ cells. The Ly6G⁻ gate was subdivided into Ly6C⁻ CCR2⁻ CD115⁻ resident monocytes (C), Ly6C^{dim} CCR2⁻ CD115⁻ monocytes (D) and Ly6C⁺ CCR2⁺ CD115⁺ inflammatory monocytes (F). **B.** Percentage of BM mononuclear phagocyte populations within the live gate (n=5 per group).

4.2.2.4 Role of NOS2 in driving macrophage differentiation *in vivo*

In the previous paragraphs we discussed how NOS2 expression is required on MSC but not on haematopoietic cells for driving macrophage differentiation. Therefore, in order to investigate the role of NOS2 expression on stromal cells *in vivo*, we chose two different approaches. The first approach was to investigate whether and how WT or Nos2^{-/-} MSC could enhance early reconstitution of haematopoiesis in myeloablated WT recipients. The rationale of the second approach, in which myeloablated and clodronate-treated WT or Nos2^{-/-} recipients received an adoptive transfer of WT BM cells, was studying the haematopoietic recovery in a NOS2 deficient mesenchymal stroma.

In both approaches, macrophage differentiation of donor cells was studied, 10 or 14 days after the transfer respectively.

4.2.2.4.1 Adoptive transfer of Nos2^{-/-} MSC results in impaired macrophage differentiation of donor cells

In order to study the role of stromal cells in supporting and inducing the differentiation of macrophages, we decided to assess the effect of MSC following HSC transplantation. Myeloablated WT Ly5.1⁺ C57Bl/6 received syngeneic WT Ly5.2⁺ BM cells either alone, with WT MSC or with Nos2^{-/-} MSC. After 10 days, BM and spleen mononuclear phagocyte populations were analysed by FACS (**Figure 4.8A**).

No significant difference was documented in the engraftment of donor CD45.2⁺ cells in the BM (BM alone=42.6%±3.24%, BM+MSC WT=33.55%±3.18% and BM+MSC Nos2^{-/-}=34.63%±5.23%) or in the spleen (BM alone=43.36%±1.54%, BM+MSC WT=41.05%±1.34% and BM+MSC Nos2^{-/-}=31.3%±9.46%) of mice injected with BM cells alone or with MSC (**Figure 4.8C**).

Following the gating strategy previously described (**Figure 4.8B**), analysis of mononuclear phagocyte populations within the donor cells (CD45.2⁺) in the BM showed that there was no difference in the proportions of granulocytes (BM alone=67.44%±4.55%; BM+MSC WT=64.95%±5.16%; BM+MSC Nos2^{-/-}=73.07%±10.55%), monocytes (Gr-1^{high}: BM alone=1.1%±0.35%; BM+MSC WT=1.42%±0.16%; BM+MSC Nos2^{-/-}=1.4%±0.62%; Gr-1^{low}: BM alone=0.39%±0.04%; BM+MSC WT=0.45%±0.13%; BM+MSC Nos2^{-/-}=0.53%±0.21%), and eosinophils (BM alone=0.32%±0.1%; BM+MSC WT=0.44%±0.16%; BM+MSC Nos2^{-/-}=0.37%±0.04%) in the three different groups (**Figure 4.9A**). An increase in the proportion of macrophages was instead documented in mice that were adoptively transferred with both BM cells and WT MSC in comparison to the ones that received only BM cells (2.46%±0.71% versus 1.17%±0.31%), and such increase was not documented in mice injected with BM cells and Nos2^{-/-} MSC (1.32%±0.44%) (**Figure 4.8D**). Analysis of the absolute numbers of these populations within the donor cells in the BM confirmed there was no difference in the proportions of granulocytes (BM alone=2.99x10⁶±1.22x10⁶; BM+MSC WT=2.16x10⁶±3.35x10⁵; BM+MSC Nos2^{-/-}=2.88x10⁶±1.24x10⁶), monocytes (Gr-1^{high}: BM alone=4.67x10⁴±1.55x10⁴; BM+MSC WT=4.66x10⁴±1.87x10³; BM+MSC Nos2^{-/-}=5.83x10⁴±4.28x10⁴; Gr-1^{low}: BM alone=1.74x10⁴±7.51x10³; BM+MSC WT=1.47x10⁴±3.02x10³; BM+MSC Nos2^{-/-}=2.19x10⁴±1.5x10⁴) and eosinophils (BM alone=1.49x10⁴±9.58x10³; BM+MSC WT=1.45x10⁴±4.25x10³; BM+MSC Nos2^{-/-}=1.39±3.34x10³) in the three groups (**Figure 4.8E**). An increase in the absolute number of macrophages was confirmed in mice that received both BM cells and WT MSC (8.04x10⁴±1.75x10⁴) as compared to mice injected with BM cells alone (5.07x10⁴±1.77x10⁴) or BM cells and Nos2^{-/-} MSC (4.82x10⁴±1.03x10⁴) (**Figure 4.8E**).

In mice injected with BM cells and MSC spleen mononuclear cells of donor origin exhibited a higher percentage of granulocytes (BM+MSC WT=32.3%±14.1%, BM+MSC Nos2^{-/-}=44.97%±13.49% versus BM alone=19.76%±2.28%), Gr-1^{high} monocytes (BM+MSC WT=10.71%±3.52%, BM+MSC Nos2^{-/-}=9.19%±3.5% versus BM alone=3.78%±0.69%), and eosinophils (BM+MSC WT=0.21%±0.04%, BM+MSC Nos2^{-/-}=0.18%±0.05% versus BM alone=0.08%±0.02%) as compared to mice that received only BM cells but no difference was observed between the groups receiving WT

or $Nos2^{-/-}$ MSC (**Figure 4.8F**). An increase in the percentage of $Gr-1^{low}$ monocytes (BM+MSC WT=2.67%±0.6% versus BM alone=1.02%±0.19% and BM+MSC $Nos2^{-/-}$ =1.67%±0.34%) and macrophages (BM+MSC WT=7.11%±1.18% versus BM alone=4.57%±0.59% and BM+MSC $Nos2^{-/-}$ =5.24%±0.73%) was documented in the group injected with BM cells and WT MSC as compared to the other two groups (**Figure 4.8F**). Analysis of the absolute number of spleen phagocyte populations revealed instead that mice that received BM cells and WT MSC had a decreased number of granulocytes (BM+MSC WT=2.5x10⁶±1.51x10⁶ versus BM alone=4.58x10⁶±1.14x10⁶, BM+MSC $Nos2^{-/-}$ =6.84x10⁶±2.45x10⁶), monocytes ($Gr-1^{high}$: BM+MSC WT=7.45x10⁵±1.91x10⁵ versus BM alone=8.59x10⁵±1.73x10⁵, BM+MSC $Nos2^{-/-}$ =1.37x10⁶±5.31x10⁵; $Gr-1^{low}$: BM+MSC WT=1.91x10⁵±6.9x10⁴ versus BM alone=2.32x10⁵±5.43x10⁴, BM+MSC $Nos2^{-/-}$ =2.47x10⁵±4.11x10⁴), eosinophils (BM+MSC WT=1.7x10⁴±1.24x10⁴ versus BM alone=1.78x10⁴±7.82x10³, BM+MSC $Nos2^{-/-}$ =2.73x10⁴±6.68x10³) and macrophages (BM+MSC WT=5.19x10⁵±2.17x10⁵ versus BM alone=1.05x10⁶±2.37x10⁵, BM+MSC $Nos2^{-/-}$ =7.85x10⁵±1.69x10⁵) as compared to the other two groups (**Figure 4.8G**).

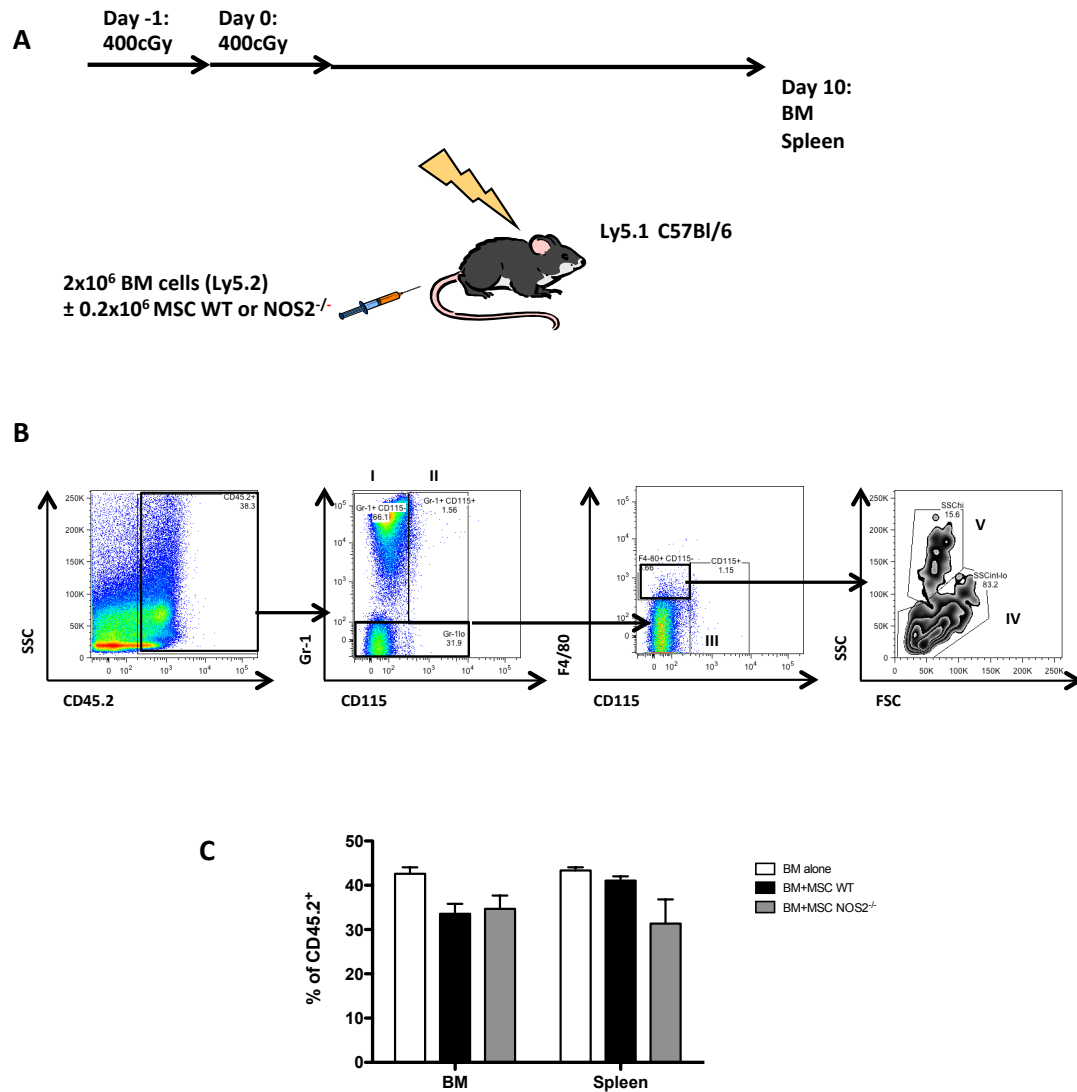


FIGURE 4.8. Nos2^{-/-} MSC exhibit an impaired ability to promote macrophage repopulation. Ly5.1 WT recipients were irradiated with a split dosage of 800cGy, and subsequently injected with 2*10⁶ Ly5.2 WT BM cells, either alone (BM alone) or in combination with 0.2*10⁶ Ly5.2 WT MSC (BM+MSC WT) or with 0.2*10⁶ Ly5.2 Nos2^{-/-} MSC (BM+MSC NOS2^{-/-}). 10 days after the transfer, BM and spleen were analysed by FACS. **A.** Scheme of experiment. **B.** Gating strategy of BM mononuclear phagocytes. Within the CD45.2⁺ cells, the Gr-1⁺ populations were divided into a CD115⁻ fraction comprised of neutrophils (I) and CD115⁺ fraction of Gr-1^{high} monocytes (II). The Gr-1^{low} fraction was further subdivided into two populations: CD115⁺ Gr-1^{low} monocytes (III) and a F4/80⁺CD115⁺ population, which can be subdivided into SSC^{high} eosinophils (V) and SSC^{int/low} macrophages (IV). **C.** Percentage of engraftment of CD45.2⁺ donor cells in BM and spleen (n=5/group).

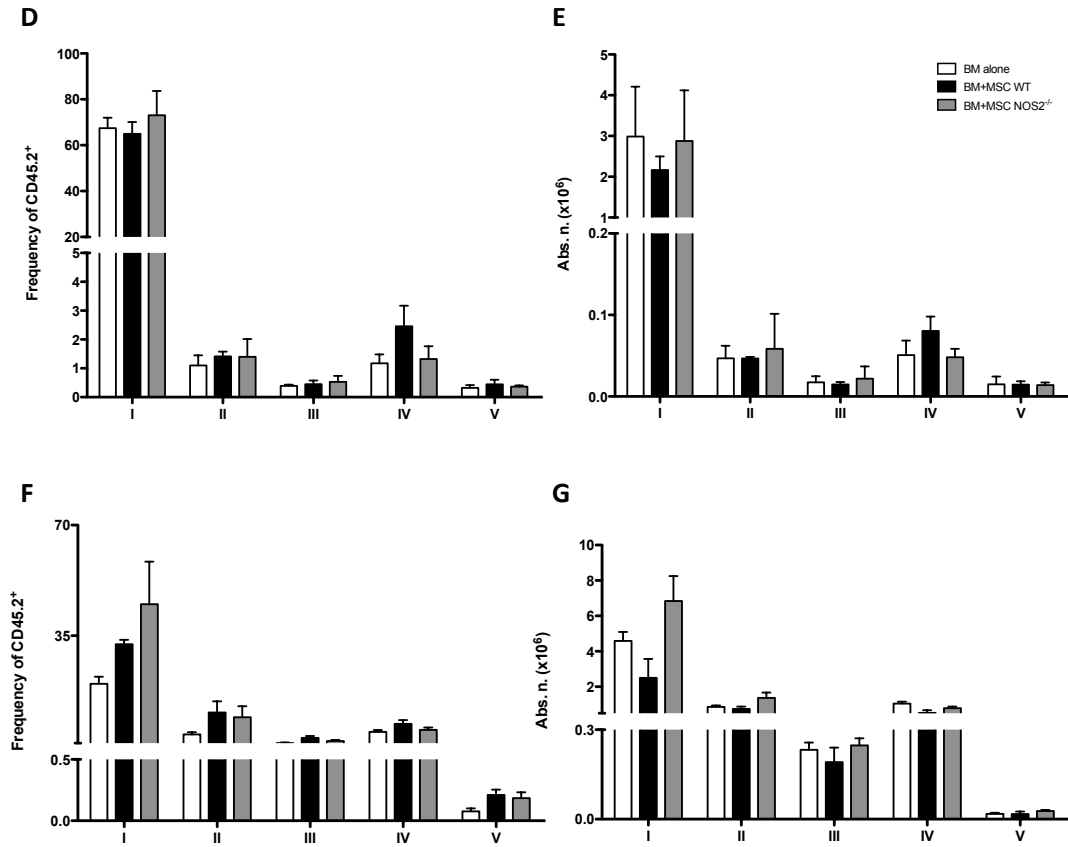


FIGURE 4.8. *Nos2*^{-/-} MSC exhibit an impaired ability to promote macrophage repopulation. Ly5.1 WT recipients were irradiated with a split dosage of 800cGy, and subsequently injected with 2*10⁶ Ly5.2 WT BM cells, either alone (BM alone) or in combination with 0.2*10⁶ Ly5.2 WT MSC (BM+MSC WT) or with 0.2*10⁶ Ly5.2 *Nos2*^{-/-} MSC (BM+MSC NOS2^{-/-}). 10 days after the transfer, BM and spleen were analysed by FACS. **D.** Percentage of BM mononuclear phagocyte populations within the donor cells (CD45.2⁺) in the BM. (n=5/group) **E.** Absolute number of BM mononuclear phagocyte populations within the gate of CD45.2⁺ cells in the BM (n=5/group). **F.** Percentage of BM mononuclear phagocyte populations within the donor cells (CD45.2⁺) in the spleen. (n=5/group) **G.** Absolute number of BM mononuclear phagocyte populations within the gate of CD45.2⁺ cells (n=5/group) in the spleen.

4.2.2.4.2 Adoptive transfer of BM cells into myeloablated and macrophage-depleted $Nos2^{-/-}$ recipient results in impaired differentiation of macrophages

In order to study the role of stromal NOS2 in driving the differentiation of macrophages, we studied the reconstitution of mononuclear phagocyte populations in $Nos2^{-/-}$ mice.

Ly5.2⁺ WT or $Nos2^{-/-}$ recipients were lethally irradiated, treated with clodronate and then transplanted with WT Ly5.1⁺ BM cells. After 14 days, BM and spleen mononuclear phagocyte populations were analysed by FACS (**Figure 4.9A**).

No significant difference was documented in the engraftment of donor CD45.1⁺ cells in the BM (WT=45.39%±9.37% versus $Nos2^{-/-}$ =55.24%±13.28%) or in the spleen (WT=32.53%±13.96% versus $Nos2^{-/-}$ =33.21%±10.54%) of WT and $Nos2^{-/-}$ mice (**Figure 4.9B**). Following the same gating strategy discussed for the previous approach (**Figure 4.8B**), analysis of mononuclear phagocyte populations within the donor cells (CD45.1⁺) in the BM showed that there was no difference in the proportions of granulocytes (WT=63.23%±6.21% versus $Nos2^{-/-}$ =57.49%±5.99%), monocytes (Gr-1^{high}: WT=0.08%±0.08% versus $Nos2^{-/-}$ =0.04%±0.02%; Gr-1^{low}: WT=0.43%±0.17% versus $Nos2^{-/-}$ =0.35%±0.18%) and eosinophils (WT=0.18%±0.14% versus $Nos2^{-/-}$ =0.16%±0.07%) in WT and $Nos2^{-/-}$ recipients (**Figure 4.9C**). A significant difference was instead documented in the percentage of macrophages within donor CD45.1⁺ cells in WT recipients (WT=1.87%±0.48% versus $Nos2^{-/-}$ =1.18%±0.22%, $p=0.0047$) as compared to $Nos2^{-/-}$ recipients (**Figure 4.9C**).

Analysis of these populations within the recipient cells (CD45.2⁺) in the BM revealed no difference in the proportions of granulocytes (WT=69.1%±8.76% versus $Nos2^{-/-}$ =59.68%±8.88%), monocytes (Gr-1^{high}: WT=0.2%±0.04% versus $Nos2^{-/-}$ =0.21%±0.14%; Gr-1^{low}: WT=0.34%±0.07% versus $Nos2^{-/-}$ =0.47%±0.21%), eosinophils (WT=0.4%±0.21% versus $Nos2^{-/-}$ =0.78%±0.59%) and macrophages (WT=1.95%±1.71% versus $Nos2^{-/-}$ =1.8%±1.32%) in WT and $Nos2^{-/-}$ recipients (**Figure 4.9D**).

Spleen mononuclear phagocyte populations were analysed in WT and $NOS2^{-/-}$ recipients. Analysis of these populations within the donor ($CD45.1^{+}$) cells showed there was no significant difference in the percentages of granulocytes (WT=31.31%±4.35 versus $Nos2^{-/-}$ =34.26%±6.86%), monocytes ($Gr-1^{high}$: WT=1.13%±1.08% versus $Nos2^{-/-}$ =0.62%±0.41%; $Gr-1^{low}$: WT=1.42%±1.16% versus $Nos2^{-/-}$ =1.06%±0.69%), eosinophils (WT=0.23%±0.21% versus $Nos2^{-/-}$ =0.3%±0.21%) and macrophages (WT=7.55%±3.95% versus $Nos2^{-/-}$ =5.81±2.67%) in WT or $Nos2^{-/-}$ recipients (**Figure 4.9E**). No difference was seen in the percentages of granulocytes (WT=7.49%±4.39% versus $Nos2^{-/-}$ =8.46%±3.99%), monocytes ($Gr-1^{high}$: WT=0.09%±0.12 versus $Nos2^{-/-}$ =0.15%±0.07%; $Gr-1^{low}$: WT=0.42%±0.29% versus $Nos2^{-/-}$ =0.2%±0.11%), eosinophils (WT=0.46%±0.46% versus $Nos2^{-/-}$ =0.31%±0.18%) and macrophages (WT=1.55%±0.97% versus $Nos2^{-/-}$ =0.67%±0.29%) within the recipient ($CD45.2^{+}$) spleen cells of WT or $Nos2^{-/-}$ mice (**Figure 4.9F**). No difference was documented in the absolute numbers of mononuclear phagocyte populations within donor or recipient cells in BM or spleen of WT or $Nos2^{-/-}$ recipients (data not shown).

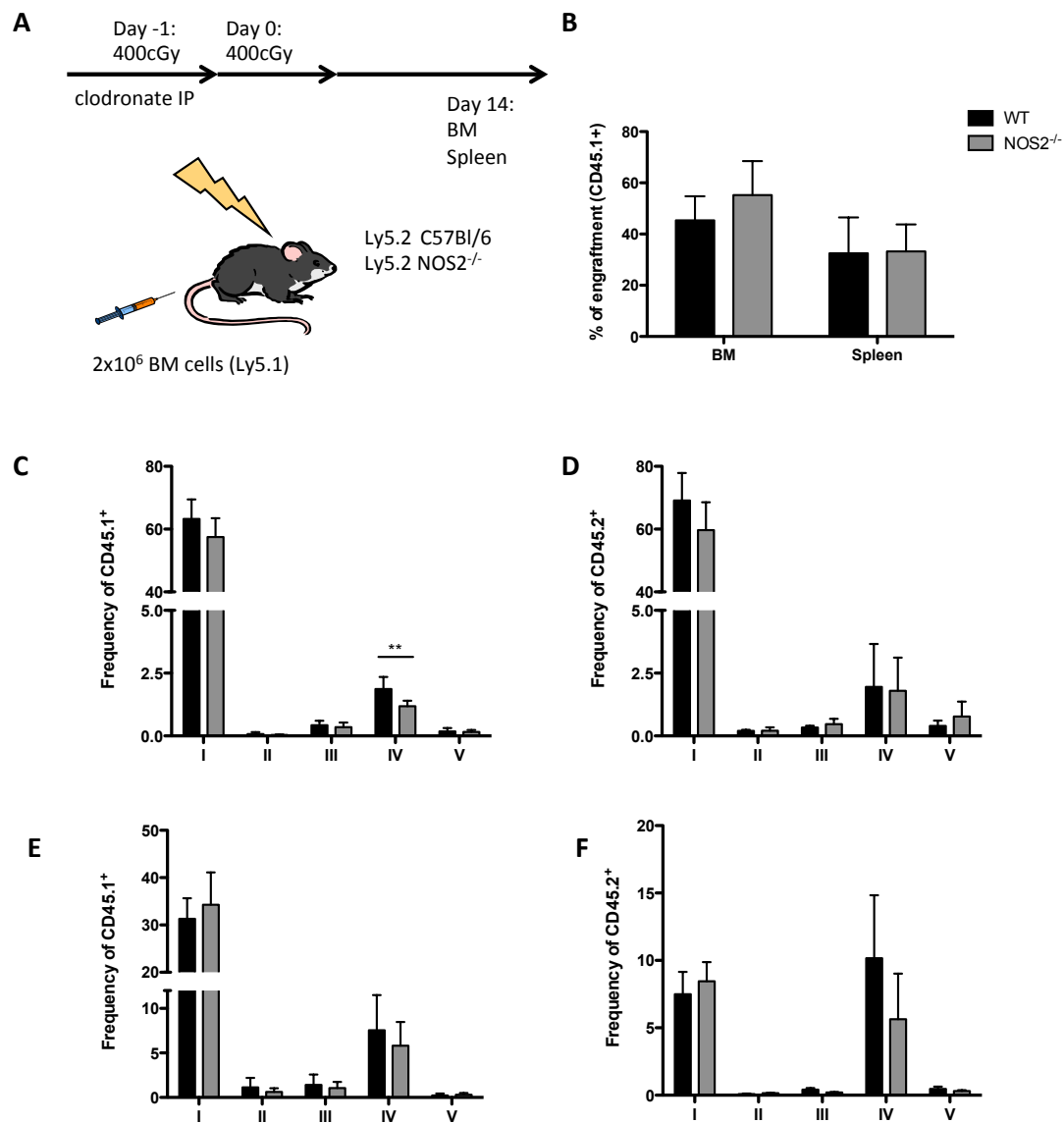


FIGURE 4.9. Macrophage reconstitution is impaired in *Nos2*^{-/-} hosts. Ly5.2 WT or *Nos2*^{-/-} recipients were irradiated with a split dosage of 800cGy, and received a clodronate injection i.p. one day previous to the transfer of 2*10⁶ Ly5.1 WT BM cells. 14 days after, BM and spleen were collected and analysed by FACS. **A.** Scheme of experiment. **B.** Percentage of engraftment (CD45.1⁺ cells) in BM and spleen. **C** and **E.** Percentage of BM mononuclear phagocyte populations within the donor cells (CD45.1⁺) in BM (**C**) and spleen (**E**). (n=8/group) **D** and **F.** Percentage of BM mononuclear phagocyte populations within the host cells (CD45.2⁺) in BM (**D**) and spleen (**F**). (n=8/group) ** p < 0.05, Mann-Whitney U test.

4.2.3 Role of TNF- α in driving MSC-mediated myeloid differentiation

We further analysed the profile of pro- and anti-inflammatory cytokines produced by BM cells and by MSC cultured alone or together. This step was fundamental to identify the mechanism underlying the interaction between MSC and myeloid cells.

4.2.3.1 TNF- α is upregulated in MSC-induced myeloid cells

Intracellular Th1 and Th2 cytokine staining of BM cells was assessed in the presence or absence of MSC. After 4 days of culture, no production of IL-12, IL-4 or IL-10 was detected in BM cultured either alone or with MSC (**Figure 4.10**). TNF- α and IFN- γ were instead increased in BM cultured with MSC as compared to the expression detected in BM cultured alone (TNF- α : MFI = 19.3 ± 1.3 versus MFI = 4.44 ± 2.7 of BM alone; IFN- γ : MFI = 3.16 ± 0.9 and MFI = 4.4 ± 2.1 of BM alone, **Figure 4.10A**). Such increase was significant only for TNF- α expression ($p=0.0096$ versus BM alone, **Figure 4.10C**). TNF- α was expressed mainly by CD11b⁺ Gr-1⁻ subset (Gr-1⁻: MFI = 21.4 ± 3.3 versus Gr-1^{high}: MFI = 10.4 ± 0.5 and Gr-1^{int-low}: MFI = 16.3 ± 4.7 , **Figure 4.10B**), whereas IFN- γ was mainly expressed by CD11b⁺ Gr-1^{int-low} and CD11b⁺ Gr-1⁻ subsets in BM cultured with MSC (Gr-1^{int-low}: MFI = 4.3 ± 2.5 , Gr-1⁻: MFI = 4.8 ± 2 and Gr-1^{high}: MFI = 2.7 ± 1 , **Figure 4.10B**). No difference was observed in cytokine production whether cells were or not stimulated with PMA-ionomycin to increase the signal.

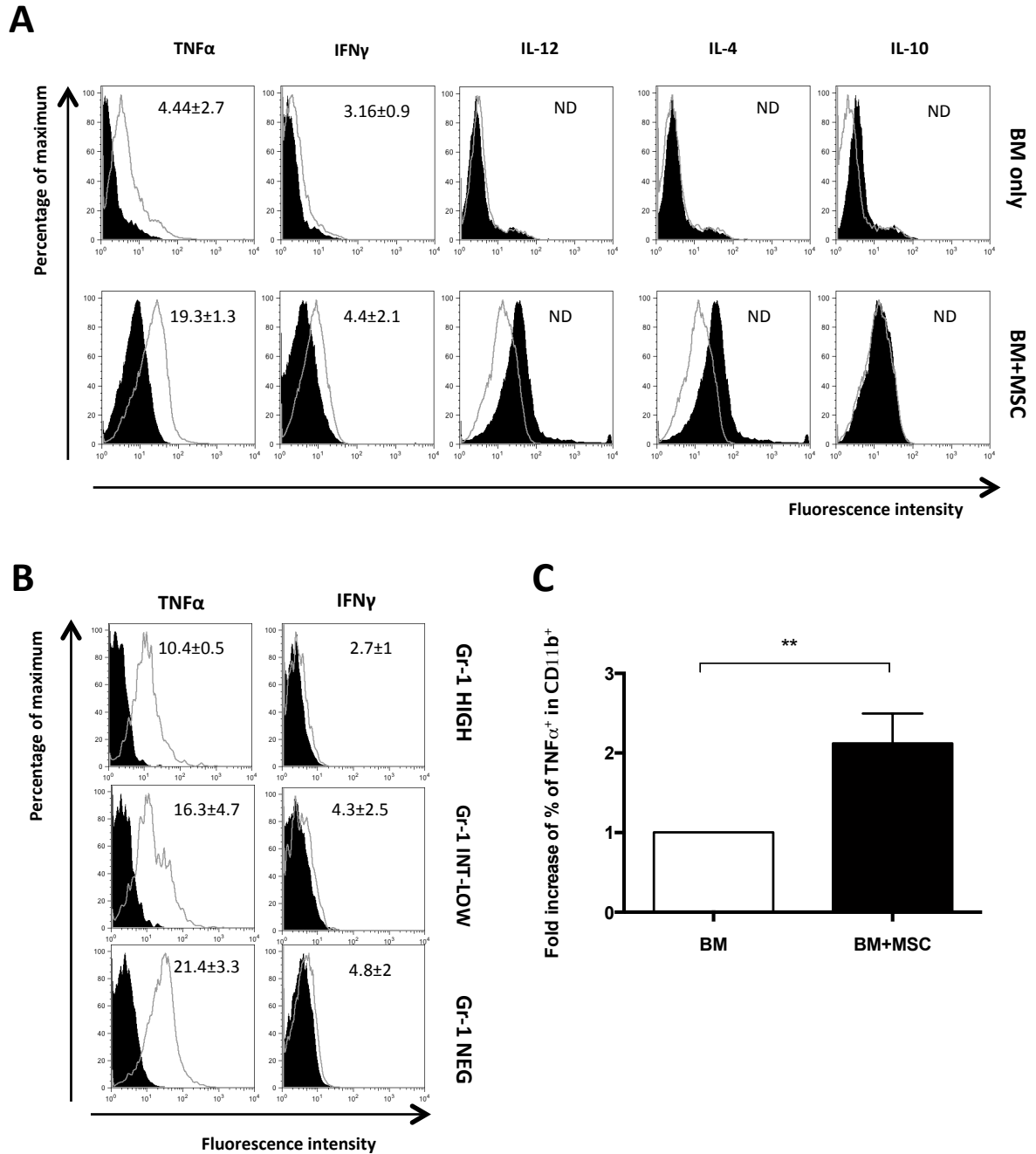


FIGURE 4.10. BM cells cultured alone or with MSC express TNF- α and IFN- γ , and TNF- α expression increases after co-culture. BM cells were cultured alone or with MSC for 4 days. Cytokine production was assessed by intracellular cytokine staining. **A.** Histogram plots represent various intracellular cytokine markers within the CD11b⁺ gated population (open histograms) against their matched isotype controls (filled histograms). Mean fluorescence intensity (MFI) of 5 independent experiments, $\pm$ SD, is represented in the histogram plots. **B.** Histogram plots represent TNF- α and IFN- γ expression within the CD11b⁺ Gr-1^{high} gate (Gr-1 HIGH), CD11b⁺ Gr-1^{int-low} gate (Gr-1 INT-LOW), and CD11b⁺ Gr-1⁻ gate (Gr-1 NEG). MFI of 5 independent experiments, $\pm$ SD, is represented in the histogram plots. **C.** Fold increase of the percentage of TNF- α expression in CD11b⁺ gated population.

The expression of pro- and anti-inflammatory cytokines was investigated through intracellular cytokine staining in BM derived MSC before and after co-culture with BM cells (**Figure 4.11**). Anti-inflammatory cytokines IL-4 and IL-10 and pro-inflammatory cytokine IL-12 were not detected before and after co-culture. IFN- γ could be detected only after co-culture (MFI = 4.59), whereas the expression of TNF- α was detected before and after co-culture (MFI = 69.7 and MFI = 31.9, **Figure 4.11**).

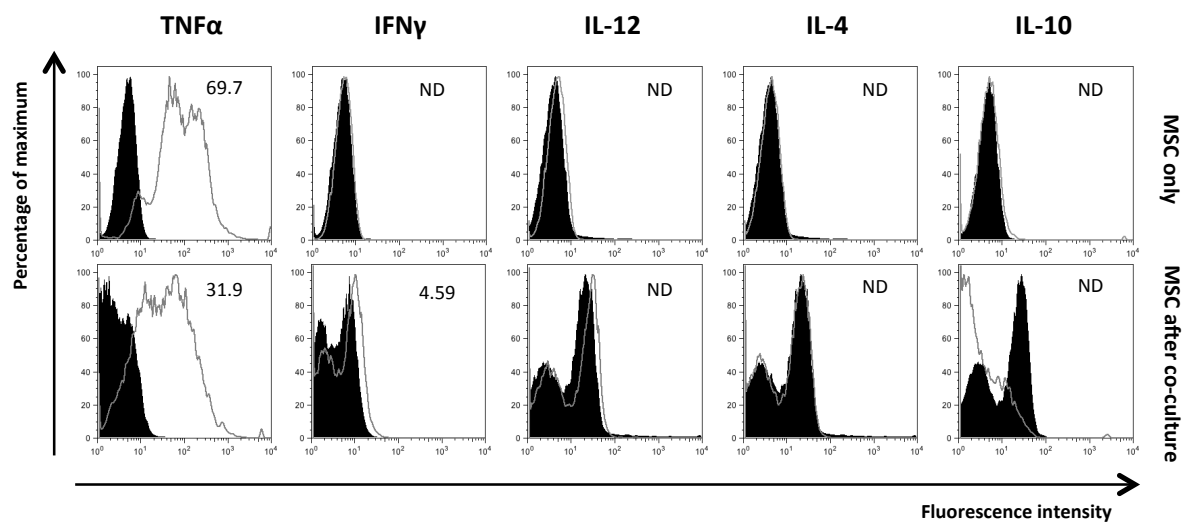


Figure 4.11. BM derived MSC express TNF- α when cultured alone and after co-culture with BM. BM derived MSC were cultured alone or with BM for 4 days. Cytokine production was assessed by intracellular cytokine staining. Histogram plots represent various intracellular cytokine markers within the CD11b⁺ gated population (open histograms) against their matched isotype controls (filled histograms). MFI of a representative experiment is shown in the histogram plots.

4.2.3.2 *TNF- α upregulates Nos2 expression on MSC*

BM cells cultured alone express the pro-inflammatory cytokines TNF- α and IFN- γ , which are upregulated after culturing BM with MSC. Several studies indicate that TNF- α and IFN- γ regulate *Nos2* expression in MSC and in macrophages [122]. Therefore, in order to confirm that these two cytokines are involved in the upregulation of *Nos2* expression, we investigated their effect, alone or in combination, on MSC after 24 or 48 hours of stimulation.

TNF- α , IFN- γ and their combination significantly upregulated *Nos2* expression in MSC after 24 and 48 hours (**Figure 4.12**). IFN- γ stimulation induced a 30-fold increase in *Nos2* after 24 hours (0.2 ± 0.09 , versus 0.007 ± 0.008 of MSC untreated at 24 hours, $p=0.0187$), and a 27-fold increase after 48 hours (0.11 ± 0.06 , versus 0.004 ± 0.004 of MSC untreated at 48 hours, $p=0.0381$). TNF- α stimulation induced a 1500-fold increase in the expression of *Nos2* after 24 hours (10.06 ± 3.7 , versus 0.007 ± 0.008 of MSC untreated at 24 hours, $p=0.0142$), and a 1200-fold increase after 48 hours (5.2 ± 1.7 , versus 0.004 ± 0.004 of MSC untreated at 48 hours, $p=0.0098$). The combination of the two cytokines determined a synergistic effect, determining a 9000-fold increase in *Nos2* expression after 24 hours (62.1 ± 33.1 , versus 0.007 ± 0.008 of MSC untreated at 24 hours, $p=0.0314$) and a 5700-fold increase after 48 hours (24.1 ± 13.9 , versus 0.004 ± 0.004 of MSC untreated at 48 hours, $p=0.0400$).

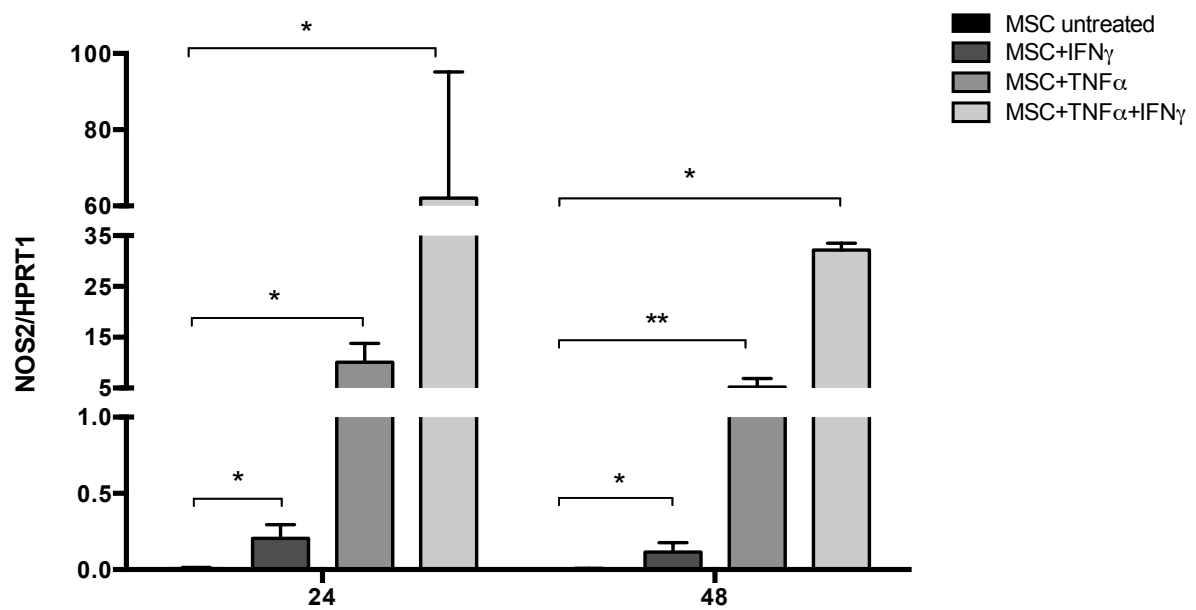


FIGURE 4.12. *Nos2* expression in MSC is up-regulated by TNF- α or the combination of TNF- α and IFN- γ . Quantitative RT-PCR data of MSC in culture alone or treated with IFN- γ (20ng/ml), TNF- α (20ng/ml), or the combination of TNF- α and IFN- γ for 24 and 48 hours. Data are expressed as normalized to *Hprt1* housekeeping gene. Mean of 5 independent experiments, $\pm$ SD. * p<0.05, ** p<0.005, Unpaired t test.

4.2.3.3 TNF- α signalling in MSC is fundamental to drive the differentiation of CD11b⁺ Gr-1⁻ cells

TNF- α induces a remarkable upregulation of *Nos2* expression in MSC, when present either alone or in combination with IFN- γ . These two cytokines have been detected in BM cells cultured alone or with MSC, and in particular TNF- α was significantly upregulated after co-culture with MSC. On the basis of these findings, we assessed the role mediated by TNF- α in MSC ability to drive myeloid differentiation by culturing BM cells with MSC lacking the expression of TNF receptor 1 and 2 (Tnfr1/r2^{-/-}).

To this purpose, unfractionated BM cells were cultured with MSC obtained from WT or Tnfr1/r2^{-/-} mice for 4 days. In order to assess whether the lack of expression of Tnfr on MSC could modify the ability to differentiate myeloid cells, expression of markers CD11b, Gr-1, F4/80, CD115, CD206, CD169 and CD68 was evaluated on cells collected after 4 days (**Figure 4.13**). Representative contour plots of expression of these markers are shown in Figure 4.9A.

Analysis of the proportions of CD11b⁺ Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ subsets in the gated CD11b⁺ population revealed a statistically significant higher percentage of CD11b⁺ Gr-1^{int-low} cells (35.6% $\pm$ 7.3% versus 22.7% $\pm$ 2.1% in BM WT+MSC WT, p=0.0428), and a significant decrease in the percentage of CD11b⁺ Gr-1⁻ cells in presence of Tnfr1/r2^{-/-} MSC (24.4% $\pm$ 7% versus 40.3% $\pm$ 5.9% in BM WT+MSC WT p=0.0136) (**Figure 4.13B**).

F4/80 was significantly decreased in the cultures with Tnfr1/r2^{-/-} MSC in the gated CD11b⁺ population (26.1% $\pm$ 12.9% versus 46.6% $\pm$ 9.1% in BM WT+MSC WT, p=0.0409, **Figure 4.13C**). A decrease in the percentage of CD206 (11.5% $\pm$ 8.3% versus 19.2% $\pm$ 8.3% in BM WT+MSC WT) and CD68 (12.4% $\pm$ 7.7% versus 16.4% $\pm$ 5.3% in BM WT+MSC WT) was also documented, although it was not significant (**Figure 4.13C**). The percentage of CD11b⁺ CD115⁺ cells was decreased in cultures with Tnfr1/r2^{-/-} MSC (9.9% $\pm$ 2.2 versus 50.3% $\pm$ 8.8% in BM WT+MSC WT, p=0.0006,

Figure 4.13C). In particular, the decrease in CD115⁺ cells was identified on the total CD11b⁺ population, whereas for all other markers the decrease was detected in the CD11b⁺ Gr-1⁻ subset (Figure 4.9A).

The presence of Tnfr1/r2^{-/-} MSC determined a reduced expansion of the myeloid cell population in comparison with WT MSC, as shown by the reduction in the absolute number of CD11b⁺ cells collected after co-culture ($4.1 \times 10^6 \pm 1 \times 10^6$ versus $6.3 \times 10^6 \pm 0.5 \times 10^6$ in BM WT+MSC WT, $p=0.0296$, **Figure 4.13D).**

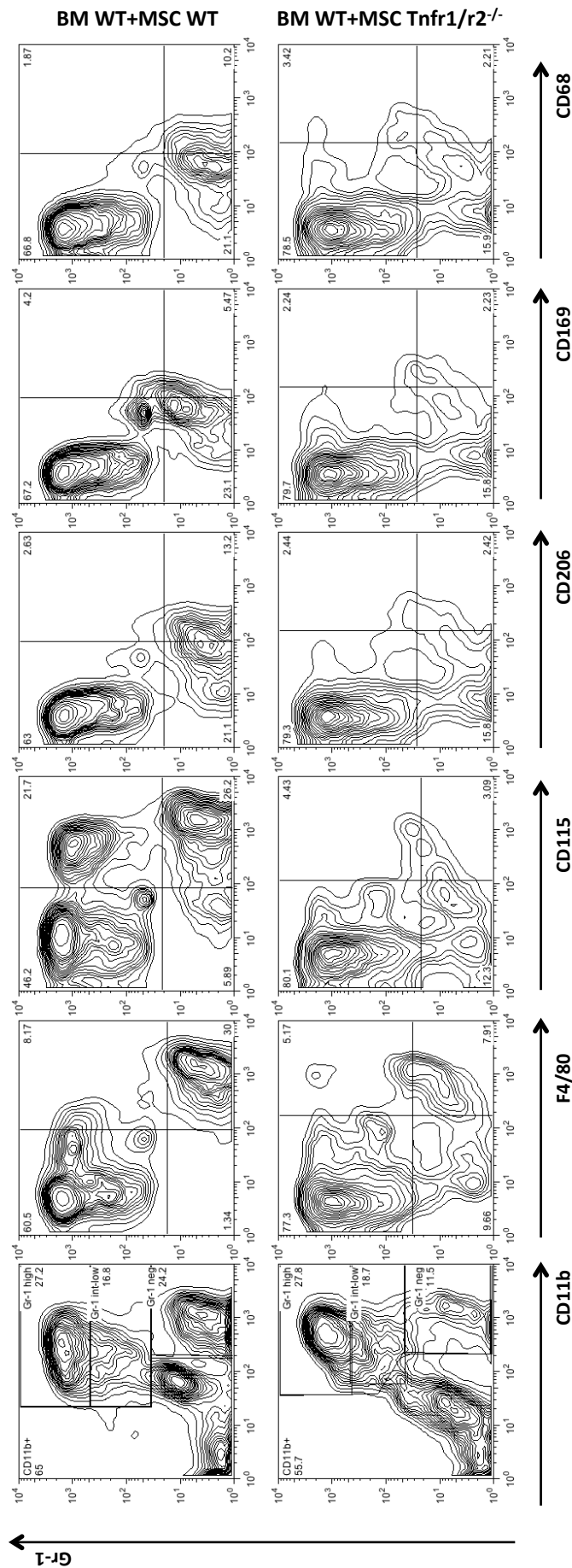


FIGURE 4.13. MSC induced differentiation of CD11b⁺ Gr-1⁻ cells requires *Tnfr1/r2* expression. BM cells were cultured with MSC isolated from WT or *Tnfr1/r2*^{-/-} mice for 4 days. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the expression of F4/80, CD115, CD206, CD169, and CD68 in the CD11b⁺ gated population.

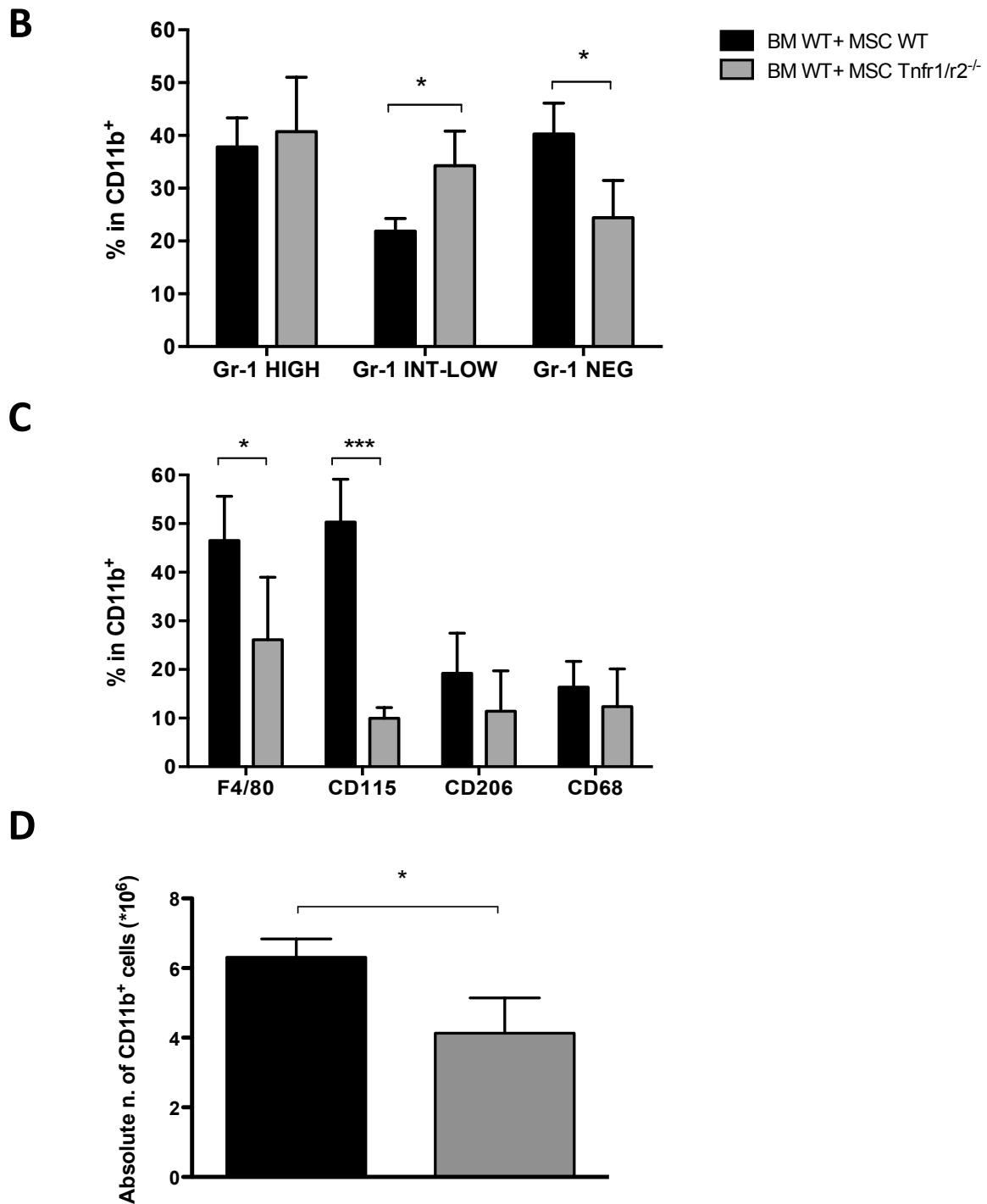


FIGURE 4.13. MSC induced differentiation of CD11b⁺ Gr-1⁻ cells requires Tnfr1/r2 expression. BM cells were cultured with MSC isolated from WT or Tnfr1/r2^{-/-} mice for 4 days. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ cells. Mean of 4 independent experiments, $\pm$ SD ** $p < 0.005$ *** $p < 0.001$ Unpaired t test. **C.** Percentage of CD11b⁺ F4/80⁺, CD115⁺, CD206⁺, CD169⁺, and CD68⁺ cells, within the CD11b⁺ population. Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ *** $p < 0.001$ Unpaired t test. **D.** Absolute number of CD11b⁺ cells recovered from BM cultured with MSC WT or Tnfr1/r2^{-/-} for 4 days. Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ Unpaired t test.

4.2.3.3.1 *CD11b⁺ cells isolated from BM cultured with WT or Tnfr1/r2^{-/-} MSC have different M1-M2 profiles*

The M1-M2 profile of the CD11b⁺ cells obtained from the co-cultures of BM cells with Tnfr1/r2^{-/-} MSC was analysed by quantitative RT-PCR (**Figure 4.14**). *Iil2b* and *Nos2* were significantly upregulated in BM cells cultured with Tnfr1/r2^{-/-} MSC (2.1±0.6, p=0.0159 and 8.4±3.1, p=0.0135 versus BM WT+MSC WT, respectively), whereas *Marco* was downregulated (0.2±0.2, p=0.0020 versus BM WT+MSC WT). *Arginase-1* was significantly upregulated in Tnfr1/r2^{-/-} co-cultures (200.8±119.6, p=0.0444 versus BM WT+MSC WT), and *Ccl12* and *Chi3l1* were upregulated although not significantly (10.7±13.5 and 2±0.8, respectively).

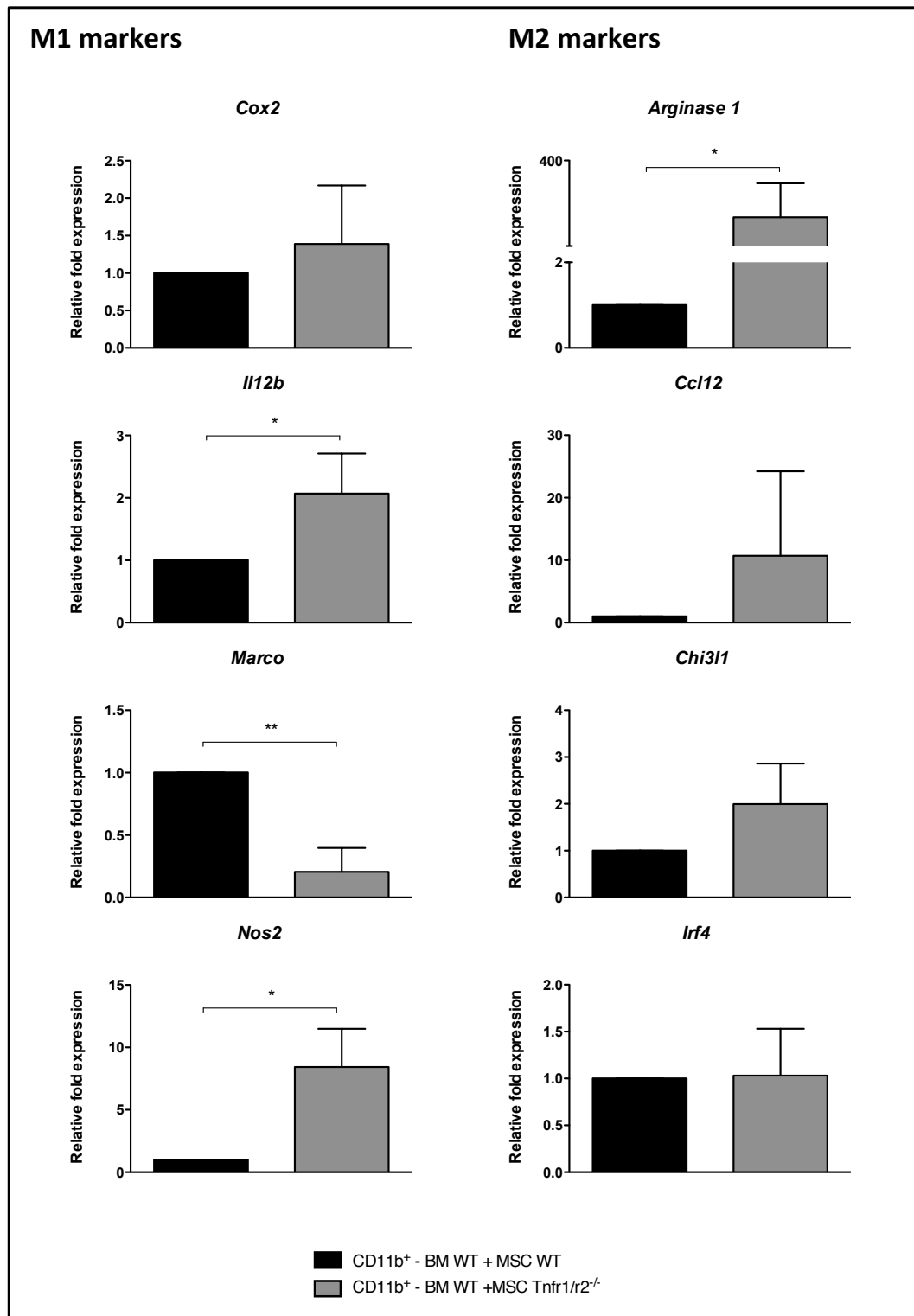


FIGURE 4.14. CD11b⁺ cells isolated from BM cultured with WT or Tnfr1/r2^{-/-} MSC have different M1-M2 profiles. Quantitative RT-PCR data of CD11b⁺ cells isolated from 4 days cultures of BM with MSC WT or Tnfr1/r2^{-/-}. Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in CD11b⁺ isolated from BM and MSC WT co-cultures. Mean of 3 independent experiments, $\pm$ SD. *Arg1*, arginase 1; *Ccl12*, CC-chemokine ligand 12; *Chi3l1*, chitinase 3-like 1; *Cox2*, cyclooxygenase 2; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; *Il12b*, interleukin 12b; *Irf4*, interferon factor 4; *Marco*, macrophage receptor with collagenous structure; *Nos2*, nitric oxide synthase 2.

4.2.3.4 Addition of TNF- α in BM and MSC co-cultures impairs the differentiation of macrophages

Nos2 expression on MSC is required for induction of myeloid differentiation, and it is induced by the presence of TNF- α alone or in combination with IFN- γ . In order to further dissect the effect of TNF- α and IFN- γ on the co-cultures of BM and MSC, the two cytokines were used alone or in combination to treat MSC before or directly added to the co-cultures (**Figure 4.15A**).

MSC were pre-treated for 24 hours with TNF- α , IFN- γ or their combination. BM cells were then added to pre-treated or untreated MSC, or to untreated MSC with the addition of the cytokines. A decrease in the percentage of CD11b⁺ Gr-1⁻ cells was detected in the presence of TNF- α or TNF- α and IFN- γ either by pre-treating MSC (26.1% with TNF- α and 21.8% with TNF- α and IFN- γ versus 36% in untreated) or directly adding the cytokines to the co-cultures (20.7% and with TNF- α and 16.3% with TNF- α and IFN- γ versus 36% in untreated) (**Figure 4.15A**). Addition or pre-treatment with IFN- γ alone had no effect on the differentiation of the three main CD11b⁺ Gr-1 subsets induced by MSC (**Figure 4.15A**).

As TNF- α addition to the co-culture induced a diverse induction of differentiation, we further investigated the expression of monocyte and macrophage markers in the co-cultures. The addition of TNF- α induced a higher percentage of CD11b⁺ Gr-1^{int-low} cells (20.9% $\pm$ 6.6% versus 11.1% $\pm$ 3.1% in BM+MSC, p=0.0356) and a decrease in the percentage of CD11b⁺ Gr-1⁻ cells in the CD11b⁺ population (25.1% $\pm$ 4.9% versus 45% $\pm$ 9.2% in BM+MSC, p=0.0089, **Figure 4.15B**). Analysis of F4/80, CD115 and CD206 revealed that addition of the cytokine induced a decrease in the percentage of CD11b⁺ F4/80⁺ cells (25.7% $\pm$ 7.8% versus 46.9% $\pm$ 5.5%, p=0.0044 versus BM WT+MSC WT), in the percentage of CD11b⁺ CD115⁺ (22.9% $\pm$ 14.1% versus 52.3% $\pm$ 9.5% in BM+MSC, p=0.0134), and in the percentage of CD206⁺ cells (11.2% $\pm$ 2.5% versus 20.8% $\pm$ 3.4% in BM+MSC, not significant) (**Figure 4.15C**). A non specific toxic effect by TNF- α could be excluded because cell

recovery was not impaired by the addition of TNF- α in the co-culture ($6.2 \times 10^6 \pm 1.9 \times 10^6$ versus $6.6 \times 10^6 \pm 1.9 \times 10^6$ of BM+MSC, **Figure 4.15D**).

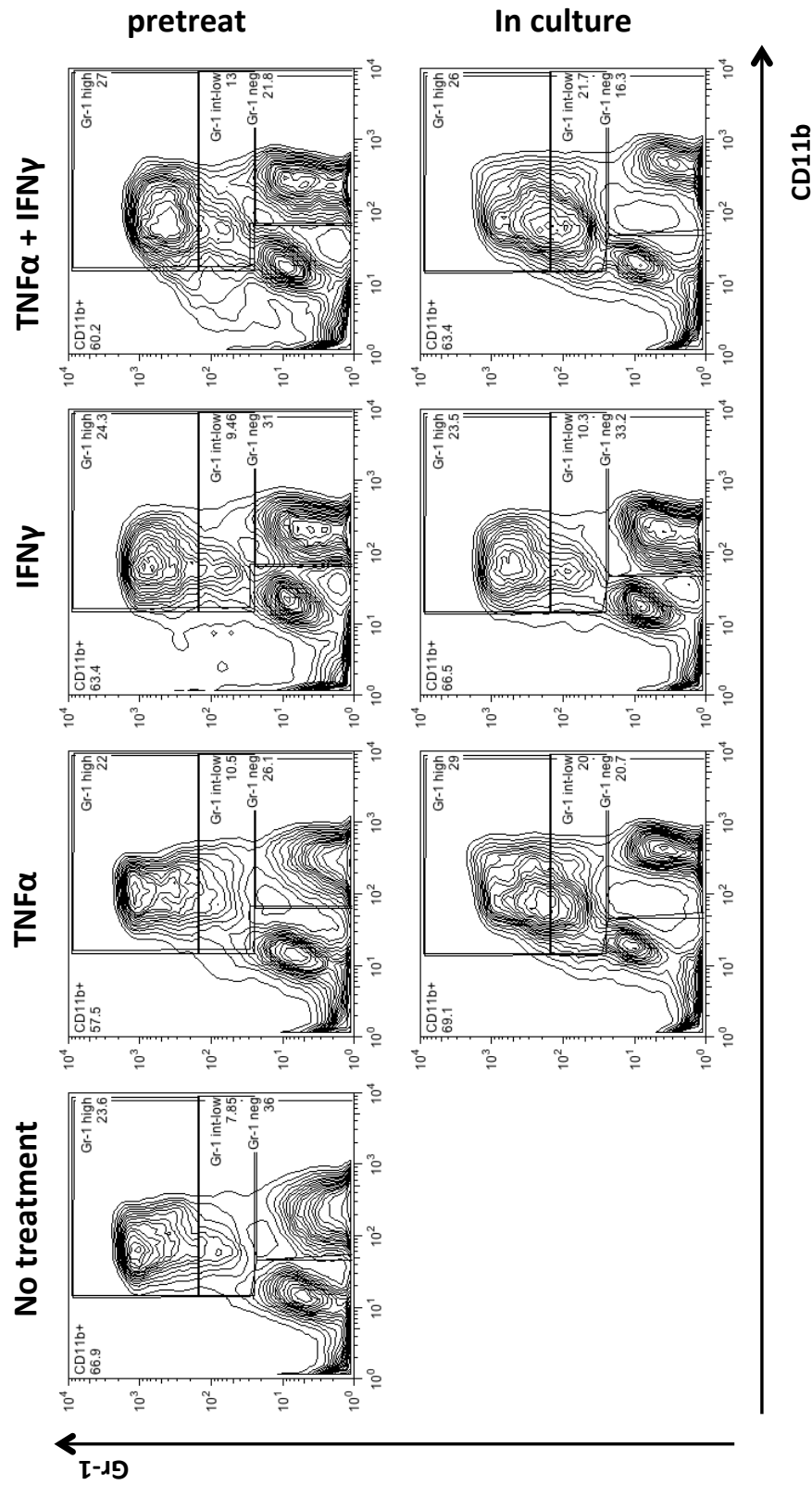


FIGURE 4.15. Augmenting TNF- α in BM and MSC cultures impairs the differentiation of CD11b⁺ Gr-1⁺ cells. A. BM cells were cultured with MSC in presence of TNF- α , IFN- γ , or the combination of TNF- α and IFN- γ (IN CULTURE), or with MSC pretreated with TNF- α , IFN- γ , or the combination of TNF- α and IFN- γ (PRETREAT), for 4 days. Contour plots represent the CD11b and Gr-1 proportion in the live gate.

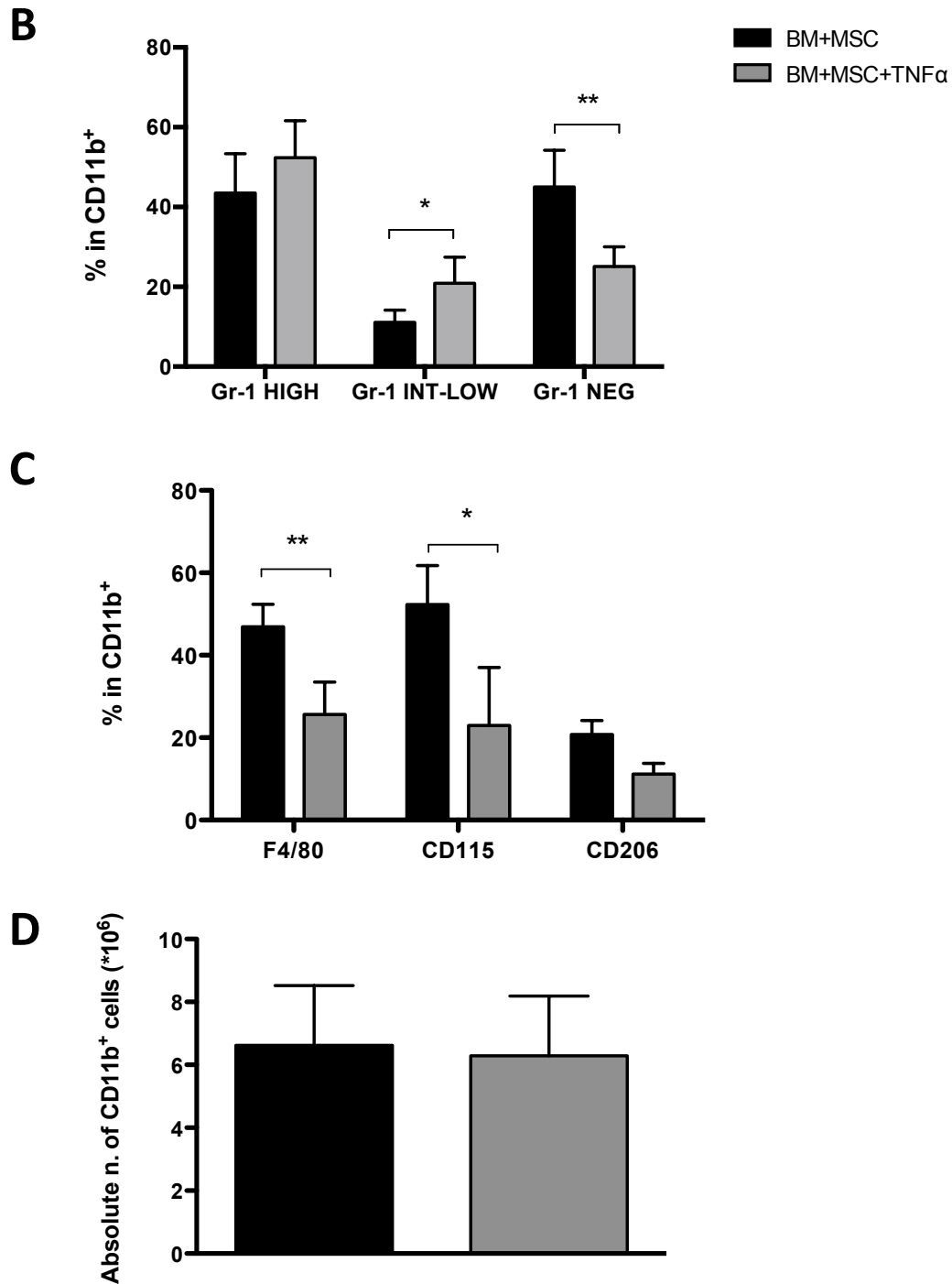


FIGURE 4.15. Augmenting TNF- α in BM and MSC cultures impairs the differentiation of CD11b⁺ Gr-1⁻ cells. B. Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ cells collected from BM cultured with MSC either alone or in presence of TNF- α . Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ ** $p < 0.005$ Unpaired t test. **C.** Percentage of CD11b⁺ F4/80⁺, CD115⁺, and CD206⁺ cells, within the CD11b⁺ cells population collected from BM cultured with MSC either alone or in presence of TNF- α . Mean of 4 independent experiments, $\pm$ SD, * $p < 0.05$, ** $p < 0.005$ Unpaired t test. **D.** Absolute number of CD11b⁺ cells recovered from BM cultured with MSC either alone or in presence of TNF- α for 4 days. Mean of 4 independent experiments, $\pm$ SD.

4.2.4 Agrin is fundamental for MSC induced myeloid differentiation

We previously showed that NOS2 expression from MSC is required for the induction of myeloid differentiation. In particular, we demonstrated that NOS2 expression could be up-regulated mainly by TNF- α , which is in turn up-regulated by BM cells cultured in presence of MSC. Nevertheless, differentiation of myeloid cells is not completely inhibited when BM cells are cultured with NOS2^{-/-} or Tnfr1/r2^{-/-} cells, and addition of TNF- α results in a similar impairment of myeloid differentiation. Therefore, other molecules might be involved in the interaction between haematopoietic and mesenchymal cells.

Extracellular matrix proteins are fundamental for organizational and functional support of cell niches. In particular, agrin has recently been described as a critical MSC-derived signal controlling the survival and proliferation of HSC [332], and as a non-redundant molecule influencing myeloid cell development and function [336].

On the basis of these notions, we investigated the ability of BM derived MSC isolated from agrin-deficient mice to drive the differentiation of myeloid cells *in vitro*.

4.2.4.1 Agrin is required for driving MSC-induced myeloid differentiation

BM derived MSC isolated from WT and Musk-L;Agrn^{-/-} mice were cultured with WT BM cells for 4 days. BM derived MSC isolated from Musk-L;Agrn^{+/-} mice were used as controls, as they share the same phenotype of WT mice [332].

Cells collected from the co-cultures were analysed for expression of markers CD11b, Gr-1, F4/80, CD115, CD206, CD169 and CD68 (**Figure 4.16**). Contour plots of Gr-1 versus CD11b, F4/80, CD115, CD206, CD169 and CD68 of a representative experiment are shown in **Figure 4.16A**.

Analysis of the expression of Gr-1 marker in the CD11b⁺ gated population revealed that Musk-L;Agrn^{-/-} MSC induced an increase in the percentage of CD11b⁺ Gr-1^{int-low} cells (27.5% ± 4.9% versus 8.6% ± 3.6% in BM WT+MSC WT, p=0.0059) and a decrease in the percentage of CD11b⁺ Gr-1⁻ cells (15.9% ± 2.2% versus 37.2% ± 6.5% in BM WT+MSC WT, p=0.0059) as compared to WT MSC (**Figure 4.16B**). Musk-L;Agrn^{+/-} MSC also induced an increase in the percentage of CD11b⁺ Gr-1^{int-low} (22.3% ± 6.3%) cells and a decrease in CD11b⁺ Gr-1⁻ cells (26.23% ± 7.4%), but the difference with BM cells cultured with WT MSC was not significant.

Expression of markers F4/80, CD115, CD206 and CD68 in the CD11b⁺ gated population was significantly down-regulated in BM cells cultured with MSC from Musk-L;Agrn^{-/-} mice (**Figure 4.16C**). A 3-fold decrease was documented in the percentage of F4/80⁺ (15.2% ± 4.1% versus 41.9% ± 8.1% in BM WT+MSC WT, p=0.0069), CD115⁺ (6.5% ± 1.1% versus 22.2% ± 7.7% in BM WT+MSC WT, p=0.0331), CD206⁺ (8.2% ± 2.3% versus 21.3% ± 2.9% in BM WT+MSC WT, p=0.0035), CD169⁺ (0.65%±0.15% versus 1.7%±0.25% in BM WT+MSC WT, p=0.0031), and CD68⁺ (7.9% ± 2.3% versus 17.8% ± 1.9% in BM WT+MSC WT, p=0.0050) cells in BM cells cultured with Musk-L;Agrn^{-/-} MSC as compared to BM cells cultured with WT MSC (Figure 4.12C). The difference in the expression of these markers in BM cultured with WT or Musk-L;Agrn^{+/-} MSC was not significant (**Figure 4.16C**). Expression of F4/80, CD206, CD169 and CD206 was mainly detected in the CD11b⁺ Gr-1⁻ population, whereas CD115 was expressed by CD11b⁺ Gr-1^{high} and CD11b⁺ Gr-1⁻ cells (**Figure 4.16A**).

The absolute number of CD11b⁺ cells recovered after 4 days of culture was decreased in BM cultured with Musk-L;Agrn^{-/-} MSC (4.1x10⁶ ± 0.5x10⁶ versus 6.5x10⁶ ± 0.8x10⁶ in BM WT+MSC WT, p=0.0101) (**Figure 4.16D**). As expected, no difference in the recovery of CD11b⁺ cells was seen between BM cultured with WT or Musk-L;Agrn^{+/-} MSC.

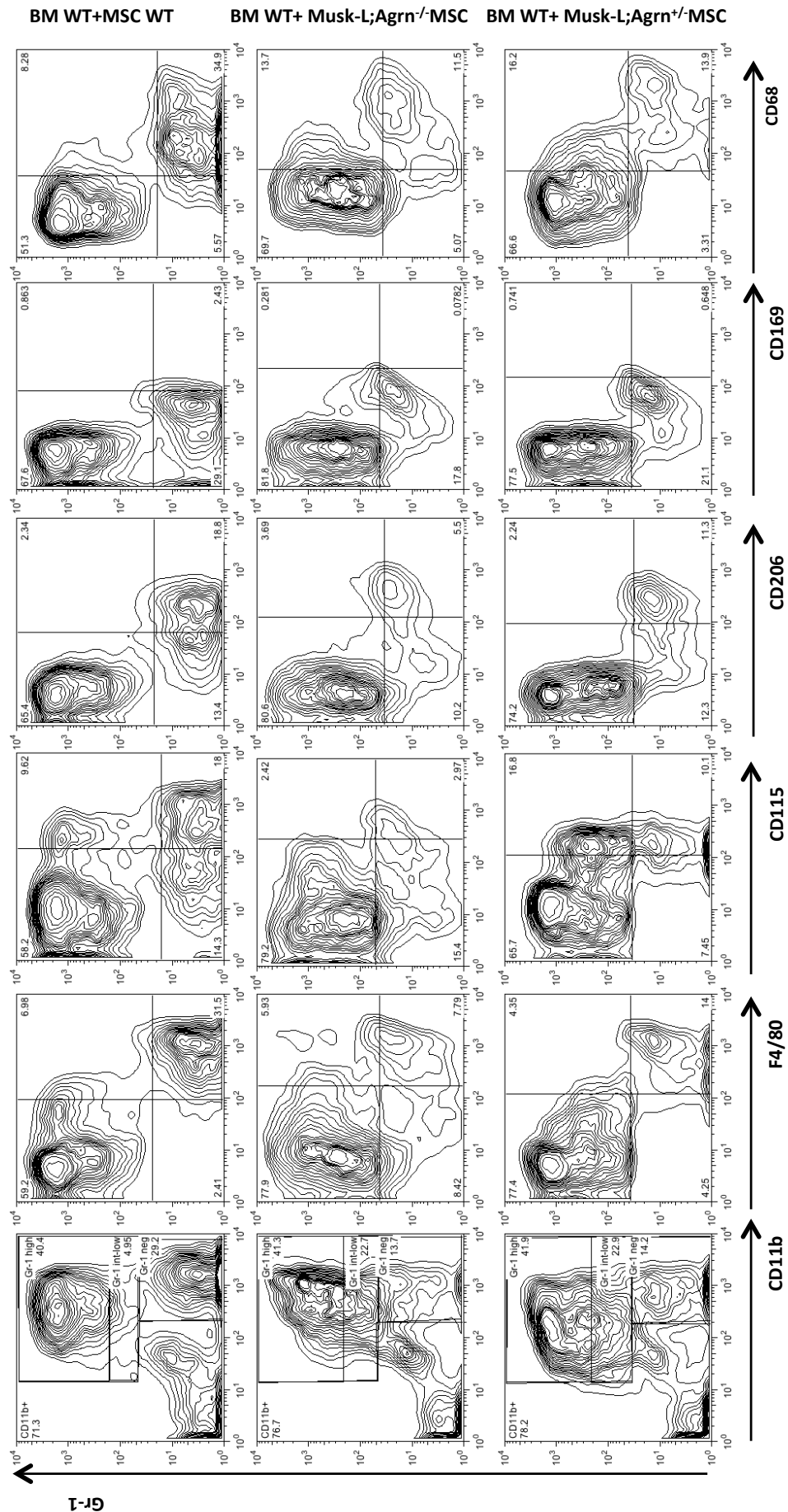


FIGURE 4.16. Expression of agrin is required for MSC-induced differentiation of myeloid cells. BM cells were cultured with MSC isolated from WT, Musk-L;Agrn^{-/-} or Musk-L;Agrn^{+/-} mice for 4 days. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the expression of F4/80, CD115, CD206, CD169, and CD68 in the CD11b⁺ gated population.

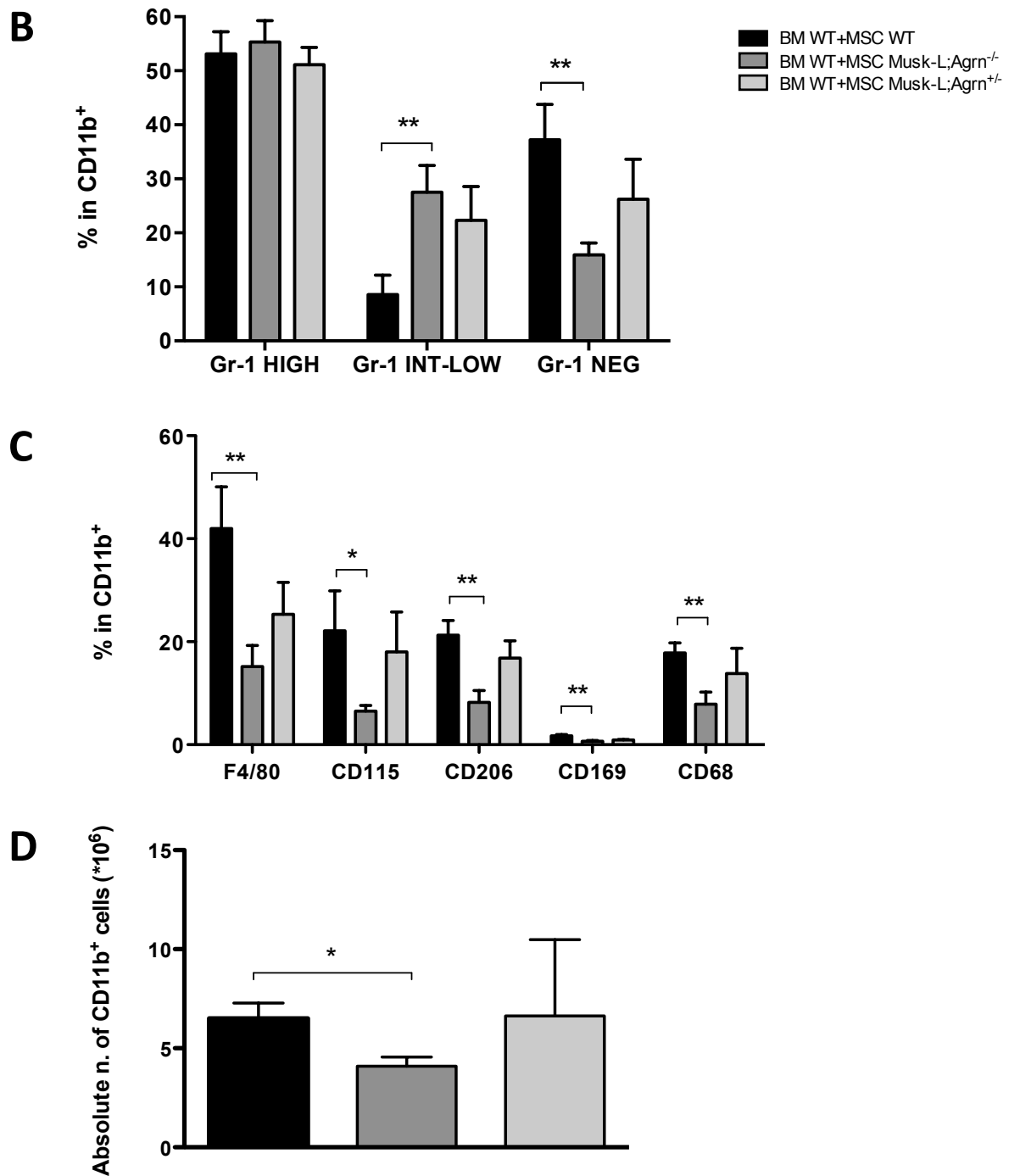


FIGURE 4.16. Expression of agrin is required for MSC-induced differentiation of myeloid cells. BM cells were cultured with MSC isolated from WT, Musk-L;Agrn^{-/-} or Musk-L;Agrn^{+/-} mice for 4 days. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ cells. Mean of 4 independent experiments, $\pm$ SD ** $p < 0.005$ Unpaired t test. **C.** Percentage of CD11b⁺ F4/80⁺, CD115⁺, CD206⁺, CD169⁺, and CD68⁺ cells, within the CD11b⁺ cells population. Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ ** $p < 0.005$ Unpaired t test. **D.** Absolute number of CD11b⁺ cells recovered from BM cultured with MSC WT, Musk-L;Agrn^{-/-} or Musk-L;Agrn^{+/-} for 4 days. Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ Unpaired t test.

4.2.4.1.1 *CD11b⁺ cells isolated from BM cultured with WT or Musk-L;Agrn^{-/-} MSC have different M1-M2 profiles*

The M1-M2 profile of CD11b⁺ cells isolated from BM cells cultured with WT, Musk-L;Agrn^{-/-} and Musk-L;Agrn^{+/-} MSC was investigated with RT-PCR (**Figure 4.17**). The analysis was run in comparison to the levels expressed by CD11b⁺ cells obtained from BM cultured with WT MSC. *Cox2* and *Il-12b* were up-regulated in cultures with Musk-L;Agrn^{-/-} MSC, although not significantly (3.3±0.9 and 1.7±0.8, respectively). Musk-L;Agrn^{-/-} MSC induced a significant down-regulation in the expression of *Marco* (0.09±0.1, p=0.0019 versus BM WT+MSC WT), and up-regulated the expression of *Nos2* (65.1±29.97, p=0.0034 versus BM WT+MSC WT). Analysis of *Arg-1*, *Ccl12*, *Chi3l1* and *Irf4* expression revealed that co-cultures of BM cells with Musk-L;Agrn^{-/-} MSC induced CD11b⁺ cells characterised by a higher expression of these markers (**Figure 4.17**). In particular, a 300-fold increase and a 4-fold increase were detected in the levels of expression of *Arg-1* (239.8±103.1, p<0.0001 versus BM WT+MSC WT) and *Chi3l1* (4.5±3.8, p=0.0137 versus BM WT+MSC WT) as compared to BM cultured with WT MSC. *Ccl12* and *Irf4* were up-regulated in presence of Musk-L;Agrn^{-/-} MSC, although not significantly (6.5±3.8 and 2.4±0.5 versus BM WT+MSC WT, respectively).

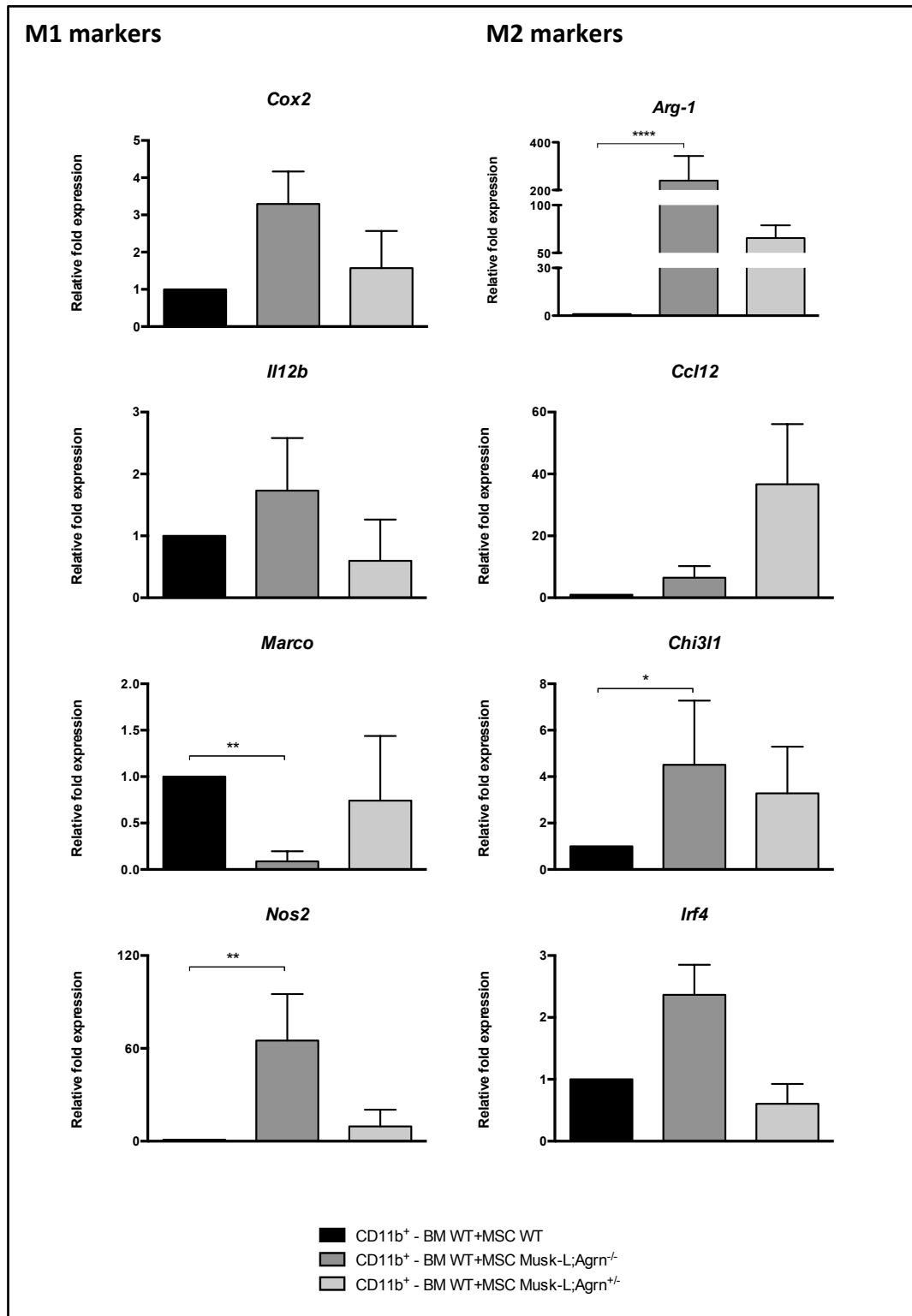


FIGURE 4.17. CD11b⁺ cells isolated from BM and MSC WT or Musk-L;Agrn^{-/-} co-cultures have a different M1-M2 profile. Quantitative RT-PCR data of CD11b⁺ cells isolated from 4 days cultures of BM with MSC WT, Musk-L;Agrn^{-/-} or Musk-L;Agrn^{+/-}. Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in CD11b⁺ isolated from BM and MSC WT co-cultures. Mean of 3 independent experiments, $\pm$ SD. * $p < 0.05$, ** $p < 0.005$, **** $p < 0.001$, Unpaired t test. *Arg1*, arginase 1; *Ccl12*, CC-chemokine ligand 12; *Chi3l1*, chitinase 3-like 1; *Cox2*, cyclooxygenase 2; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; *Il12b*, interleukin 12b; *Irf4*, interferon factor 4; *Marco*, macrophage receptor with collagenous structure; *Nos2*, nitric oxide synthase 2.

4.2.4.1.2 *Nos2* expression in Agrin-deficient MSC is strongly downregulated

We then investigated whether *Nos2* and agrin expression on MSC were correlated.

MSC obtained from WT or Musk-L;*Agrn*^{-/-} mice were examined for the expression of *Nos2* with or without TNF- α stimulation for 24 hours. MSC isolated from Musk-L;*Agrn*^{+/-} were used as control. RT-PCR data show that *Nos2* expression is significantly impaired in MSC derived from mice that are null for Agrin (0.16 ± 0.1 , $p=0.0002$ versus MSC WT), being downregulated 6 times (**Figure 4.18**). After TNF- α stimulation, a 288-fold decrease in *Nos2* expression is detected in Musk-L;*Agrn*^{-/-} MSC as compared to WT MSC (10.7 ± 1.4 versus 3076 ± 85.6 in MSC WT, $p=0.0004$) (**Figure 4.18**).

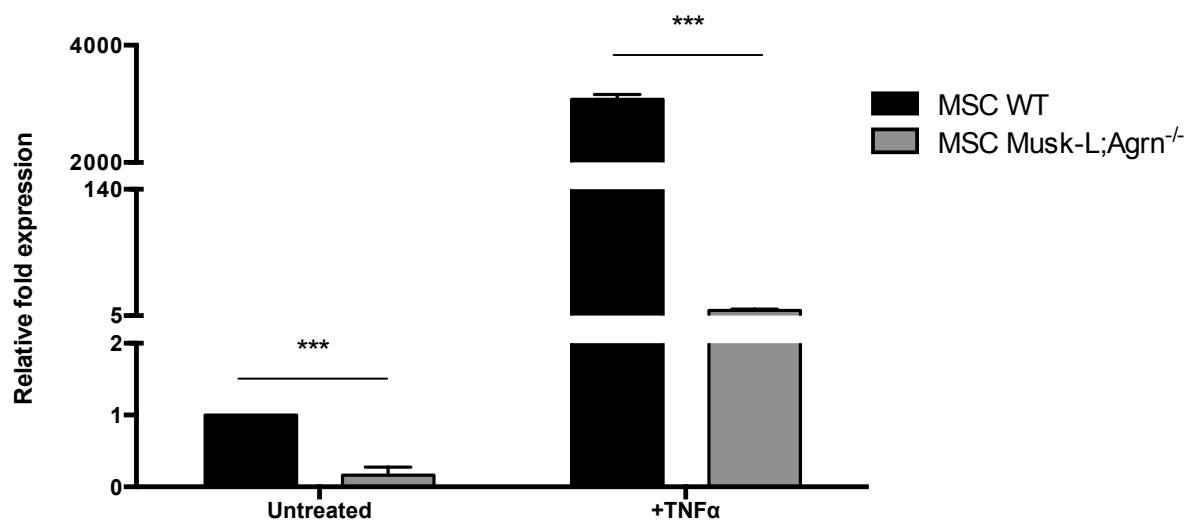


FIGURE 4.18. Musk-L;*Agrn*^{-/-} MSC express less *Nos2* and are less responsive to TNF- α for *Nos2* up-regulation. Quantitative RT-PCR data of MSC isolated from WT or Musk-L;*Agrn*^{-/-} mice, in culture alone or treated with TNF- α (20ng/ml) for 24 hours. Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in untreated MSC WT. Mean of 3 independent experiments, $\pm$ SD. *** $p<0.001$, Unpaired t test.

4.2.5 C3 complement is required for driving the differentiation of CD11b⁺ Gr-1^{int-low} cells

The complement cascade is a fundamental first line of defence during infections and inflammation. Recent investigations shed a new light on the role of complement in regulating HSPC mobilization and egress from the niche. In particular, a lack of expression of C3 in niche stromal cells impairs normal recovery of haematopoiesis after transplantation [354]. Furthermore, a recent study suggests that another complement factor, C5a, favours the recruitment of myeloid cells with immunosuppressive properties (MDSC) into solid tumours [382]. Taken altogether, these data prompted us to investigate whether a lack of C3 complement component on MSC could determine an impaired myeloid differentiation.

BM derived MSC isolated from WT or C3^{-/-} mice were cultured with WT BM cells. Representative contour plots of Gr-1 versus CD11b and F4/80 are shown in Figure 4.15A. A decreased percentage of CD11b⁺ Gr-1^{int-low} cells was found in the co-cultures of BM cells with C3^{-/-} MSC (16.5% ± 2.5% versus 29.2% ± 4.9% in BM WT+MSC WT, p=0.0035) (**Figure 4.19B**). Analysis of F4/80 showed no difference between BM cells cultured with WT or C3^{-/-} MSC (**Figure 4.19A**), as confirmed by a similar percentage of CD11b⁺ Gr-1⁻ cells in both cultures (45.5% ± 13.7% versus 32.9% ± 16.5% in BM WT+MSC WT, **Figure 4.19B**). To understand whether lack of expression of C3 on haematopoietic cells could determine an impaired myeloid differentiation, BM cells isolated from C3^{-/-} mice were co-cultured either with MSC from WT or C3^{-/-} mice. No difference was seen in the co-cultures (data not shown), further confirming that myeloid differentiation requires expression of C3 on stromal cells and not on haematopoietic cells.

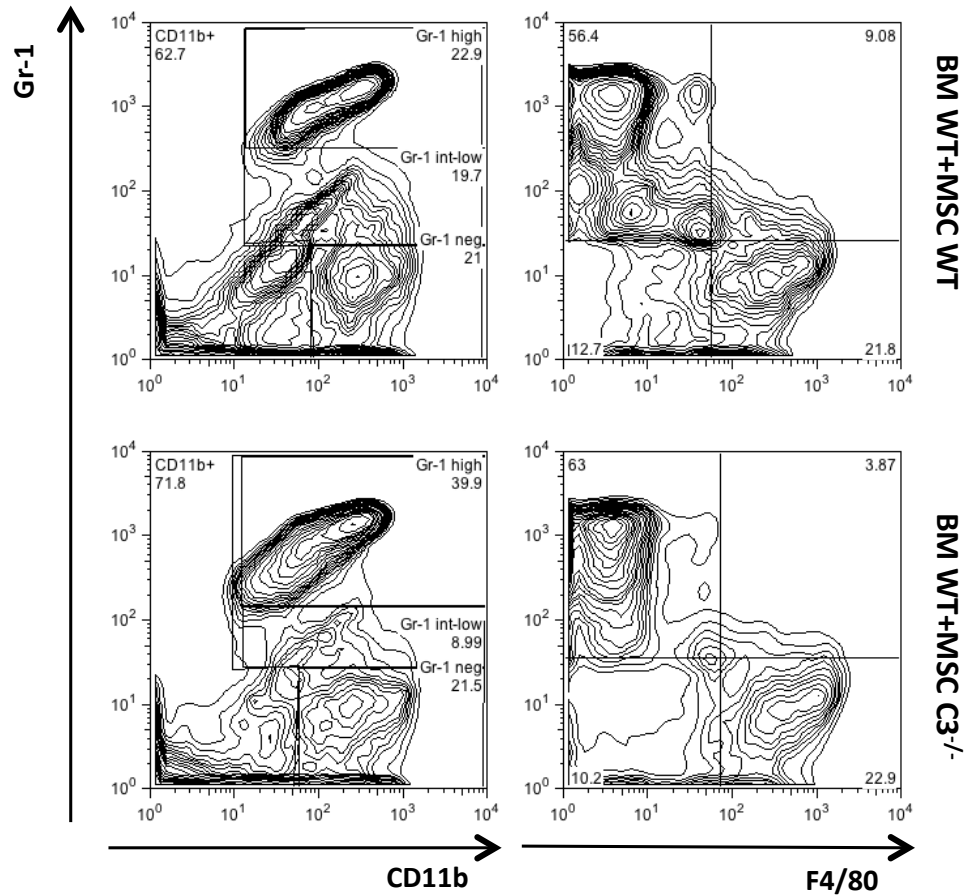
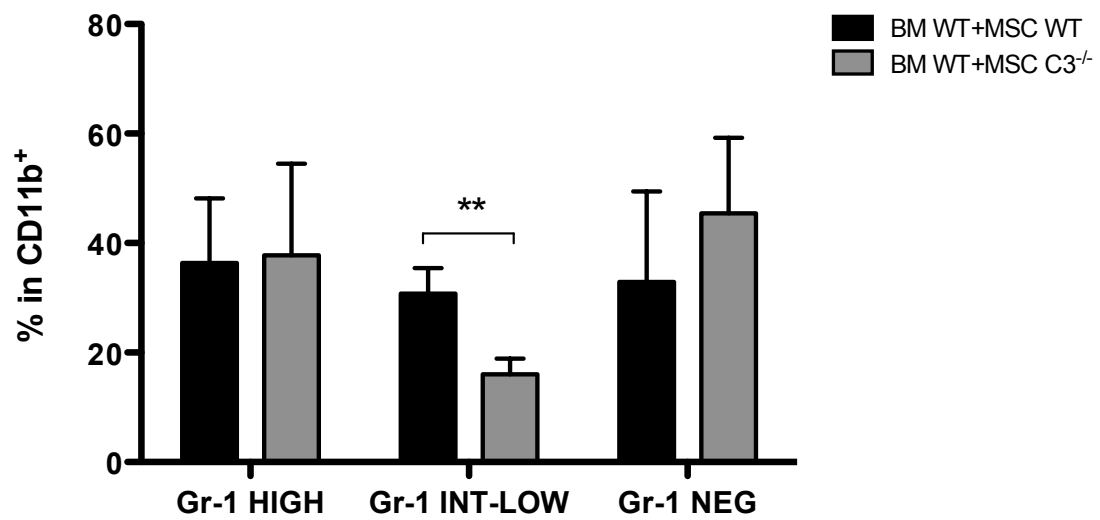
A**B**

FIGURE 4.19. C3^{-/-} MSC ability to induce CD11b⁺ Gr-1^{int-low} cells is impaired. BM cells isolated from WT mice were cultured alone or with MSC from WT or C3^{-/-} mice. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the F4/80 versus Gr-1 proportion in the CD11b⁺ gate. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ gated cells. Mean of 3 independent experiments, $\pm$ SD ** p < 0.005 Unpaired t test.

4.2.5.1 The role of soluble factors in MSC-induced $CD11b^+$ $Gr-1^{int-low}$ cells

Since $C3^{-/-}$ MSC are less able to induce the differentiation of $CD11b^+$ $Gr-1^{int-low}$ cells, we analysed the expression of several soluble factors produced by these cells by ELISA. In particular, the production of cytokines GM-CSF and IL-6 were investigated on the basis of their ability to expand a myeloid population characterised by expression of CD11b and differential expression of Gr-1 [259].

In order to mimic the experimental settings in which BM cells are cultured together with MSC for 4 days, supernatants were collected from BM derived MSC isolated from WT or $C3^{-/-}$ mice plated for 24, 48, 72 and 96 hours. GM-CSF was not produced by WT or $C3^{-/-}$ MSC (data not shown), whereas IL-6 was produced by WT MSC but not from $C3^{-/-}$ MSC (**Figure 4.20B**).

The next step was to investigate the amount of complement C3 produced by WT BM cells, WT or $C3^{-/-}$ MSC, either in co-culture or alone at 24, 48 and 96 hours (**Figure 4.20A**). MSC produced C3 in increasing amounts with time, whereas BM cells from WT mice and $C3^{-/-}$ MSC did not express it. The levels of expression of C3 from BM cells cultured with MSC were similar to the ones detected in MSC WT alone, thus suggesting that MSC cannot induce production of C3 from BM cells.

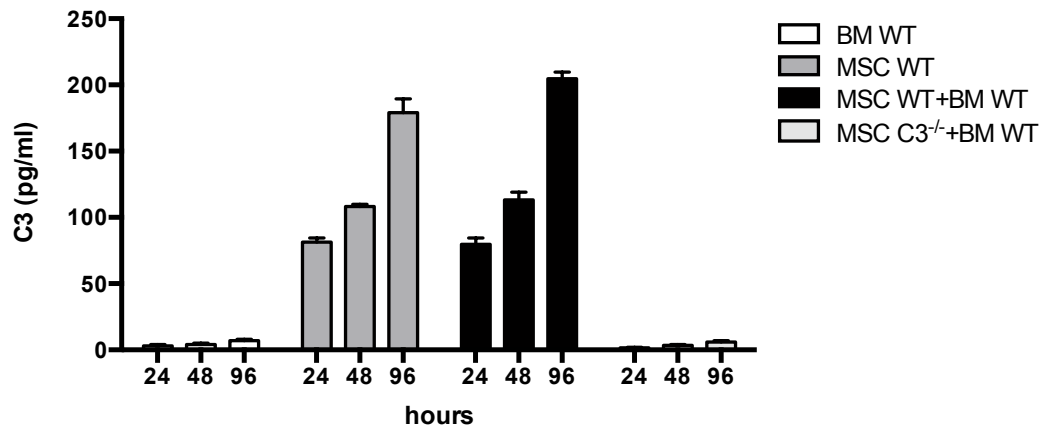
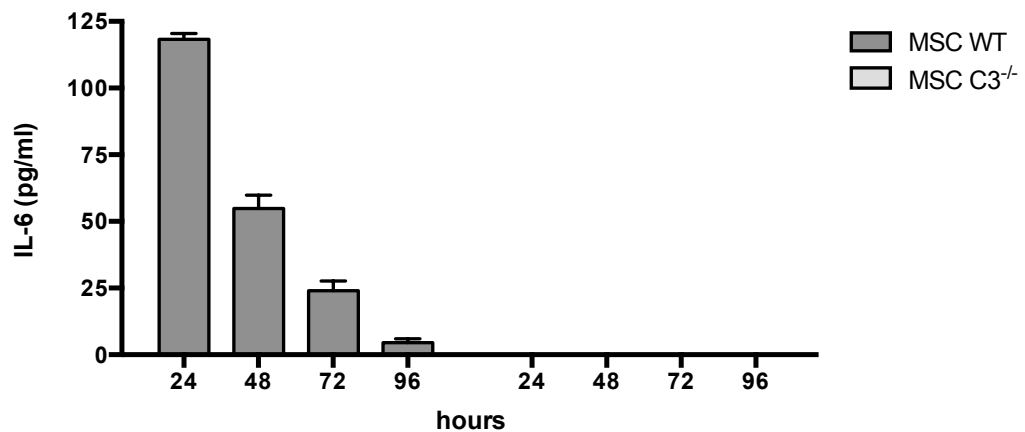
A**B**

FIGURE 4.20. C3^{-/-} MSC do not produce C3 and IL-6. **A.** BM cells isolated from WT mice were cultured alone or with MSC isolated from WT or C3^{-/-} mice for 4 days. Supernatants were collected at 24, 48 and 96 hours, and tested for the production of C3 with ELISA. Mean of 2 independent experiments, $\pm$ SD. **B.** MSC from WT or C3^{-/-} mice were cultured alone as described in Materials and Methods for 4 days. Supernatants were collected at 24, 48, 72 and 96 hours, and tested for the production of IL-6 with ELISA. Mean of 2 independent experiments, $\pm$ SD.

C3^{-/-} MSC induced a decreased percentage of CD11b⁺ Gr-1^{int-low} cells when cultured with BM cells, and did not produce C3 and IL-6 as compared to MSC WT. Therefore we investigated the effect of addition of C3 or IL-6 to co-cultures of BM cells derived from WT or C3^{-/-} mice with C3^{-/-} MSC (Figure 4.21). Cytokines were added at day 1 and day 3. Although a small increase in the percentage of CD11b⁺ Gr-1^{int-low} cells could be detected with the addition of C3 or IL-6, the amount was not restored to the levels expressed from BM cells cultured with WT MSC (Figure 4.21).

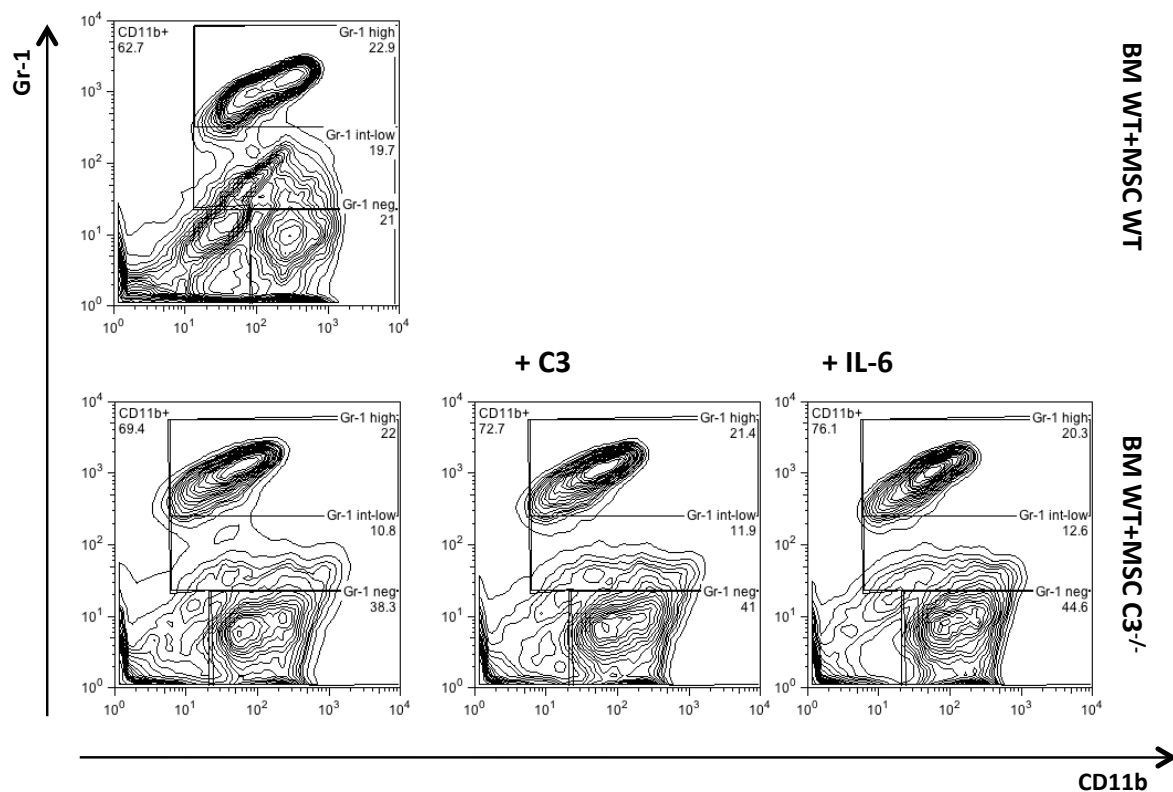


FIGURE 4.21. Addition of C3 or IL-6 to BM and MSC C3^{-/-} co-cultures does not determine an increase in the percentage of CD11b⁺ Gr-1^{int-low} cells. BM cells isolated from WT or C3^{-/-} mice were cultured with MSC isolated from WT or C3^{-/-} mice or 4 days either without or with the addition of C3 (150ng/ml) or IL-6 (150ng/ml). Contour plots represent the CD11b and Gr-1 proportion in the live gate. A representative example of 2 independent experiments is shown.

4.3 DISCUSSION

In this chapter, we describe the investigations on the mechanisms involved in MSC-induced myeloid differentiation. We have observed that macrophages are induced through cell contact, whereas the differentiation of immature myeloid cells is mediated by soluble factors (**Figure 4.1**). We studied therefore the role of some candidate molecules involved in haematopoietic niche functions.

Several studies have investigated the role of NO in different settings, such as immunosuppression, tumour progression, and HSC self-renewal. In our *in vitro* system, the lack of NOS2 in the stromal compartment, but not in haematopoietic cells (**Figure 4.3**), impaired the ability of MSC to expand and differentiate the macrophage population, and induced a higher proportion of granulocytes (**Figure 4.2**). The myeloid population induced by *Nos2*^{-/-} MSC showed a slightly reduced ability to suppress the proliferation of mitogen-induced splenocytes, and a concomitant lower expression of M1 and M2 markers as compared to myeloid cells induced by WT MSC (**Figure 4.5** and **4.4**).

Interestingly, we found that *Nos2*^{-/-} mice did not have constitutive significant abnormalities in the proportions of mononuclear phagocyte populations (**Figure 4.6** and **4.7**) as compared to WT mice both in BM and spleen. Although NOS2 may not indeed have any substantial effect, in the *in vivo* steady-state condition there can be pathways compensating for the defect. Therefore, we thought to test the role of NOS2 during myeloid reconstitution adopting two different strategies.

The adoptive transfer of WT MSC induced a higher percentage of macrophages in BM donor cells, but not in spleen, whereas such ability was impaired in *Nos2*^{-/-} MSC (**Figure 4.8D** and **4.8F**). In contrast with the known ability of MSC in favouring HSC engraftment, no difference was documented between mice adoptively transferred with BM alone or with WT or *Nos2*^{-/-} MSC (**Figure 4.8C**). Such discrepancy can find a potential explanation in the different experimental systems used. The engraftment enhancing effect of MSC on HSC engraftment was observed using human MSC in NOD/SCID mice after six weeks from transplant [63]. An effect on early HSC engraftment was

documented twelve days after syngeneic transplantation when murine MSC were injected intra-femur [64]. These results might suggest that, in early phases of reconstitution, MSC preferentially induce the repopulation of short-term progenitors (mature myeloid cells) in which NOS2 may play a fundamental role. Alternatively, but not exclusively, the myeloid population, supported by MSC, could be one involved in HSC long-term survival [262].

Further work is required to confirm whether MSC have a favouring activity on HSC long-term engraftment, and whether such activity requires early reconstitution of mature myeloid cells in the early stages of engraftment.

The selective effect of MSC on BM but not on spleen macrophage repopulation could be explained by the intrinsic tissue macrophage sensitivity to the irradiation dose. Therefore, in order to eliminate any confusing effect given by the WT recipient environment, and to eliminate the competition with host tissue macrophages, *Nos2*^{-/-} recipients were treated with clodronate, irradiated and then received WT BM cells (**Figure 4.9**). Whilst WT recipients promoted early macrophage repopulation in BM (**Figure 4.9**), such ability was impaired in NOS2 deficient hosts. These data suggest that a defect in the haematopoietic environment of *Nos2*^{-/-} mice exists, and impacts on donor macrophage differentiation. We cannot exclude that other cells (endothelial cells or pericytes) contribute to the macrophage defect via NOS2 deficiency .

These *in vivo* experiments have not addressed which population in the unfractionated BM is interacting with MSC although the *in vitro* data suggest to be a committed myeloid progenitors (CMP or GMP).

As *Nos2* expression on MSC is mainly up-regulated by TNF- α , or its combination with IFN- γ (**Figure 4.12**), we hypothesized that TNF- α produced by BM cells in culture with MSC (**Figure 4.10**) could drive the up-regulation of *Nos2*, and in turn the differentiation of macrophages. However, the high concentrations used to assay the role of these cytokines in MSC up-regulation of NOS2 cannot reflect

their physiological relevance in inducing myeloid differentiation ability. When added to the co-cultures, the same concentrations gave the paradoxical effect of impairing macrophage differentiation (**Figure 4.15**), confirming their inappropriateness. Further experiments are needed to understand the role of BM derived TNF- α in the co-cultures, and whether macrophage differentiation is impaired by blocking or neutralising its effect. However, the impaired differentiation ability seen in *Tnfr1/r2^{-/-}* MSC seems to indicate that TNF- α might play a role (**Figure 4.13**). Further investigation is therefore required to understand how other forms of TNF- α (such as trans-membrane TNF- α) on BM cells could mediate such effect, possibly corroborating the importance of cell contact in MSC-induced macrophage differentiation.

Agrin-deficient MSC were also shown to induce a reduction in CD11b⁺ cells absolute number, in particular in the macrophage subset, and, similarly to the effect of *Tnfr1/r2^{-/-}* MSC, they induced a higher proportion of early myeloid cells (CD11b⁺ Gr-1^{int-low}) (**Figure 4.16**). Such effect was not unexpected, as agrin deficiency has been shown to correlate with impaired development of monocytes and macrophages, but not of neutrophils [336]. Although agrin-deficient MSC had a lower expression of *Nos2*, the existence of a relationship between agrin and NO has not been demonstrated yet (**Figure 4.17**). However, in the neuromuscular junction, NOS activity is required for agrin-induced aggregation of postsynaptic acetylcholine receptors at the embryonic neuromuscular junction *in vivo* [383]. It has been suggested that an increase in NO production is part of the agrin/MuSK signal transduction pathway, but such hypothesis has not yet been confirmed [384].

Whilst NOS2, TNFR1/R2 and agrin seemed to play a crucial role in MSC induced differentiation of macrophages, complement C3 was found to be necessary to drive the differentiation of immature myeloid cells (**Figure 4.19**). However, adding back C3 to the co-cultures did not restore the differentiation of this subset (**Figure 4.21**). Further investigation is therefore required to determine the soluble factor mediating the differentiation of CD11b⁺ Gr-1^{int-low} cells.

These results could be interpreted in light of a possible role of BM MSC in driving macrophage differentiation in the haematopoietic BM niche, whereby several molecules involved in HSPC regulation can modulate such activity.

The inflammatory environment has been shown to produce contrasting effects on HSPC functions. TNF- α has been associated with apoptosis and inhibition of HSC self-renewal [385, 386]. On the other hand, TNF- α signalling through TNFR1 appears to be fundamental for the maintenance of HSC functions [347]. Another inflammatory component, represented by the complement system, has been postulated as one of the major players in HSPC mobilization. C3 cleavage fragments enhance the responsiveness of HSPC to a CXCL12 gradient, thus promoting retention of these cells in the BM [387, 388].

Hypoxia is another important regulator of niche activity, whereby low oxygen levels play a fundamental role in the maintenance of self-renewal for dormant HSC [358]. Together with inflammatory cytokines, hypoxia has been associated with up-regulation of NOS2 and ROS levels, which also have a role in regulating dormancy and mobilization of HSC [369].

MSC-macrophage interaction in the niche is fundamental to control the retention of HSPC in the BM [261, 262]. In this perspective, we could argue that hypoxic and/or inflammatory microenvironments can shape the ability of MSC to differentiate myeloid cells, thus resulting in a differential modulation of HSPC self-renewal and mobilization.

CHAPTER 5.

MSC-INDUCED MYELOID DIFFERENTIATION: IDENTIFICATION OF THE CELLULAR PLAYERS

5.1 INTRODUCTION

In the previous chapters we have described the ability of MSC to expand and differentiate unfractionated BM into three subsets of CD11b⁺ cells according to differing expression of Gr-1. In this section, our aims have been directed at identifying, amongst the haematopoietic hierarchy, the cellular target(s) of MSC driven myeloid differentiation.

Intimately linked to this question, is the debatable origin of MSC and whether its source of origin impacts on their functions. In fact, there is still no consensus regarding the definition of stromal cells, which have been isolated from several tissues and given various names, such as fibroblasts, reticular cells, and mesenchymal stem/stromal cells. Several lines of evidence show that BM derived MSC share the same phenotype and immunosuppressive functions of the commonly defined fibroblasts [13, 14]. Furthermore, a number of studies indicate that an interaction between MSC and macrophages exists also in peripheral tissues. In particular, stem cell niches present in the skin, kidney and liver are supported by the interaction between MSC and tissue-macrophages.

Therefore, it was fundamental to understand whether the phenomenon we observed using cells of BM origin can also be reproduced: 1. using MSC from other tissues, 2. using haematopoietic cells at different differentiation stages. The information would be invaluable to understand the dynamics of tissue homeostasis in normal and inflammatory conditions.

5.2 RESULTS

5.2.1 MSC target committed myeloid progenitors but not primitive HSC

In an effort to understand the target cells of MSC-induced myeloid differentiation, FACS sorted HSC, common myeloid progenitors (CMP) and granulocyte/macrophage progenitors (GMP) were cultured with MSC in the usual conditions. Isolated megakaryocyte/erythroid progenitors (MEP) and unfractionated BM were used as negative or positive control of differentiation, respectively. Unfractionated BM cells were stained for Lineage (Lin), IL-7R α , c-kit, Sca-1, CD16/32 (Fc γ R), CD34 and fetal liver kinase 2 (Flk2, FLT3). Because the expression of IL-7R α chain marks the common lymphoid progenitors (CLP) and other downstream lymphoid progenitors, IL-7R α positive cells were excluded, as well as all the Lineage positive cells. Following the isolation method proposed by Weissman group [160], c-kit⁺ and Sca-1⁻ cells were subdivided in CD16/32^{low} CD34⁺, CD16/32^{low} CD34⁻, and CD16/32^{high} CD34⁺ populations (MEP, CMP, GMP respectively). HSC were instead defined as c-kit⁺ Sca-1⁺ Flk-2⁻ cells (**Figure 5.1A**). Analysis of the sorted populations revealed a high purity (83.3% of MEP, 94.1% of CMP, 100% of GMP and 100% of HSC) (**Figure 5.1B**).

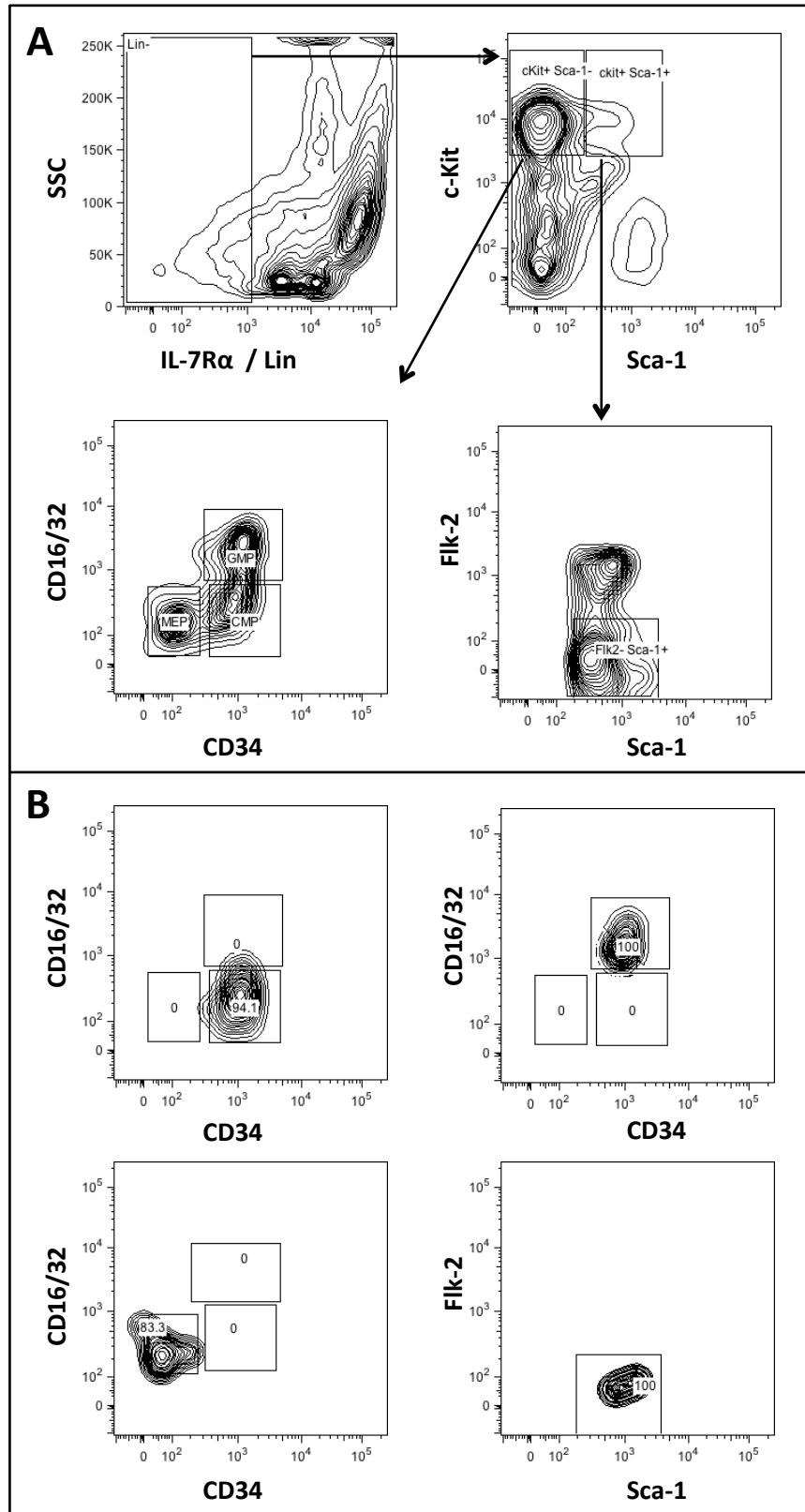


FIGURE 5.1. Sorting of myeloid progenitors in mouse BM. **A.** BM cell suspension was prepared as described in Materials and Methods. The IL-7R α Lin⁻ Sca-1⁻c-Kit⁺ fraction of BM cells was subdivided into CD16/32^{low} CD34⁺, CD16/32^{low} CD34⁻, and CD16/32^{high} CD34⁺ populations (MEP, CMP, GMP respectively), whereas the IL-7R α Lin⁻Sca-1⁺c-Kit⁺ was subdivided into Flk2⁻Sca-1⁺ (HSC). **B.** Re-analysis of the sorted CD16/32^{low} CD34⁺ (MEP), CD16/32^{low} CD34⁻ (CMP), and CD16/32^{high} CD34⁺ (GMP) populations.

Sorted HSC, CMP, GMP and MEP were co-cultured with MSC for 4 days and cells collected and stained for expression of CD11b, Gr-1, F4/80 and CD115 (**Figure 5.2**). The presence of MSC in the cultures induced the differentiation of the three CD11b⁺ subsets from CMP and GMP, but not HSC and MEP (**Figure 5.2A**, left panel).

Analysis of the Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ subsets in the gated CD11b⁺ population revealed that co-cultures of CMP and MSC induced a 2-fold decreased proportion of Gr-1^{high} cells in comparison to unfractionated BM cultured with MSC (24% ± 6.9% versus 41.8% ± 10.8% in BM+MSC, p=0.0283), and a 2-fold increased percentage of Gr-1⁻ cells as compared to unfractionated BM cultured with MSC (60.1% ± 8.9% versus 35% ± 12.8% in BM+MSC, p=0.0345) (**Figure 5.2B**). No statistically significant difference was found between GMP or unfractionated BM cultured with MSC (**Figure 5.2B**).

A 2-fold increase was documented in the percentage of CD11b⁺ F4/80⁺ cells in the co-cultures of CMP and MSC, in comparison to BM cultured with MSC (63.6% ± 9.8% versus 36.8% ± 13.7% in BM+MSC, p=0.0352) (**Figure 5.2C** and **Figure 5.2A**, middle panel). Furthermore, analysis of the percentage of CD115⁺ cells in the gated CD11b⁺ population showed that CMP cultured with MSC were able to induce a higher percentage of CD115⁺ cells as compared to BM cultured with MSC (85.8% ± 1.3% versus 38.6% ± 18.9% in BM+MSC, p=0.0155) (**Figure 5.2C** and **Figure 5.2A**, right panel). No statistically significant difference was documented between the co-cultures of MSC with GMP or with unfractionated BM (**Figure 5.2C**).



FIGURE 5.2. Committed myeloid progenitors, but not primitive HSC, generate myeloid cells when cultured with MSC. Unfractionated BM or sorted CMP, GMP, MEP and HSC were co-cultured with MSC for 4 days. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the expression of F4/80 and CD115 in the CD11b⁺ gated population. ND= not determined.

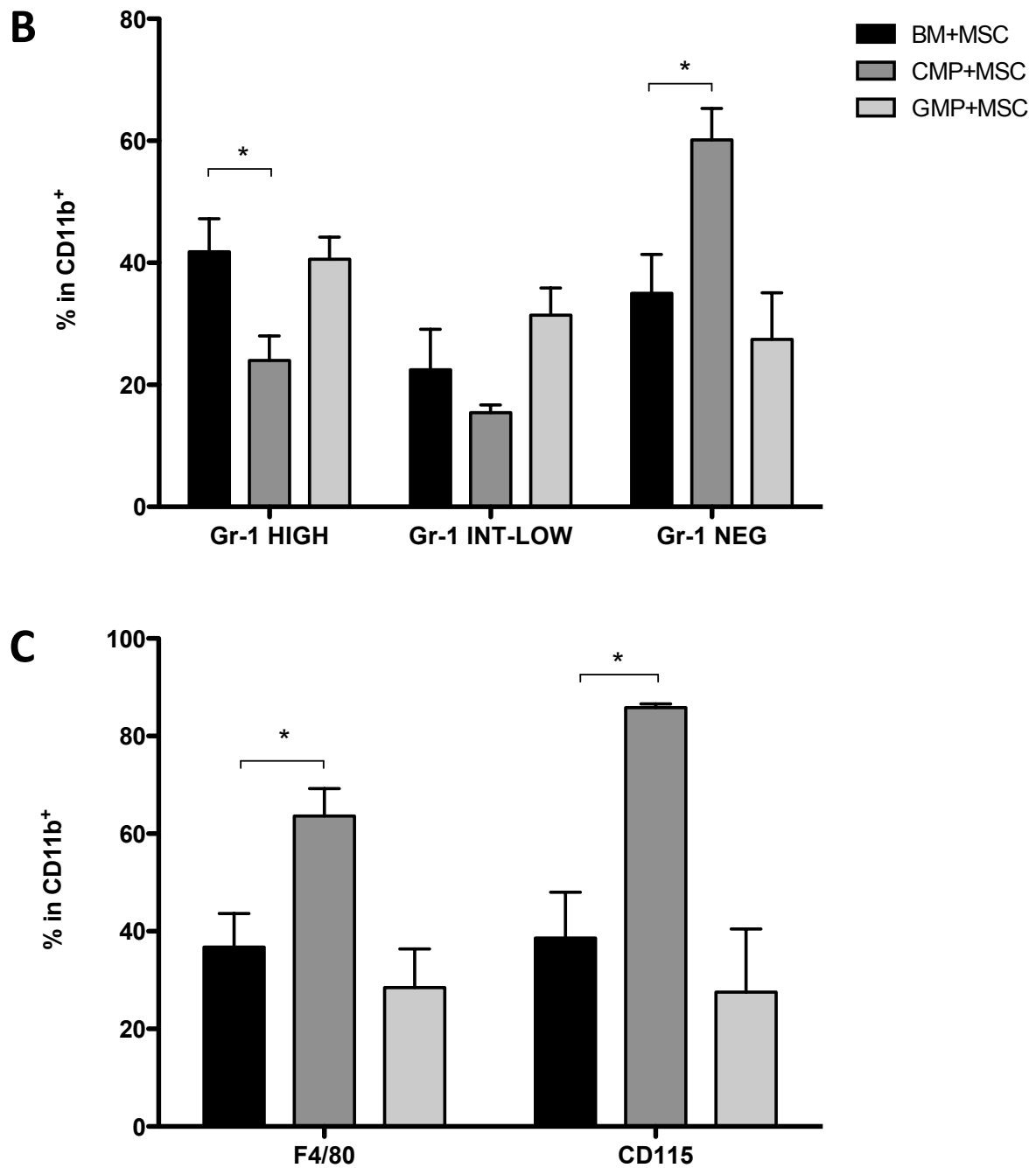


FIGURE 5.2. Committed myeloid progenitors, but not primitive HSC, generate myeloid cells when cultured with MSC. Unfractionated BM or sorted CMP, GMP, MEP and HSC were co-cultured with MSC for 4 days. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1^{neg} in CD11b⁺ cells. Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ Unpaired t test. **C.** Percentage of CD11b⁺ F4/80⁺ and CD115⁺ cells in the CD11b⁺ gated population. Mean of 4 independent experiments, $\pm$ SD * $p < 0.05$ Unpaired t test.

To further confirm the hypothesis that committed cells would have an impaired differentiation ability as compared to myeloid precursors, we evaluated the ability of CD11b⁺ sorted cells from BM and from spleen of C57Bl/6 mice to differentiate into the three subsets of CD11b⁺ cells after culture with MSC.

Unfractionated or CD11b⁺ sorted BM or spleen cells were cultured with MSC and myeloid differentiation analysed (**Figure 5.3**). A 4-fold decrease in the percentage of CD11b⁺ Gr-1⁻ cells (8.74% versus 35% of unfractionated BM+MSC control in the live gated population) and a 2.5-fold increase in the percentage of CD11b⁺ Gr-1^{high} cells (60.1% versus 24% of unfractionated BM+MSC control) were documented in the cultures of MSC and CD11b⁺ cells isolated from the BM.

No differentiation was recorded in co-cultures of unfractionated spleen and MSC (Figure 5.3), whilst CD11b⁺ splenocytes showed minimal ability to differentiate, with only 16.8% of CD11b⁺ cells detected after the co-culture (**Figure 5.3**).

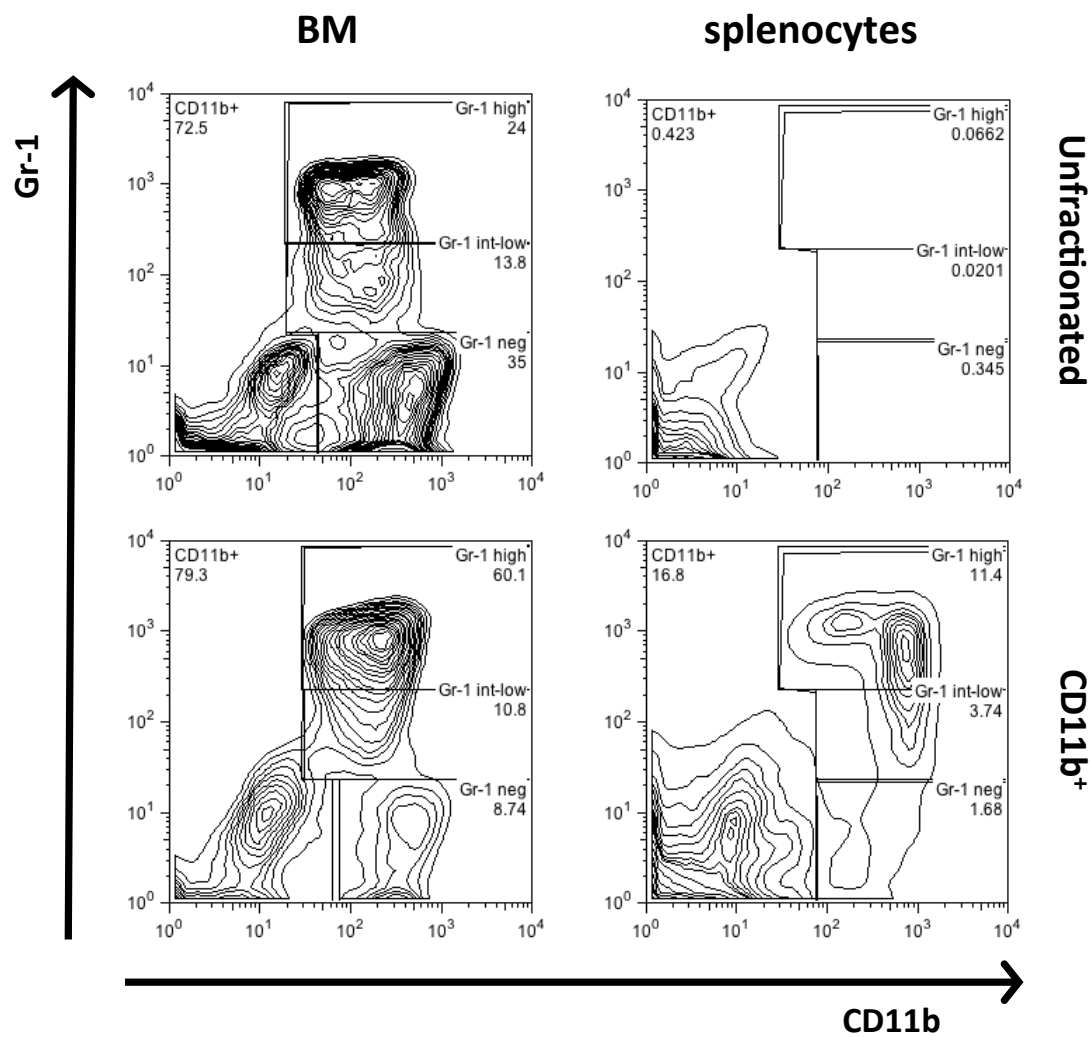


FIGURE 5.3. MSC do not differentiate spleen or BM CD11b⁺ cells. Unfractionated or CD11b⁺ BM or spleen cells were cultured with MSC for 4 days. Contour plots represent the CD11b and Gr-1 proportion in the live gate. A representative example of 3 independent experiments is shown.

5.2.2 MSC from peripheral tissues have the ability to differentiate myeloid cells

We asked whether MSC isolated from skin and kidney glomeruli (mesangial cells) could drive the differentiation of myeloid cells from unfractionated BM.

MSC isolated from the skin of C57Bl/6 mice exhibited a similar phenotype of BM derived MSC, with a proportion of PDGFR- α^+ /Sca-1 $^+$ cells of 40.2%. Cells did not express c-Kit, and the percentage of CD90 $^+$ cells was 80% (**Figure 5.4A**).

In order to test their immunosuppressive activity, escalating concentrations of skin MSC were cultured with ConA-stimulated splenocytes (**Figure 5.4B**). The proliferation of activated splenocytes was completely abolished when skin stromal cells were present up to a 1:80 ratio, with a 6-fold reduction of proliferation still detectable at a 1:160 ratio.

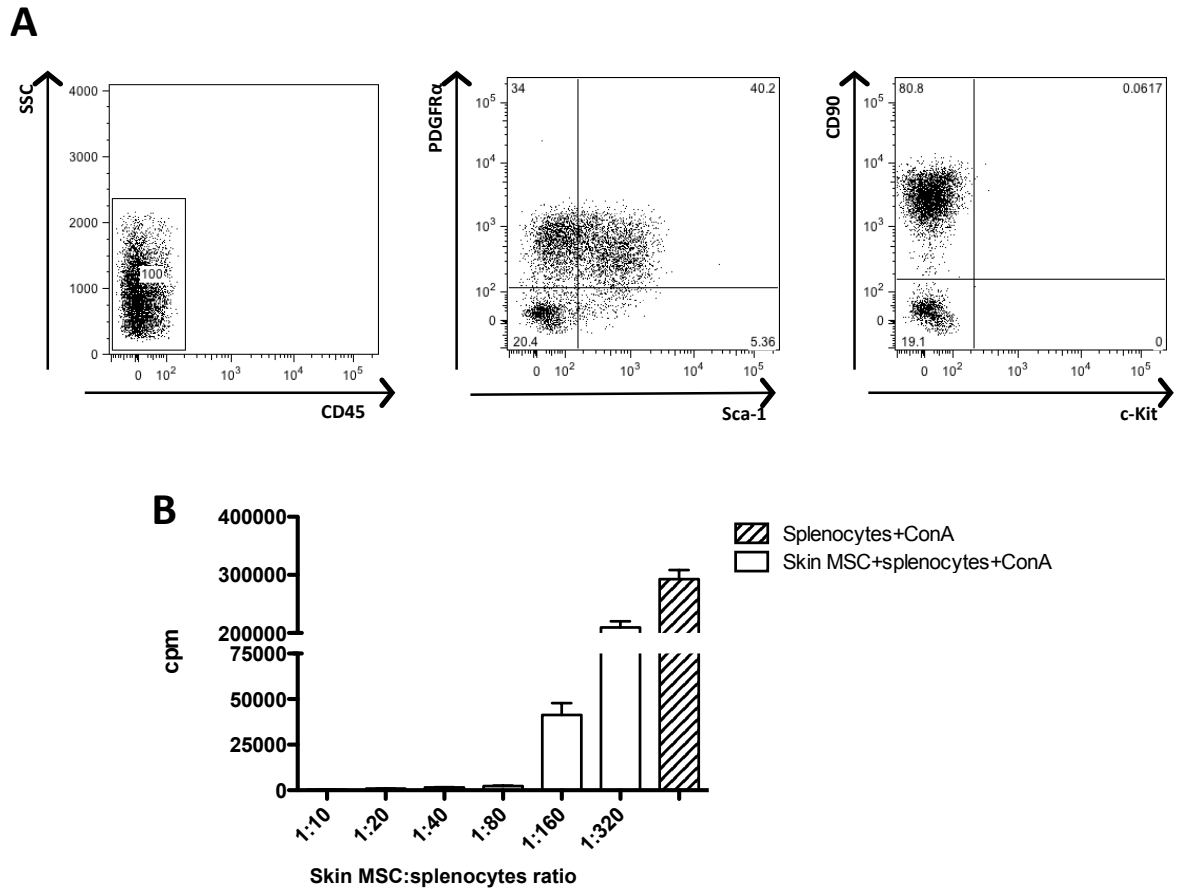


FIGURE 5.4: Characterization of skin MSC preparation. **A.** FACS dot plots of skin MSC. Proportion of CD45⁻ cells is represented in the live gate, and the proportions of PDGFRα⁺ Sca-1⁺ cells and CD90⁺ c-Kit⁺ cells are represented in the CD45⁻ gate. A representative example from 3 independently derived preparations of skin MSC is represented. **B.** Skin MSC were plated at 5x10⁴ cells/well, with 1:2 serial dilutions, and splenocytes were added at 5x10⁵ cells/well and stimulated with 3μg/ml of ConA for 3 days. ³H-thymidine was added for the last 18h. The figure reports a representative experiment, and each column represents the mean±SD of triplicates of cultures. (cpm: count per minute). Results are expressed as the value obtained by subtracting the (Skin MSC+Splenocytes) value from the (Skin MSC+Splenocytes ConA) value.

We then assessed the ability of skin MSC and mesangial cells to induce the differentiation of unfractionated BM cells. Both cells were able to induce the differentiation of the three myeloid subsets (**Figure 5.5B**).

However, the recovery of CD11b⁺ cells was different between BM cultured with cutaneous MSC or mesangial cells and BM MSC (**Figure 5.5C**). In particular, skin MSC induced a 2-fold increase in the expansion of CD11b⁺ cells as compared to BM cultured alone ($2.4 \times 10^6 \pm 0.6 \times 10^6$ versus $0.7 \times 10^6 \pm 0.09 \times 10^6$ of BM alone, $p=0.0058$), but such expansion was reduced when compared to the effect of BM MSC ($6.1 \times 10^6 \pm 0.8 \times 10^6$ in BM+MSC, $p=0.0031$). Similarly, mesangial cells induced an expansion of CD11b⁺ cells ($1.5 \times 10^6 \pm 0.15 \times 10^6$, $p=0.0018$ versus BM alone) but it was lower than that produced by BM MSC ($p=0.0007$ versus BM+MSC).

The proportion of the myeloid subsets was quantitatively different using different MSC preparations. Mesangial cells induced a pattern of differentiation similar to the one induced by BM derived MSC, whereas skin MSC induced a 2-fold increase in the percentage of CD11b⁺ Gr-1^{int-low} cells ($38.7\% \pm 12.6\%$ versus $12.4\% \pm 1.1\%$ in BM+MSC, not significant), and a 2-fold decrease in the percentage of CD11b⁺ Gr-1⁻ cells as compared to BM cultured with BM MSC ($26.8\% \pm 2.4\%$ versus $52.1\% \pm 1.6\%$ in BM+MSC, $p=0.0065$, **Figure 5.5B**).

The phenotype of the myeloid components was further characterised by F4/80, CD115 and CD206 expression. A 4- and a 2-fold decrease in the percentage of CD11b⁺ Gr-1⁻ F4/80⁺ cells was documented in the co-cultures of BM with skin MSC and mesangial cells compared to BM MSC (13.3% and 23% versus 46.3% of BM MSC, respectively) (**Figure 5.5**). Notably, little or no expression of CD115 was detected in the co-cultures of BM with skin MSC or mesangial cells. Mesangial cells were able to induce only 8.76% of CD11b⁺ Gr-1⁻ CD115⁺ cells, whereas no expression of CD115 was detected in CD11b⁺ cells from the cultures with skin MSC. A 3-fold reduction in the percentage of CD11b⁺ Gr-1⁻ CD206⁺ cells was documented in BM cultured with skin

MSC or mesangial cells, as compared to BM cultured with BM MSC (6.56% and 6.98% versus 21.7%, respectively).

A

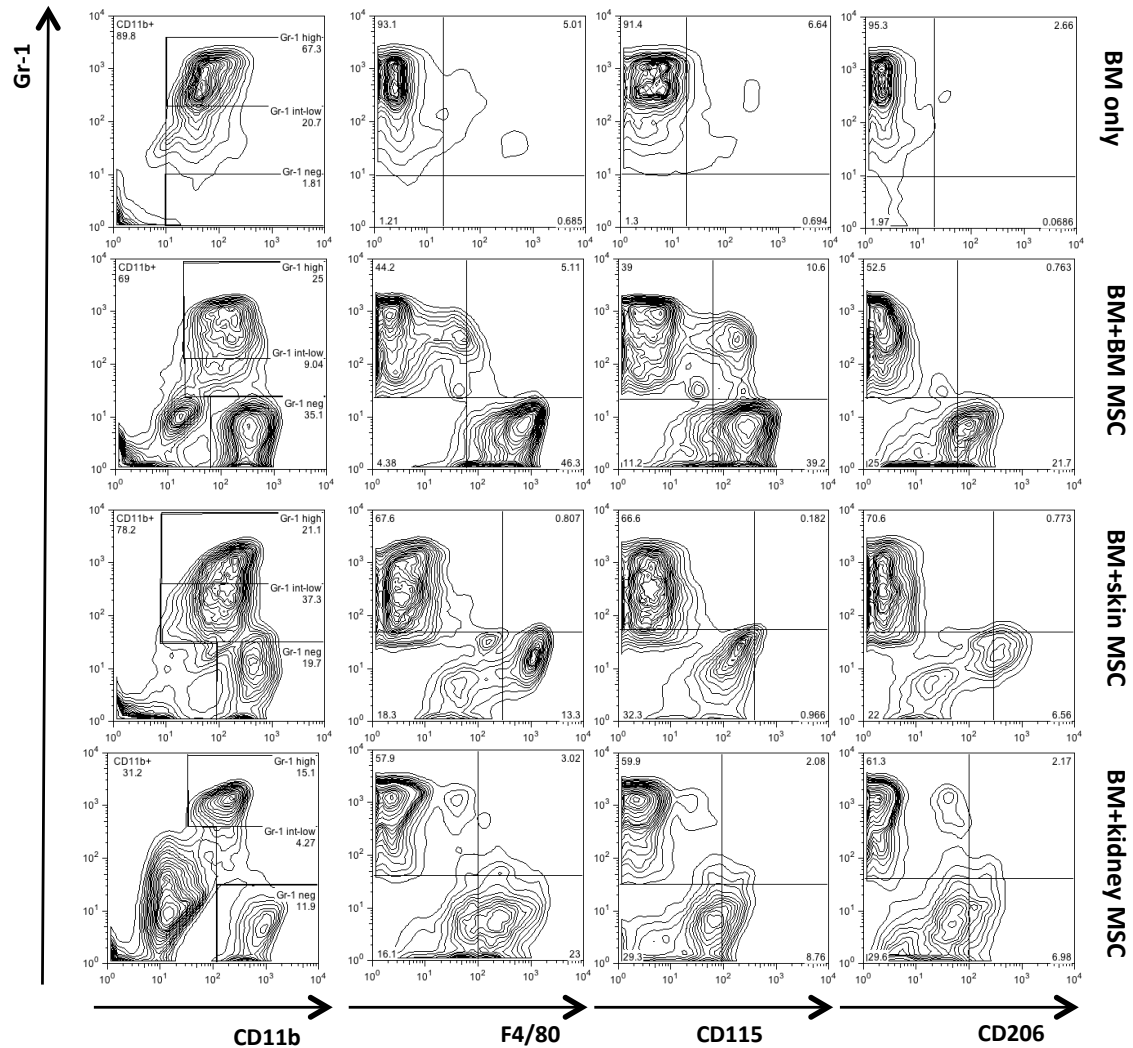


FIGURE 5.5. MSC of peripheral tissues are able to induce the differentiation of CD11b⁺ Gr-1⁻ cells. BM was cultured with MSC isolated from BM or skin or mesangial cells (kidney MSC) for 4 days. **A.** Contour plots represent the CD11b and Gr-1 proportion in the live gate, and the expression of F4/80, CD115 and CD206 in the CD11b⁺ gated population. Data are representative of 2 independent experiments.

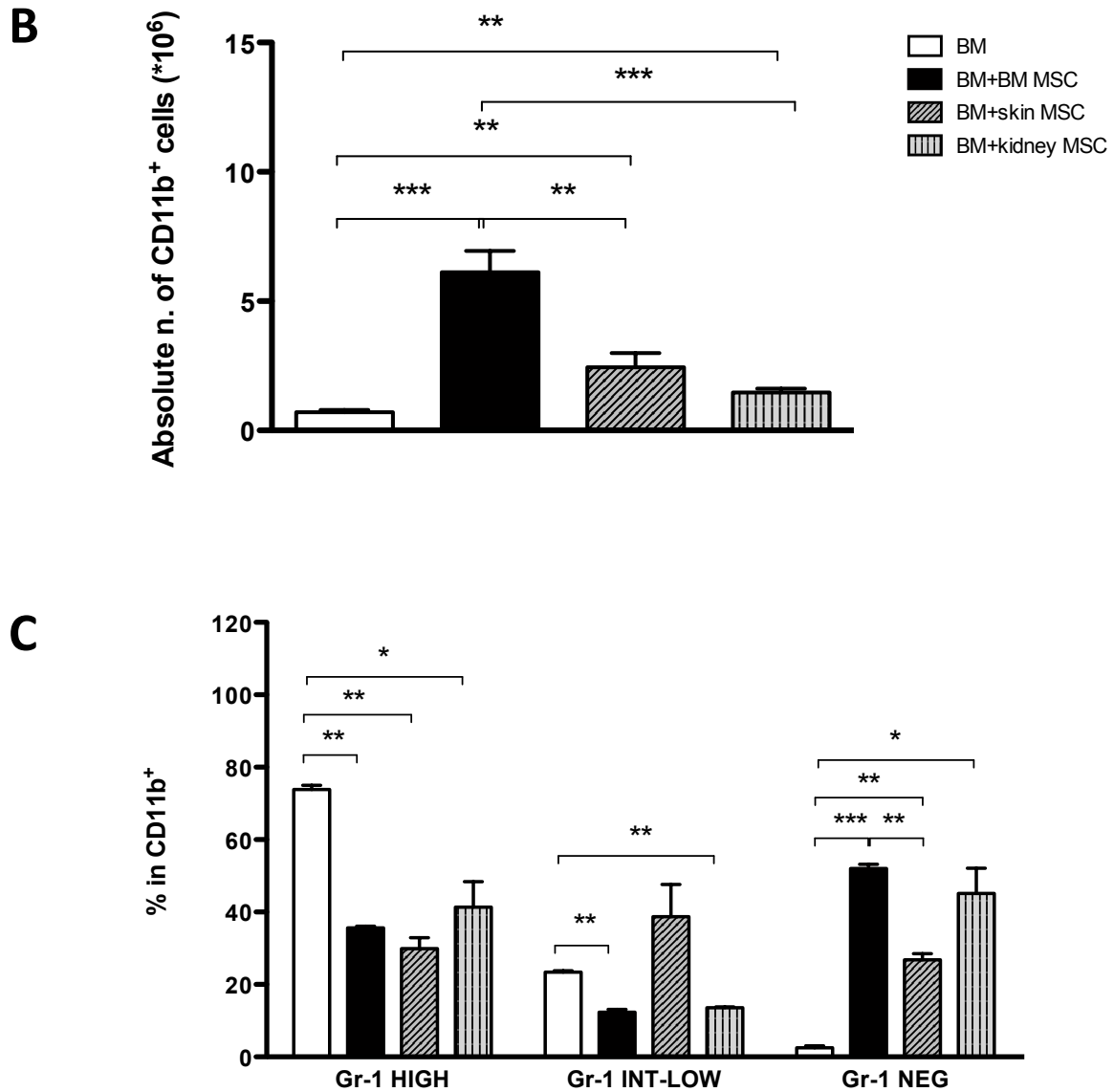


FIGURE 5.5. MSC of peripheral tissues are able to induce the differentiation of CD11b⁺ Gr-1⁻ cells. BM was cultured with BM or skin-derived MSC or mesangial cells (kidney MSC) for 4 days. **B.** Percentage of Gr-1^{high}, Gr-1^{int-low} and Gr-1⁻ in CD11b⁺ cells. Mean of 2 independent experiments, $\pm$ SD * $p < 0.05$ ** $p < 0.005$ *** $p < 0.001$, Unpaired t test. **C.** Absolute number of CD11b⁺ cells recovered from BM cultured alone, with MSC, with skin MSC or with mesangial cells for 4 days. Mean of 2 independent experiments, $\pm$ SD. ** $p < 0.005$ *** $p < 0.001$, Unpaired t test.

The next step was to understand whether the CD11b⁺ induced cells from the co-cultures of BM cells and MSC isolated from peripheral tissues shared a similar M1-M2 profile with CD11b⁺ cells obtained from BM cultured with BM MSC.

CD11b⁺ cells obtained from the co-cultures were isolated and RT-PCR analysis was performed on RNA extracted from these cells (**Figure 5.6**). *Cox2* expression was similar in all of the co-cultures. *Il12b* expression from co-cultures of BM with skin MSC was similar to the expression induced by MSC, whereas mesangial cells induced a 2-fold increase in the expression of *Il12b*. A 2-fold increase and a 2-fold decrease were documented in the expression of *Marco* from CD11b⁺ cells isolated from BM cultured with skin MSC or mesangial cells, respectively. *Nos2* was up-regulated by 20-folds and 80-folds in BM cultured with skin MSC and mesangial cells, respectively, as compared to the expression induced by BM MSC.

The M2 markers *Arg-1* and *Ccl12* were highly up-regulated in BM cultured with skin MSC (40-folds and 25-folds increase, respectively). Mesangial cells induced a similar expression of *Arg-1* and *Ccl12* to the one induced by BM MSC.

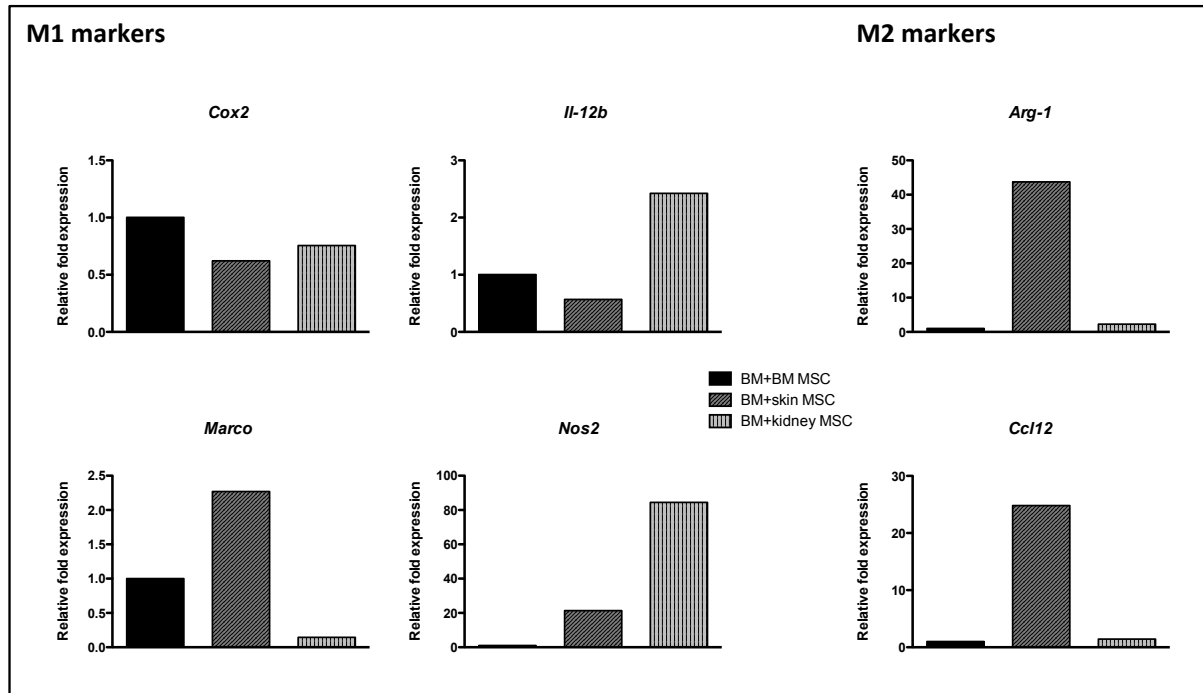


FIGURE 5.6. M1-M2 profile of CD11b⁺ cells from MSC cultures. Quantitative RT-PCR data of CD11b⁺ cells sorted from 4 days cultures of BM with MSC isolated from BM or skin or mesangial cells (kidney MSC). Data are expressed as normalized to *Hprt1* housekeeping gene, and compared to abundance in CD11b⁺ isolated from BM cultured with BM MSC. A representative example of 2 independent experiments is shown. *Arg-1*, arginase 1; *Ccl12*, CC-chemokine ligand 12; *Cox2*, cyclooxygenase 2; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; *Il12b*, interleukin 12b; *Marco*, macrophage receptor with collagenous structure; *Nos2*, nitric oxide synthase 2.

5.3 DISCUSSION

In this chapter, the cellular players involved in the interaction between myeloid cells and MSC were investigated.

Common myeloid progenitors (CMP), and granulocyte/macrophage progenitors (GMP), but not HSC or megakaryocyte/erythrocyte progenitors (MEP) were able to differentiate into macrophages when in contact with MSC (**Figure 5.2**). The results were not surprising, as several studies have shown that MSC can maintain long-term culture-initiating cell frequency and HSC primitive profile via mechanisms involving cell contact [389, 390].

Consistent with these data, CD11b⁺ cells obtained from either the BM or the spleen of WT mice have an impaired ability to differentiate into macrophages (**Figure 5.3**). Spleen myeloid cells, such as marginal zone and red pulp macrophages, are probably already fully differentiated, hence unable to respond to MSC-derived differentiation stimulus. Furthermore, myeloid cells isolated from spleen and cultured with MSC seemed to lose the expression of CD11b after 4 days of culture.

The lack of differentiation ability seen in the CD11b⁺ Gr-1^{int-low} subset after co-culture with MSC could be explained by two possibilities. Firstly, CD11b⁺ Gr-1^{int-low} are induced by MSC to have a fully committed phenotype that would impede the further differentiation into other types of myeloid cells. Secondly, the Gr-1^{int-low} subset represents an immature myeloid population the survival of which depends on soluble factors provided by stromal cells. Further investigation is required to elucidate this issue.

We observed that not only BM derived, but also skin MSC and mesangial cells were able to drive the differentiation of myeloid cells. Mesangial cells represent the stromal component of kidney glomeruli, and have been defined as specialised pericytes of the glomeruli [391]. Their osteogenic and adipogenic differentiation capabilities, along with their self-renewal capacity, led some investigators

to define them as mesenchymal stem cells [392]. The ability to differentiate mature and immature myeloid populations adds therefore on to the list of common features shared between MSC of BM origin and those isolated from peripheral tissues (**Figure 5.4** and [14]), thus revealing new perspectives in the functional notion of tissue mesenchyma.

Taken together, these data suggest a scenario in which tissue MSC can recruit and promote the maturation of myeloid precursors that recirculate in peripheral blood.

However, the composition of the three myeloid subsets differs with the source of MSC. This is in line with the current notions on tissue-specific macrophages, which are characterised by different histomorphological features and functions. The differences documented in BM cells and cutaneous MSC or mesangial cells co-cultures could therefore account for a different ability of these cells to differentiate tissue specific macrophages. Further investigation is required to understand the impact of this *in vitro* property in the physiology of the recruitment and differentiation of peripheral tissue-specific macrophages.

CHAPTER 6.

GENERAL DISCUSSION

The findings presented in this thesis highlight a new role for stromal cells in the differentiation of circulating progenitors into macrophages in peripheral tissues. Myeloid progenitors differentiate into granulocytes and macrophages when in contact with or in proximity to MSC from different tissue sources. The molecular mechanisms underlying this effect appear to differ depending on the myeloid subset. Whilst NOS2 contribute to macrophage differentiation, complement C3 fraction drives the generation of early myeloid cells. *Nos2*^{-/-} recipients are haematologically normal under steady-state conditions, yet they show a significant impairment in the ability to reconstitute macrophages in the BM after irradiation and transplantation of WT BM cells. Although limited to *in vitro* evidence, we showed that BM cells from *Nos2*^{-/-} mice could differentiate into macrophages when in contact with WT MSC, thus suggesting that there is a defect in the haematopoietic environment of *Nos2*^{-/-} mice.

We have shown that MSC mediated *Nos2* expression is dependent on TNF- α and IFN- γ , thus indicating that inflammatory molecules play an important role. Similarly, MSC immunomodulatory activity is susceptible to environmental cues. In fact, two opposing functional profiles have been identified for MSC immune regulation, whereby different TLR stimulation polarises MSC towards a pro- or alternatively an anti-inflammatory activity [135].

During inflammation, cells of the innate immune system infiltrate tissues and their activation results into the release of chemokines and cytokines. Innate immunity is not only critical for removing dead cells and fight infections, but also to ignite a series of processes that limit inflammation and, as a consequence, promote tissue repair. Macrophage polarization has been shown to have a fundamental role in this, as inhibition of the transcription machinery driving the reprogramming of macrophage

function leads to impaired tissue regeneration [145]. Under these circumstances, ‘licensed’ tissue MSC may be instrumental at orchestrating resolution of inflammation not only by suppressing immune effectors, but also – and possibly more importantly – by recruiting and activating cellular networks of ‘innate (M2 macrophages) and adaptive (T_{REGS}) immune tolerance’ [393]. Therefore, in the early stages of inflammation, MSC-induced differentiation of immature neutrophils and macrophages could be fundamental to initiate such a process by increasing the number of myeloid cells susceptible to acquire this anti-inflammatory profile at the site of inflammation.

The interaction between MSC and macrophages is also central in the BM niche to regulate HSPC mobilization and egress [262]. However, the nature of MSC-macrophage interaction as ‘inflammatory unit’ in the stem cell niche has never been addressed or surmised. The notion of ‘stem cell niche’ traditionally refers to an environment that assures stem cell self-renewal and dormancy, and regulates differentiation. However, very recent results have suggested it may also be ‘immunoprivileged’ because T_{REGS} have been identified in discrete areas of the BM niche [394]. On the other side, it has been observed that inflammation, transplantations, bleeding, infection and chemotherapeutic agents stress the dormant HSC and induce it to proliferate and differentiate. Therefore, it is plausible that the immunomodulatory activity of MSC in the BM niche may be subject to inflammatory cues enabling them to regulate HSC responses to stress.

There is also evidence that the modalities by which MSC control inflammation might overlap with those regulating stem cell quiescence. Several metabolic pathways have been identified that can control T cell proliferation and that prominently involve the depletion of amino acids like tryptophan and arginine [378]. Similar mechanisms have been identified to regulate HSC self-renewal. Furthermore, m-TOR independent pathways, involving lipid and nucleotide metabolism, restrict HSC entry into cell cycle [395]. Dormant and self-renewing HSC reside in hypoxic niches, whereby they generate energy by maintaining a high rate of glycolysis, whereas the differentiated progeny has opposite metabolic needs. Finally, fibroblast growth factors have been extensively documented to act as metabolic regulators [396].

These considerations suggest the existence of common pathways between inflammation and tissue repair in which the interaction between tissue MSC and myeloid cells play a crucial role. Our observation that MSC can differentially drive the generation of myeloid cells with different functions depending on the environmental signals is therefore a step forward in understanding tissue homeostasis through inflammation.

It should be noted that a parallel can be drawn also between the stem cell and the tumour niche in which much more information about the interaction between inflammation and the cancer self-renewing process is available. For the vast amount of chemokines and cytokines released by tumour cells, tumour has indeed been defined as ‘a wound that never heals’. Tumour cells hijack normal tissue niches for their own survival by reconfiguring the host microenvironment. In solid tumours, cancer cells are embedded in a framework of tumour associated macrophages and fibroblasts that foster their growth and promote immune evasion by inducing an immunosuppressive environment. In the field of haematopoietic malignancies, it is also well established that leukaemia cells exploit the niche ability to maintain HSC quiescence to evade the anti-proliferative and anti-apoptotic effects of chemotherapeutic agents, thus accounting for disease relapse.

New tools and investigations are therefore required along the concept that inflammation is intrinsically linked to tissue repair. Our data on the ability of tissue MSC to drive the differentiation of circulating progenitors into tissue macrophages are possibly fundamental to produce vital information in stem cell biology and cancer treatment.

CHAPTER 7.

FUTURE PLANS

We have observed that MSC are able to induce the differentiation and expansion of three different subsets of myeloid cells. These observations may have profound implications for understanding the biology of the interaction between haematopoietic and mesenchymal stroma. However, the fundamental step is to investigate whether such phenomenon really occurs *in vivo*.

Since we have identified a number of molecules that appear to be selectively involved in the differentiation of CD11b⁺ cell subsets, we can address whether the absence of these molecules determines an impairment of myeloid subsets differentiation *in vivo*.

In order to do this, lethally irradiated CD45.2 WT recipients will receive a congenic CD45.1 BM transplantation and adoptive transfer of MSC from WT or knockout (Tnfr1/r2^{-/-}, C3^{-/-} and Musk-L;Agrn^{-/-}) mice. The relative abundance of the three different subsets induced by WT or knockout MSC will be evaluated in peripheral blood, BM and peripheral organs after 3, 7 and 10 days post-transplantation.

In order to understand whether the deficiency of NOS2 in the haematopoietic niche impairs not only the early reconstitution of macrophages but also the long term engraftment of HSPC, we will study the proportions of mononuclear phagocyte populations and their progenitors (HSC, CMP and GMP) in BM and spleen 10, 20 and 30 days post-transplantation of WT BM cells into clodronate-depleted and lethally irradiated Nos2^{-/-} mice.

Furthermore, we will investigate if and how the different proportion of macrophages found in the BM niche of WT and Nos2^{-/-} recipients modulates the mobilization and egress of HSPC under

physiological and inflammatory conditions (G-CSF induced mobilization or infection). The proportion of stem cells and neutrophils will be evaluated in peripheral blood and BM under such conditions.

In order to further unravel the mechanism involved in MSC-induced differentiation of myeloid cells, and to identify if there is a common pathway impairing the ability of *Nos2*^{-/-}, *Tnfr1/r2*^{-/-} and *Musk-L;Agrn*^{-/-} MSC to differentiate macrophages, we will perform a proteomic analysis on MSC isolated from WT and from knockout mice. If a discrete number of effector molecules arise, their precise involvement in MSC driven myeloid differentiation will be validated by means of the most appropriate technology, such as knockout models, RNA interfering or metabolic inhibitors.

CHAPTER 8.

REFERENCES

- [1] P.J. Murray, T.A. Wynn, Protective and pathogenic functions of macrophage subsets, *Nat Rev Immunol* 11 (2011) 723-737.
- [2] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, *Cytotherapy* 8 (2006) 315-317.
- [3] E.A. Luria, A.F. Panasyuk, A.Y. Friedenstein, Fibroblast colony formation from monolayer cultures of blood cells, *Transfusion* 11 (1971) 345-349.
- [4] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, *Science* 284 (1999) 143-147.
- [5] A. Erices, P. Conget, J.J. Minguell, Mesenchymal progenitor cells in human umbilical cord blood, *Br J Haematol* 109 (2000) 235-242.
- [6] A.C. Zannettino, S. Paton, A. Arthur, F. Khor, S. Itescu, J.M. Gimble, S. Gronthos, Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo, *J Cell Physiol* 214 (2008) 413-421.
- [7] K. Igura, X. Zhang, K. Takahashi, A. Mitsuru, S. Yamaguchi, T.A. Takashi, Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta, *Cytotherapy* 6 (2004) 543-553.
- [8] C. Campagnoli, I.A. Roberts, S. Kumar, P.R. Bennett, I. Bellantuono, N.M. Fisk, Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow, *Blood* 98 (2001) 2396-2402.
- [9] P.S. in 't Anker, W.A. Noort, S.A. Scherjon, C. Kleijburg-van der Keur, A.B. Kruisselbrink, R.L. van Bezooijen, W. Beekhuizen, R. Willemze, H.H. Kanhai, W.E. Fibbe, Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential, *Haematologica* 88 (2003) 845-852.
- [10] M. Reyes, A. Dudek, B. Jahagirdar, L. Koodie, P.H. Marker, C.M. Verfaillie, Origin of endothelial progenitors in human postnatal bone marrow, *J Clin Invest* 109 (2002) 337-346.

- [11] D. Woodbury, E.J. Schwarz, D.J. Prockop, I.B. Black, Adult rat and human bone marrow stromal cells differentiate into neurons, *J Neurosci Res* 61 (2000) 364-370.
- [12] Y. Sato, H. Araki, J. Kato, K. Nakamura, Y. Kawano, M. Kobune, T. Sato, K. Miyanishi, T. Takayama, M. Takahashi, R. Takimoto, S. Iyama, T. Matsunaga, S. Ohtani, A. Matsuura, H. Hamada, Y. Niitsu, Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion, *Blood* 106 (2005) 756-763.
- [13] M.A. Haniffa, X.N. Wang, U. Holtick, M. Rae, J.D. Isaacs, A.M. Dickinson, C.M. Hilkens, M.P. Collin, Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells, *J Immunol* 179 (2007) 1595-1604.
- [14] S. Jones, N. Horwood, A. Cope, F. Dazzi, The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells, *J Immunol* 179 (2007) 2824-2831.
- [15] M. Reyes, T. Lund, T. Lenvik, D. Aguiar, L. Koodie, C.M. Verfaillie, Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells, *Blood* 98 (2001) 2615-2625.
- [16] G. D'Ippolito, S. Diabira, G.A. Howard, P. Menei, B.A. Roos, P.C. Schiller, Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential, *J Cell Sci* 117 (2004) 2971-2981.
- [17] M. Kucia, R. Reca, F.R. Campbell, E. Zuba-Surma, M. Majka, J. Ratajczak, M.Z. Ratajczak, A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow, *Leukemia* 20 (2006) 857-869.
- [18] M. Serafini, S.J. Dylla, M. Oki, Y. Heremans, J. Tolar, Y. Jiang, S.M. Buckley, B. Pelacho, T.C. Burns, S. Frommer, D.J. Rossi, D. Bryder, A. Panoskaltsis-Mortari, M.J. O'Shaughnessy, M. Nelson-Holte, G.C. Fine, I.L. Weissman, B.R. Blazar, C.M. Verfaillie, Hematopoietic reconstitution by multipotent adult progenitor cells: precursors to long-term hematopoietic stem cells, *J Exp Med* 204 (2007) 129-139.
- [19] M.A. Vodyanik, J. Yu, X. Zhang, S. Tian, R. Stewart, J.A. Thomson, Slukvin, II, A mesoderm-derived precursor for mesenchymal stem and endothelial cells, *Cell Stem Cell* 7 (2010) 718-729.
- [20] M. Crisan, S. Yap, L. Casteilla, C.W. Chen, M. Corselli, T.S. Park, G. Andriolo, B. Sun, B. Zheng, L. Zhang, C. Norotte, P.N. Teng, J. Traas, R. Schugar, B.M. Deasy, S. Badyrak, H.J. Buhring, J.P. Giacobino, L. Lazzari, J. Huard, B. Peault, A perivascular origin for mesenchymal stem cells in multiple human organs, *Cell Stem Cell* 3 (2008) 301-313.
- [21] S. Shi, S. Gronthos, Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp, *J Bone Miner Res* 18 (2003) 696-704.
- [22] D.T. Covas, R.A. Panepucci, A.M. Fontes, W.A. Silva, Jr., M.D. Orellana, M.C. Freitas, L. Neder, A.R. Santos, L.C. Peres, M.C. Jamur, M.A. Zago, Multipotent mesenchymal stromal

- cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts, *Exp Hematol* 36 (2008) 642-654.
- [23] S. Bruno, B. Bussolati, C. Grange, F. Collino, L.V. di Cantogno, M.B. Herrera, L. Biancone, C. Tetta, G. Segoloni, G. Camussi, Isolation and characterization of resident mesenchymal stem cells in human glomeruli, *Stem Cells Dev* 18 (2009) 867-880.
 - [24] W. Tang, D. Zeve, J.M. Suh, D. Bosnakovski, M. Kyba, R.E. Hammer, M.D. Tallquist, J.M. Graff, White fat progenitor cells reside in the adipose vasculature, *Science* 322 (2008) 583-586.
 - [25] B. Sacchetti, A. Funari, S. Michienzi, S. Di Cesare, S. Piersanti, I. Saggio, E. Tagliafico, S. Ferrari, P.G. Robey, M. Riminucci, P. Bianco, Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment, *Cell* 131 (2007) 324-336.
 - [26] P.J. Simmons, B. Torok-Storb, Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1, *Blood* 78 (1991) 55-62.
 - [27] S. Gronthos, A.C. Zannettino, S.J. Hay, S. Shi, S.E. Graves, A. Kortesidis, P.J. Simmons, Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow, *J Cell Sci* 116 (2003) 1827-1835.
 - [28] N. Quirici, D. Soligo, P. Bossolasco, F. Servida, C. Lumini, G.L. Delilieri, Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies, *Exp Hematol* 30 (2002) 783-791.
 - [29] C. Martinez, T.J. Hofmann, R. Marino, M. Dominici, E.M. Horwitz, Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs, *Blood* 109 (2007) 4245-4248.
 - [30] Y. Takashima, T. Era, K. Nakao, S. Kondo, M. Kasuga, A.G. Smith, S. Nishikawa, Neuroepithelial cells supply an initial transient wave of MSC differentiation, *Cell* 129 (2007) 1377-1388.
 - [31] F. Anjos-Afonso, D. Bonnet, Nonhematopoietic/endothelial SSEA-1+ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment, *Blood* 109 (2007) 1298-1306.
 - [32] S. Morikawa, Y. Mabuchi, Y. Kubota, Y. Nagai, K. Niibe, E. Hiratsu, S. Suzuki, C. Miyauchi-Hara, N. Nagoshi, T. Sunabori, S. Shimmura, A. Miyawaki, T. Nakagawa, T. Suda, H. Okano, Y. Matsuzaki, Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow, *J Exp Med* 206 (2009) 2483-2496.
 - [33] A.J. Leo, D.A. Grande, Mesenchymal stem cells in tissue engineering, *Cells Tissues Organs* 183 (2006) 112-122.
 - [34] A.L. Ponte, E. Marais, N. Gallay, A. Langonne, B. Delorme, O. Herault, P. Charbord, J. Domenech, The in vitro migration capacity of human bone marrow mesenchymal stem cells:

- comparison of chemokine and growth factor chemotactic activities, *Stem Cells* 25 (2007) 1737-1745.
- [35] M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady, L. Rouso-Noori, A. Zanin-Zhorov, S. Cohen, I.R. Cohen, D. Zipori, Toll-like receptors and their ligands control mesenchymal stem cell functions, *Blood* 109 (2007) 1422-1432.
 - [36] M. Honczarenko, Y. Le, M. Swierkowski, I. Ghiran, A.M. Glodek, L.E. Silberstein, Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors, *Stem Cells* 24 (2006) 1030-1041.
 - [37] M.K. Majumdar, M. Keane-Moore, D. Buyaner, W.B. Hardy, M.A. Moorman, K.R. McIntosh, J.D. Mosca, Characterization and functionality of cell surface molecules on human mesenchymal stem cells, *J Biomed Sci* 10 (2003) 228-241.
 - [38] D. Docheva, C. Popov, W. Mutschler, M. Schieker, Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system, *J Cell Mol Med* 11 (2007) 21-38.
 - [39] J. Croitoru-Lamoury, F.M. Lamoury, J.J. Zaunders, L.A. Veas, B.J. Brew, Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone, *J Interferon Cytokine Res* 27 (2007) 53-64.
 - [40] V. Sordi, M.L. Malosio, F. Marchesi, A. Mercalli, R. Melzi, T. Giordano, N. Belmonte, G. Ferrari, B.E. Leone, F. Bertuzzi, G. Zerbini, P. Allavena, E. Bonifacio, L. Piemonti, Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets, *Blood* 106 (2005) 419-427.
 - [41] J.L. Curry, J.J. Trentin, Hemopoietic spleen colony studies. IV. Phytohemagglutinin and hemopoietic regeneration, *J Exp Med* 126 (1967) 819-832.
 - [42] R. Schofield, The relationship between the spleen colony-forming cell and the haemopoietic stem cell, *Blood Cells* 4 (1978) 7-25.
 - [43] A. Wilson, A. Trumpp, Bone-marrow haematopoietic-stem-cell niches, *Nat Rev Immunol* 6 (2006) 93-106.
 - [44] M.J. Kiel, S.J. Morrison, Uncertainty in the niches that maintain haematopoietic stem cells, *Nat Rev Immunol* 8 (2008) 290-301.
 - [45] H.M. Patt, M.A. Maloney, Bone formation and resorption as a requirement for marrow development, *Proc Soc Exp Biol Med* 140 (1972) 205-207.
 - [46] M.A. Maloney, H.M. Patt, On the origin of hematopoietic stem cells after local marrow extirpation, *Proc Soc Exp Biol Med* 149 (1975) 94-97.
 - [47] B.I. Lord, N.G. Testa, J.H. Hendry, The relative spatial distributions of CFUs and CFUc in the normal mouse femur, *Blood* 46 (1975) 65-72.

- [48] R.S. Taichman, S.G. Emerson, The role of osteoblasts in the hematopoietic microenvironment, *Stem Cells* 16 (1998) 7-15.
- [49] L.M. Calvi, G.B. Adams, K.W. Weibrecht, J.M. Weber, D.P. Olson, M.C. Knight, R.P. Martin, E. Schipani, P. Divieti, F.R. Bringhurst, L.A. Milner, H.M. Kronenberg, D.T. Scadden, Osteoblastic cells regulate the haematopoietic stem cell niche, *Nature* 425 (2003) 841-846.
- [50] J. Zhang, C. Niu, L. Ye, H. Huang, X. He, W.G. Tong, J. Ross, J. Haug, T. Johnson, J.Q. Feng, S. Harris, L.M. Wiedemann, Y. Mishina, L. Li, Identification of the haematopoietic stem cell niche and control of the niche size, *Nature* 425 (2003) 836-841.
- [51] Y. Xie, T. Yin, W. Wiegand, X.C. He, D. Miller, D. Stark, K. Perko, R. Alexander, J. Schwartz, J.C. Grindley, J. Park, J.S. Haug, J.P. Wunderlich, H. Li, S. Zhang, T. Johnson, R.A. Feldman, L. Li, Detection of functional haematopoietic stem cell niche using real-time imaging, *Nature* 457 (2009) 97-101.
- [52] S. Lympieri, N. Horwood, S. Marley, M.Y. Gordon, A.P. Cope, F. Dazzi, Strontium can increase some osteoblasts without increasing hematopoietic stem cells, *Blood* 111 (2008) 1173-1181.
- [53] S.T. Avezilla, K. Hattori, B. Heissig, R. Tejada, F. Liao, K. Shido, D.K. Jin, S. Dias, F. Zhang, T.E. Hartman, N.R. Hackett, R.G. Crystal, L. Witte, D.J. Hicklin, P. Bohlen, D. Eaton, D. Lyden, F. de Sauvage, S. Rafii, Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis, *Nat Med* 10 (2004) 64-71.
- [54] S. Rafii, F. Shapiro, R. Pettengell, B. Ferris, R.L. Nachman, M.A. Moore, A.S. Asch, Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors, *Blood* 86 (1995) 3353-3363.
- [55] P. Bianco, A. Boyde, Confocal images of marrow stromal (Westen-Bainton) cells, *Histochemistry* 100 (1993) 93-99.
- [56] M.J. Kiel, O.H. Yilmaz, T. Iwashita, C. Terhorst, S.J. Morrison, SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells, *Cell* 121 (2005) 1109-1121.
- [57] A.J. Friedenstein, R.K. Chailakhyan, N.V. Latsinik, A.F. Panasyuk, I.V. Keiliss-Borok, Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo, *Transplantation* 17 (1974) 331-340.
- [58] T. Sugiyama, H. Kohara, M. Noda, T. Nagasawa, Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches, *Immunity* 25 (2006) 977-988.

- [59] Y. Omatsu, T. Sugiyama, H. Kohara, G. Kondoh, N. Fujii, K. Kohno, T. Nagasawa, The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche, *Immunity* 33 (2010) 387-399.
- [60] S. Mendez-Ferrer, T.V. Michurina, F. Ferraro, A.R. Mazloom, B.D. Macarthur, S.A. Lira, D.T. Scadden, A. Ma'ayan, G.N. Enikolopov, P.S. Frenette, Mesenchymal and haematopoietic stem cells form a unique bone marrow niche, *Nature* 466 (2010) 829-834.
- [61] F.E. Mercier, C. Ragu, D.T. Scadden, The bone marrow at the crossroads of blood and immunity, *Nat Rev Immunol* 12 (2012) 49-60.
- [62] J. Isern, S. Mendez-Ferrer, Stem cell interactions in a bone marrow niche, *Curr Osteoporos Rep* 9 (2011) 210-218.
- [63] W.A. Noort, A.B. Kruisselbrink, P.S. in't Anker, M. Kruger, R.L. van Bezooijen, R.A. de Paus, M.H. Heemskerk, C.W. Lowik, J.H. Falkenburg, R. Willemze, W.E. Fibbe, Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice, *Exp Hematol* 30 (2002) 870-878.
- [64] Y. Zhang, Y. Adachi, Y. Suzuki, K. Minamino, M. Iwasaki, H. Hisha, C.Y. Song, K. Kusafuka, K. Nakano, Y. Koike, J. Wang, E. Koh, Y. Cui, C. Li, S. Ikehara, Simultaneous injection of bone marrow cells and stromal cells into bone marrow accelerates hematopoiesis in vivo, *Stem Cells* 22 (2004) 1256-1262.
- [65] K. Sugiura, H. Hisha, J. Ishikawa, Y. Adachi, S. Taketani, S. Lee, T. Nagahama, S. Ikehara, Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vitro, *Stem Cells* 19 (2001) 46-58.
- [66] M.E. Bernardo, A.M. Cometa, F. Locatelli, Mesenchymal stromal cells: a novel and effective strategy for facilitating engraftment and accelerating hematopoietic recovery after transplantation?, *Bone Marrow Transplant* 47 (2012) 323-329.
- [67] O.N. Koc, S.L. Gerson, B.W. Cooper, S.M. Dyhouse, S.E. Haynesworth, A.I. Caplan, H.M. Lazarus, Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy, *J Clin Oncol* 18 (2000) 307-316.
- [68] H.M. Lazarus, O.N. Koc, S.M. Devine, P. Curtin, R.T. Maziarz, H.K. Holland, E.J. Shpall, P. McCarthy, K. Atkinson, B.W. Cooper, S.L. Gerson, M.J. Laughlin, F.R. Loberiza, Jr., A.B. Moseley, A. Bacigalupo, Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients, *Biol Blood Marrow Transplant* 11 (2005) 389-398.
- [69] K. Rieger, O. Marinets, T. Fietz, S. Korper, D. Sommer, C. Mucke, B. Reufi, W.I. Blau, E. Thiel, W.U. Knauf, Mesenchymal stem cells remain of host origin even a long time after allogeneic peripheral blood stem cell or bone marrow transplantation, *Exp Hematol* 33 (2005) 605-611.

- [70] M. Di Nicola, C. Carlo-Stella, M. Magni, M. Milanesi, P.D. Longoni, P. Matteucci, S. Grisanti, A.M. Gianni, Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli, *Blood* 99 (2002) 3838-3843.
- [71] K. Le Blanc, L. Tammik, B. Sundberg, S.E. Haynesworth, O. Ringden, Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex, *Scand J Immunol* 57 (2003) 11-20.
- [72] J.A. Potian, H. Aviv, N.M. Ponzio, J.S. Harrison, P. Rameshwar, Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens, *J Immunol* 171 (2003) 3426-3434.
- [73] W.T. Tse, J.D. Pendleton, W.M. Beyer, M.C. Egalka, E.C. Guinan, Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation, *Transplantation* 75 (2003) 389-397.
- [74] A. Bartholomew, C. Sturgeon, M. Siatskas, K. Ferrer, K. McIntosh, S. Patil, W. Hardy, S. Devine, D. Ucker, R. Deans, A. Moseley, R. Hoffman, Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo, *Exp Hematol* 30 (2002) 42-48.
- [75] F. Djouad, P. Ponce, C. Bony, P. Tropel, F. Apparailly, J. Sany, D. Noel, C. Jorgensen, Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals, *Blood* 102 (2003) 3837-3844.
- [76] M. Krampera, S. Glennie, J. Dyson, D. Scott, R. Laylor, E. Simpson, F. Dazzi, Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide, *Blood* 101 (2003) 3722-3729.
- [77] K. Le Blanc, I. Rasmusson, C. Gotherstrom, C. Seidel, B. Sundberg, M. Sundin, K. Rosendahl, C. Tammik, O. Ringden, Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohemagglutinin-activated lymphocytes, *Scand J Immunol* 60 (2004) 307-315.
- [78] B. Maitra, E. Szekely, K. Gjini, M.J. Laughlin, J. Dennis, S.E. Haynesworth, O.N. Koc, Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation, *Bone Marrow Transplant* 33 (2004) 597-604.
- [79] S. Aggarwal, M.F. Pittenger, Human mesenchymal stem cells modulate allogeneic immune cell responses, *Blood* 105 (2005) 1815-1822.
- [80] R. Maccario, M. Podesta, A. Moretta, A. Cometa, P. Comoli, D. Montagna, L. Daudt, A. Ibatici, G. Piaggio, S. Pozzi, F. Frassoni, F. Locatelli, Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4⁺ T-cell subsets expressing a regulatory/suppressive phenotype, *Haematologica* 90 (2005) 516-525.

- [81] I. Rasmusson, O. Ringden, B. Sundberg, K. Le Blanc, Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells, *Transplantation* 76 (2003) 1208-1213.
- [82] E. Klyushnenkova, J.D. Mosca, V. Zernetkina, M.K. Majumdar, K.J. Beggs, D.W. Simonetti, R.J. Deans, K.R. McIntosh, T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression, *J Biomed Sci* 12 (2005) 47-57.
- [83] K. Le Blanc, C. Tammik, K. Rosendahl, E. Zetterberg, O. Ringden, HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells, *Exp Hematol* 31 (2003) 890-896.
- [84] S. Glennie, I. Soeiro, P.J. Dyson, E.W. Lam, F. Dazzi, Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells, *Blood* 105 (2005) 2821-2827.
- [85] J. Plumas, L. Chaperot, M.J. Richard, J.P. Molens, J.C. Bensa, M.C. Favrot, Mesenchymal stem cells induce apoptosis of activated T cells, *Leukemia* 19 (2005) 1597-1604.
- [86] E. Zappia, S. Casazza, E. Pedemonte, F. Benvenuto, I. Bonanni, E. Gerdoni, D. Giunti, A. Ceravolo, F. Cazzanti, F. Frassoni, G. Mancardi, A. Uccelli, Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy, *Blood* 106 (2005) 1755-1761.
- [87] A. Augello, R. Tasso, S.M. Negrini, A. Amateis, F. Indiveri, R. Cancedda, G. Pennesi, Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway, *Eur J Immunol* 35 (2005) 1482-1490.
- [88] W. Deng, Q. Han, L. Liao, S. You, H. Deng, R.C. Zhao, Effects of allogeneic bone marrow-derived mesenchymal stem cells on T and B lymphocytes from BXSb mice, *DNA Cell Biol* 24 (2005) 458-463.
- [89] A. Corcione, F. Benvenuto, E. Ferretti, D. Giunti, V. Cappiello, F. Cazzanti, M. Risso, F. Gualandi, G.L. Mancardi, V. Pistoia, A. Uccelli, Human mesenchymal stem cells modulate B-cell functions, *Blood* 107 (2006) 367-372.
- [90] E. Gerdoni, B. Gallo, S. Casazza, S. Musio, I. Bonanni, E. Pedemonte, R. Mantegazza, F. Frassoni, G. Mancardi, R. Pedotti, A. Uccelli, Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis, *Ann Neurol* 61 (2007) 219-227.
- [91] E. Traggiai, S. Volpi, F. Schena, M. Gattorno, F. Ferlito, L. Moretta, A. Martini, Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients, *Stem Cells* 26 (2008) 562-569.
- [92] P.A. Sotiropoulou, S.A. Perez, A.D. Gritzapis, C.N. Baxevanis, M. Papamichail, Interactions between human mesenchymal stem cells and natural killer cells, *Stem Cells* 24 (2006) 74-85.

- [93] G.M. Spaggiari, A. Capobianco, S. Becchetti, M.C. Mingari, L. Moretta, Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation, *Blood* 107 (2006) 1484-1490.
- [94] M. Krampera, L. Cosmi, R. Angeli, A. Pasini, F. Liotta, A. Andreini, V. Santarlasci, B. Mazzinghi, G. Pizzolo, F. Vinante, P. Romagnani, E. Maggi, S. Romagnani, F. Annunziato, Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells, *Stem Cells* 24 (2006) 386-398.
- [95] X.X. Jiang, Y. Zhang, B. Liu, S.X. Zhang, Y. Wu, X.D. Yu, N. Mao, Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells, *Blood* 105 (2005) 4120-4126.
- [96] R. Ramasamy, H. Fazekasova, E.W. Lam, I. Soeiro, G. Lombardi, F. Dazzi, Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle, *Transplantation* 83 (2007) 71-76.
- [97] Y.P. Li, S. Paczesny, E. Lauret, S. Poirault, P. Bordigoni, F. Mekhloufi, O. Hequet, Y. Bertrand, J.P. Ou-Yang, J.F. Stoltz, P. Miossec, A. Eljaafari, Human mesenchymal stem cells license adult CD34⁺ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway, *J Immunol* 180 (2008) 1598-1608.
- [98] A.J. Nauta, A.B. Kruisselbrink, E. Lurvink, R. Willemze, W.E. Fibbe, Mesenchymal stem cells inhibit generation and function of both CD34⁺-derived and monocyte-derived dendritic cells, *J Immunol* 177 (2006) 2080-2087.
- [99] S. Beyth, Z. Borovsky, D. Mevorach, M. Liebergall, Z. Gazit, H. Aslan, E. Galun, J. Rachmilewitz, Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness, *Blood* 105 (2005) 2214-2219.
- [100] A. Aldinucci, L. Rizzetto, L. Pieri, D. Nosi, P. Romagnoli, T. Biagioli, B. Mazzanti, R. Saccardi, L. Beltrame, L. Massacesi, D. Cavalieri, C. Ballerini, Inhibition of immune synapse by altered dendritic cell actin distribution: a new pathway of mesenchymal stem cell immune regulation, *J Immunol* 185 (2010) 5102-5110.
- [101] S. Chiesa, S. Morbelli, S. Morando, M. Massollo, C. Marini, A. Bertoni, F. Frassoni, S.T. Bartolome, G. Sambuceti, E. Traggiai, A. Uccelli, Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells, *Proc Natl Acad Sci U S A* 108 (2011) 17384-17389.
- [102] C. Shi, T. Jia, S. Mendez-Ferrer, T.M. Hohl, N.V. Serbina, L. Lipuma, I. Leiner, M.O. Li, P.S. Frenette, E.G. Pamer, Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands, *Immunity* 34 (2011) 590-601.

- [103] L. Chen, E.E. Tredget, P.Y. Wu, Y. Wu, Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing, *PLoS One* 3 (2008) e1886.
- [104] J. Kim, P. Hematti, Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages, *Exp Hematol* 37 (2009) 1445-1453.
- [105] J. Maggini, G. Mirkin, I. Bognanni, J. Holmberg, I.M. Piazzon, I. Nepomnaschy, H. Costa, C. Canones, S. Raiden, M. Vermeulen, J.R. Geffner, Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile, *PLoS One* 5 (2010) e9252.
- [106] M. Francois, R. Romieu-Mourez, M. Li, J. Galipeau, Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation, *Mol Ther* 20 (2012) 187-195.
- [107] K. Nemeth, A. Leelahavanichkul, P.S. Yuen, B. Mayer, A. Parmelee, K. Doi, P.G. Robey, K. Leelahavanichkul, B.H. Koller, J.M. Brown, X. Hu, I. Jelinek, R.A. Star, E. Mezey, Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production, *Nat Med* 15 (2009) 42-49.
- [108] N. Gupta, X. Su, B. Popov, J.W. Lee, V. Serikov, M.A. Matthay, Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice, *J Immunol* 179 (2007) 1855-1863.
- [109] H. Choi, R.H. Lee, N. Bazhanov, J.Y. Oh, D.J. Prockop, Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-kappaB signaling in resident macrophages, *Blood* 118 (2011) 330-338.
- [110] L.A. Ortiz, M. Dutreil, C. Fattman, A.C. Pandey, G. Torres, K. Go, D.G. Phinney, Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury, *Proc Natl Acad Sci U S A* 104 (2007) 11002-11007.
- [111] L. Raffaghello, G. Bianchi, M. Bertolotto, F. Montecucco, A. Busca, F. Dallegri, L. Ottonello, V. Pistoia, Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche, *Stem Cells* 26 (2008) 151-162.
- [112] M.A. Cassatella, F. Mosna, A. Micheletti, V. Lisi, N. Tamassia, C. Cont, F. Calzetti, M. Pelletier, G. Pizzolo, M. Krampera, Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils, *Stem Cells* 29 (2011) 1001-1011.
- [113] S. Brandau, M. Jakob, H. Hemeda, K. Bruderek, S. Janeschik, F. Bootz, S. Lang, Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge, *J Leukoc Biol* 88 (2010) 1005-1015.

- [114] R. Romieu-Mourez, M. Francois, M.N. Boivin, M. Bouchentouf, D.E. Spaner, J. Galipeau, Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype, *J Immunol* 182 (2009) 7963-7973.
- [115] R. Meisel, A. Zibert, M. Laryea, U. Gobel, W. Daubener, D. Dilloo, Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation, *Blood* 103 (2004) 4619-4621.
- [116] K. Sato, K. Ozaki, I. Oh, A. Meguro, K. Hatanaka, T. Nagai, K. Muroi, K. Ozawa, Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells, *Blood* 109 (2007) 228-234.
- [117] D. Chabannes, M. Hill, E. Merieau, J. Rossignol, R. Brion, J.P. Soulillou, I. Anegon, M.C. Cuturi, A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells, *Blood* 110 (2007) 3691-3694.
- [118] F. Gieseke, B. Schutt, S. Viebahn, E. Koscielniak, W. Friedrich, R. Handgretinger, I. Muller, Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFN γ signaling and IDO expression, *Blood* 110 (2007) 2197-2200.
- [119] P. Hwu, M.X. Du, R. Lapointe, M. Do, M.W. Taylor, H.A. Young, Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation, *J Immunol* 164 (2000) 3596-3599.
- [120] D.H. Munn, M. Zhou, J.T. Attwood, I. Bondarev, S.J. Conway, B. Marshall, C. Brown, A.L. Mellor, Prevention of allogeneic fetal rejection by tryptophan catabolism, *Science* 281 (1998) 1191-1193.
- [121] G. Frumento, R. Rotondo, M. Tonetti, G. Damonte, U. Benatti, G.B. Ferrara, Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase, *J Exp Med* 196 (2002) 459-468.
- [122] G. Ren, L. Zhang, X. Zhao, G. Xu, Y. Zhang, A.I. Roberts, R.C. Zhao, Y. Shi, Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide, *Cell Stem Cell* 2 (2008) 141-150.
- [123] R.M. Bingisser, P.A. Tilbrook, P.G. Holt, U.R. Kees, Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway, *J Immunol* 160 (1998) 5729-5734.
- [124] I. Rasmusson, O. Ringden, B. Sundberg, K. Le Blanc, Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms, *Exp Cell Res* 305 (2005) 33-41.
- [125] K. Tashiro, H. Tada, R. Heilker, M. Shirozu, T. Nakano, T. Honjo, Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins, *Science* 261 (1993) 600-603.

- [126] M.C. Poznansky, I.T. Olszak, R. Foxall, R.H. Evans, A.D. Luster, D.T. Scadden, Active movement of T cells away from a chemokine, *Nat Med* 6 (2000) 543-548.
- [127] J.L. Chan, K.C. Tang, A.P. Patel, L.M. Bonilla, N. Pierobon, N.M. Ponzio, P. Rameshwar, Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma, *Blood* 107 (2006) 4817-4824.
- [128] J. Stagg, S. Pommey, N. Eliopoulos, J. Galipeau, Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell, *Blood* 107 (2006) 2570-2577.
- [129] H. Hwa Cho, Y.C. Bae, J.S. Jung, Role of toll-like receptors on human adipose-derived stromal cells, *Stem Cells* 24 (2006) 2744-2752.
- [130] G. Raicevic, R. Rouas, M. Najar, P. Stordeur, H.I. Boufker, D. Bron, P. Martiat, M. Goldman, M.T. Nevessignsky, L. Lagneaux, Inflammation modifies the pattern and the function of Toll-like receptors expressed by human mesenchymal stromal cells, *Hum Immunol* 71 (2010) 235-244.
- [131] Z.J. Wang, F.M. Zhang, L.S. Wang, Y.W. Yao, Q. Zhao, X. Gao, Lipopolysaccharides can protect mesenchymal stem cells (MSCs) from oxidative stress-induced apoptosis and enhance proliferation of MSCs via Toll-like receptor(TLR)-4 and PI3K/Akt, *Cell Biol Int* 33 (2009) 665-674.
- [132] E. Lombardo, O. DelaRosa, P. Mancheno-Corvo, R. Menta, C. Ramirez, D. Buscher, Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential, *Tissue Eng Part A* 15 (2009) 1579-1589.
- [133] S.L. Tomchuck, K.J. Zvezdaryk, S.B. Coffelt, R.S. Waterman, E.S. Danka, A.B. Scandurro, Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses, *Stem Cells* 26 (2008) 99-107.
- [134] M. Sioud, A. Mobergslien, A. Boudabous, Y. Floisand, Evidence for the involvement of galectin-3 in mesenchymal stem cell suppression of allogeneic T-cell proliferation, *Scand J Immunol* 71 (2010) 267-274.
- [135] R.S. Waterman, S.L. Tomchuck, S.L. Henkle, A.M. Betancourt, A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype, *PLoS One* 5 (2010) e10088.
- [136] A.J. Nauta, G. Westerhuis, A.B. Kruisselbrink, E.G. Lurvink, R. Willemze, W.E. Fibbe, Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting, *Blood* 108 (2006) 2114-2120.
- [137] S. Itakura, S. Asari, J. Rawson, T. Ito, I. Todorov, C.P. Liu, N. Sasaki, F. Kandeel, Y. Mullen, Mesenchymal stem cells facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in the rat, *Am J Transplant* 7 (2007) 336-346.

- [138] F. Djouad, V. Fritz, F. Apparailly, P. Louis-Plence, C. Bony, J. Sany, C. Jorgensen, D. Noel, Reversal of the immunosuppressive properties of mesenchymal stem cells by tumor necrosis factor alpha in collagen-induced arthritis, *Arthritis Rheum* 52 (2005) 1595-1603.
- [139] A. Augello, R. Tasso, S.M. Negrini, R. Cancedda, G. Pennesi, Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis, *Arthritis Rheum* 56 (2007) 1175-1186.
- [140] E. Gonzalez-Rey, P. Anderson, M.A. Gonzalez, L. Rico, D. Buscher, M. Delgado, Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis, *Gut* 58 (2009) 929-939.
- [141] R. Ramasamy, E.W. Lam, I. Soeiro, V. Tisato, D. Bonnet, F. Dazzi, Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth, *Leukemia* 21 (2007) 304-310.
- [142] R.H. Lee, M.J. Seo, R.L. Reger, J.L. Spees, A.A. Pulin, S.D. Olson, D.J. Prockop, Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice, *Proc Natl Acad Sci U S A* 103 (2006) 17438-17443.
- [143] V.S. Urban, J. Kiss, J. Kovacs, E. Gocza, V. Vas, E. Monostori, F. Uher, Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes, *Stem Cells* 26 (2008) 244-253.
- [144] F. Togel, Z. Hu, K. Weiss, J. Isaac, C. Lange, C. Westenfelder, Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms, *Am J Physiol Renal Physiol* 289 (2005) F31-42.
- [145] D. Ruffell, F. Mourkioti, A. Gambardella, P. Kirstetter, R.G. Lopez, N. Rosenthal, C. Nerlov, A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair, *Proc Natl Acad Sci U S A* 106 (2009) 17475-17480.
- [146] S. Meirelles Lda, A.M. Fontes, D.T. Covas, A.I. Caplan, Mechanisms involved in the therapeutic properties of mesenchymal stem cells, *Cytokine Growth Factor Rev* 20 (2009) 419-427.
- [147] K. Le Blanc, I. Rasmusson, B. Sundberg, C. Gotherstrom, M. Hassan, M. Uzunel, O. Ringden, Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells, *Lancet* 363 (2004) 1439-1441.
- [148] K. Le Blanc, F. Frassoni, L. Ball, F. Locatelli, H. Roelofs, I. Lewis, E. Lanino, B. Sundberg, M.E. Bernardo, M. Remberger, G. Dini, R.M. Egeler, A. Bacigalupo, W. Fibbe, O. Ringden, Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study, *Lancet* 371 (2008) 1579-1586.
- [149] M. Sudres, F. Norol, A. Trenado, S. Gregoire, F. Charlotte, B. Levacher, J.J. Lataillade, P. Bourin, X. Holy, J.P. Vernant, D. Klatzmann, J.L. Cohen, Bone marrow mesenchymal stem

- cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice, *J Immunol* 176 (2006) 7761-7767.
- [150] V. Tisato, K. Naresh, J. Girdlestone, C. Navarrete, F. Dazzi, Mesenchymal stem cells of cord blood origin are effective at preventing but not treating graft-versus-host disease, *Leukemia* 21 (2007) 1992-1999.
 - [151] D. Polchert, J. Sobinsky, G. Douglas, M. Kidd, A. Moadsiri, E. Reina, K. Genrich, S. Mehrotra, S. Setty, B. Smith, A. Bartholomew, IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease, *Eur J Immunol* 38 (2008) 1745-1755.
 - [152] L. Marinova-Mutafchieva, R.O. Williams, K. Funa, R.N. Maini, N.J. Zvaifler, Inflammation is preceded by tumor necrosis factor-dependent infiltration of mesenchymal cells in experimental arthritis, *Arthritis Rheum* 46 (2002) 507-513.
 - [153] F.M. Marelli-Berg, M.J. James, J. Dangerfield, J. Dyson, M. Millrain, D. Scott, E. Simpson, S. Nourshargh, R.I. Lechler, Cognate recognition of the endothelium induces HY-specific CD8⁺ T-lymphocyte transendothelial migration (diapedesis) in vivo, *Blood* 103 (2004) 3111-3116.
 - [154] F. Dazzi, F.M. Marelli-Berg, Mesenchymal stem cells for graft-versus-host disease: close encounters with T cells, *Eur J Immunol* 38 (2008) 1479-1482.
 - [155] G.J. Spangrude, S. Heimfeld, I.L. Weissman, Purification and characterization of mouse hematopoietic stem cells, *Science* 241 (1988) 58-62.
 - [156] D. Metcalf, N. Di Nicola, The hemopoietic colony-stimulating factors, Cambridge University Press, New York, 1995.
 - [157] S.J. Morrison, I.L. Weissman, The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype, *Immunity* 1 (1994) 661-673.
 - [158] M. Osawa, K. Hanada, H. Hamada, H. Nakauchi, Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell, *Science* 273 (1996) 242-245.
 - [159] S.K. Bhatia, L.T. Tygrett, K.H. Grabstein, T.J. Waldschmidt, The effect of in vivo IL-7 deprivation on T cell maturation, *J Exp Med* 181 (1995) 1399-1409.
 - [160] K. Akashi, D. Traver, T. Miyamoto, I.L. Weissman, A clonogenic common myeloid progenitor that gives rise to all myeloid lineages, *Nature* 404 (2000) 193-197.
 - [161] T.N. Nakorn, T. Miyamoto, I.L. Weissman, Characterization of mouse clonogenic megakaryocyte progenitors, *Proc Natl Acad Sci U S A* 100 (2003) 205-210.
 - [162] C.J. Pronk, D.J. Rossi, R. Månsson, J.L. Attema, G.L. Norddahl, C.K. Chan, M. Sigvardsson, I.L. Weissman, D. Bryder, Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy, *Cell Stem Cell* 1 (2007) 428-442.

- [163] C. Auffray, M.H. Sieweke, F. Geissmann, Blood monocytes: development, heterogeneity, and relationship with dendritic cells, *Annu Rev Immunol* 27 (2009) 669-692.
- [164] D.K. Fogg, C. Sibon, C. Miled, S. Jung, P. Aucouturier, D.R. Littman, A. Cumano, F. Geissmann, A clonogenic bone marrow progenitor specific for macrophages and dendritic cells, *Science* 311 (2006) 83-87.
- [165] C. Varol, L. Landsman, D.K. Fogg, L. Greenshtein, B. Gildor, R. Margalit, V. Kalchenko, F. Geissmann, S. Jung, Monocytes give rise to mucosal, but not splenic, conventional dendritic cells, *J Exp Med* 204 (2007) 171-180.
- [166] S.H. Naik, P. Sathe, H.Y. Park, D. Metcalf, A.I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J.Y. Kwak, L. Wu, K. Shortman, Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo, *Nat Immunol* 8 (2007) 1217-1226.
- [167] S.I. Kim, E.H. Bresnick, Transcriptional control of erythropoiesis: emerging mechanisms and principles, *Oncogene* 26 (2007) 6777-6794.
- [168] S.H. Orkin, Diversification of haematopoietic stem cells to specific lineages, *Nat Rev Genet* 1 (2000) 57-64.
- [169] K. Kumano, S. Chiba, A. Kunisato, M. Sata, T. Saito, E. Nakagami-Yamaguchi, T. Yamaguchi, S. Masuda, K. Shimizu, T. Takahashi, S. Ogawa, Y. Hamada, H. Hirai, Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells, *Immunity* 18 (2003) 699-711.
- [170] A.V. Krivtsov, D. Twomey, Z. Feng, M.C. Stubbs, Y. Wang, J. Faber, J.E. Levine, J. Wang, W.C. Hahn, D.G. Gilliland, T.R. Golub, S.A. Armstrong, Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9, *Nature* 442 (2006) 818-822.
- [171] C. Nerlov, T. Graf, PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors, *Genes Dev* 12 (1998) 2403-2412.
- [172] H. Iwasaki, C. Somoza, H. Shigematsu, E.A. Duprez, J. Iwasaki-Arai, S. Mizuno, Y. Arinobu, K. Geary, P. Zhang, T. Dayaram, M.L. Fenyus, S. Elf, S. Chan, P. Kastner, C.S. Huettner, R. Murray, D.G. Tenen, K. Akashi, Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation, *Blood* 106 (2005) 1590-1600.
- [173] J.C. Walsh, R.P. DeKoter, H.J. Lee, E.D. Smith, D.W. Lancki, M.F. Gurish, D.S. Friend, R.L. Stevens, J. Anastasi, H. Singh, Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates, *Immunity* 17 (2002) 665-676.
- [174] R. Dahl, J.C. Walsh, D. Lancki, P. Laslo, S.R. Iyer, H. Singh, M.C. Simon, Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor, *Nat Immunol* 4 (2003) 1029-1036.

- [175] P. Laslo, C.J. Spooner, A. Warmflash, D.W. Lancki, H.J. Lee, R. Sciammas, B.N. Gantner, A.R. Dinner, H. Singh, Multilineage transcriptional priming and determination of alternate hematopoietic cell fates, *Cell* 126 (2006) 755-766.
- [176] T. Tamura, T. Nagamura-Inoue, Z. Shmeltzer, T. Kuwata, K. Ozato, ICSBP directs bipotential myeloid progenitor cells to differentiate into mature macrophages, *Immunity* 13 (2000) 155-165.
- [177] M.W. Feinberg, A.K. Wara, Z. Cao, M.A. Lebedeva, F. Rosenbauer, H. Iwasaki, H. Hirai, J.P. Katz, R.L. Haspel, S. Gray, K. Akashi, J. Segre, K.H. Kaestner, D.G. Tenen, M.K. Jain, The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation, *EMBO J* 26 (2007) 4138-4148.
- [178] W. Wiktor-Jedrzejczak, S. Gordon, Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse, *Physiol Rev* 76 (1996) 927-947.
- [179] K.P. MacDonald, V. Rowe, H.M. Bofinger, R. Thomas, T. Sasmono, D.A. Hume, G.R. Hill, The colony-stimulating factor 1 receptor is expressed on dendritic cells during differentiation and regulates their expansion, *J Immunol* 175 (2005) 1399-1405.
- [180] M.A. Reddy, B.S. Yang, X. Yue, C.J. Barnett, I.L. Ross, M.J. Sweet, D.A. Hume, M.C. Ostrowski, Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes, *J Exp Med* 180 (1994) 2309-2319.
- [181] R.P. DeKoter, J.C. Walsh, H. Singh, PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors, *EMBO J* 17 (1998) 4456-4468.
- [182] Y. Bakri, S. Sarrazin, U.P. Mayer, S. Tillmanns, C. Nerlov, A. Boned, M.H. Sieweke, Balance of MafB and PU.1 specifies alternative macrophage or dendritic cell fate, *Blood* 105 (2005) 2707-2716.
- [183] S. Jung, J. Aliberti, P. Graemmel, M.J. Sunshine, G.W. Kreutzberg, A. Sher, D.R. Littman, Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion, *Mol Cell Biol* 20 (2000) 4106-4114.
- [184] F. Geissmann, S. Jung, D.R. Littman, Blood monocytes consist of two principal subsets with distinct migratory properties, *Immunity* 19 (2003) 71-82.
- [185] F. Geissmann, C. Auffray, R. Palframan, C. Wirrig, A. Ciocca, L. Campisi, E. Narni-Mancinelli, G. Lauvau, Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses, *Immunol Cell Biol* 86 (2008) 398-408.
- [186] C. Sunderkötter, T. Nikolic, M.J. Dillon, N. Van Rooijen, M. Stehling, D.A. Drevets, P.J. Leenen, Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response, *J Immunol* 172 (2004) 4410-4417.

- [187] U. Yrliid, C.D. Jenkins, G.G. MacPherson, Relationships between distinct blood monocyte subsets and migrating intestinal lymph dendritic cells in vivo under steady-state conditions, *J Immunol* 176 (2006) 4155-4162.
- [188] J.C. Scatizzi, J. Hutcheson, E. Bickel, J.M. Woods, K. Klosowska, T.L. Moore, G.K. Haines, H. Perlman, p21Cip1 is required for the development of monocytes and their response to serum transfer-induced arthritis, *Am J Pathol* 168 (2006) 1531-1541.
- [189] J.K. Alder, R.W. Georgantas, R.L. Hildreth, I.M. Kaplan, S. Morisot, X. Yu, M. McDevitt, C.I. Civin, Kruppel-like factor 4 is essential for inflammatory monocyte differentiation in vivo, *J Immunol* 180 (2008) 5645-5652.
- [190] C. Qu, E.W. Edwards, F. Tacke, V. Angeli, J. Llodrá, G. Sanchez-Schmitz, A. Garin, N.S. Haque, W. Peters, N. van Rooijen, C. Sanchez-Torres, J. Bromberg, I.F. Charo, S. Jung, S.A. Lira, G.J. Randolph, Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes, *J Exp Med* 200 (2004) 1231-1241.
- [191] M. Nahrendorf, F.K. Swirski, E. Aikawa, L. Stangenberg, T. Wurdinger, J.L. Figueiredo, P. Libby, R. Weissleder, M.J. Pittet, The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions, *J Exp Med* 204 (2007) 3037-3047.
- [192] N.V. Serbina, T. Jia, T.M. Hohl, E.G. Pamer, Monocyte-mediated defense against microbial pathogens, *Annu Rev Immunol* 26 (2008) 421-452.
- [193] R.T. Palframan, S. Jung, G. Cheng, W. Weninger, Y. Luo, M. Dorf, D.R. Littman, B.J. Rollins, H. Zweerink, A. Rot, U.H. von Andrian, Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues, *J Exp Med* 194 (2001) 1361-1373.
- [194] N.V. Serbina, W. Kuziel, R. Flavell, S. Akira, B. Rollins, E.G. Pamer, Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection, *Immunity* 19 (2003) 891-901.
- [195] N.V. Serbina, T.P. Salazar-Mather, C.A. Biron, W.A. Kuziel, E.G. Pamer, TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection, *Immunity* 19 (2003) 59-70.
- [196] N.V. Serbina, E.G. Pamer, Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2, *Nat Immunol* 7 (2006) 311-317.
- [197] C.L. Tsou, W. Peters, Y. Si, S. Slaymaker, A.M. Aslanian, S.P. Weisberg, M. Mack, I.F. Charo, Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites, *J Clin Invest* 117 (2007) 902-909.
- [198] F. Ginhoux, F. Tacke, V. Angeli, M. Bogunovic, M. Loubreau, X.M. Dai, E.R. Stanley, G.J. Randolph, M. Merad, Langerhans cells arise from monocytes in vivo, *Nat Immunol* 7 (2006) 265-273.

- [199] L. Landsman, C. Varol, S. Jung, Distinct differentiation potential of blood monocyte subsets in the lung, *J Immunol* 178 (2007) 2000-2007.
- [200] C. Jakubzick, F. Tacke, F. Ginhoux, A.J. Wagers, N. van Rooijen, M. Mack, M. Merad, G.J. Randolph, Blood monocyte subsets differentially give rise to CD103⁺ and CD103⁻ pulmonary dendritic cell populations, *J Immunol* 180 (2008) 3019-3027.
- [201] K. Movahedi, M. Guillemins, J. Van den Bossche, R. Van den Bergh, C. Gysemans, A. Beschinn, P. De Baetselier, J.A. Van Ginderachter, Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity, *Blood* 111 (2008) 4233-4244.
- [202] B. Huang, P.Y. Pan, Q. Li, A.I. Sato, D.E. Levy, J. Bromberg, C.M. Divino, S.H. Chen, Gr-1⁺CD115⁺ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host, *Cancer Res* 66 (2006) 1123-1131.
- [203] V. Bronte, E. Apolloni, A. Cabrelle, R. Ronca, P. Serafini, P. Zamboni, N.P. Restifo, P. Zanovello, Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells, *Blood* 96 (2000) 3838-3846.
- [204] S. Kusmartsev, Y. Nefedova, D. Yoder, D.I. Gabrilovich, Antigen-specific inhibition of CD8⁺ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species, *J Immunol* 172 (2004) 989-999.
- [205] C. Auffray, D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, F. Geissmann, Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior, *Science* 317 (2007) 666-670.
- [206] F.O. Martinez, S. Gordon, M. Locati, A. Mantovani, Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression, *J Immunol* 177 (2006) 7303-7311.
- [207] P.R. Taylor, S. Gordon, Monocyte heterogeneity and innate immunity, *Immunity* 19 (2003) 2-4.
- [208] F. Geissmann, C. Prost, J.P. Monnet, M. Dy, N. Brousse, O. Hermine, Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells, *J Exp Med* 187 (1998) 961-966.
- [209] F.O. Martinez, A. Sica, A. Mantovani, M. Locati, Macrophage activation and polarization, *Front Biosci* 13 (2008) 453-461.
- [210] F.O. Martinez, L. Helming, S. Gordon, Alternative activation of macrophages: an immunologic functional perspective, *Annu Rev Immunol* 27 (2009) 451-483.
- [211] L.E. Smythies, M. Sellers, R.H. Clements, M. Mosteller-Barnum, G. Meng, W.H. Benjamin, J.M. Orenstein, P.D. Smith, Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity, *J Clin Invest* 115 (2005) 66-75.

- [212] K. Asano, A. Nabeyama, Y. Miyake, C.H. Qiu, A. Kurita, M. Tomura, O. Kanagawa, S. Fujii, M. Tanaka, CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens, *Immunity* 34 (2011) 85-95.
- [213] S. Gordon, Alternative activation of macrophages, *Nat Rev Immunol* 3 (2003) 23-35.
- [214] F.S. Sutterwala, G.J. Noel, P. Salgame, D.M. Mosser, Reversal of proinflammatory responses by ligating the macrophage Fcgamma receptor type I, *J Exp Med* 188 (1998) 217-222.
- [215] J.J. O'Shea, P.J. Murray, Cytokine signaling modules in inflammatory responses, *Immunity* 28 (2008) 477-487.
- [216] D.C. Dale, L. Boxer, W.C. Liles, The phagocytes: neutrophils and monocytes, *Blood* 112 (2008) 935-945.
- [217] Z. Szekanecz, A.E. Koch, Macrophages and their products in rheumatoid arthritis, *Curr Opin Rheumatol* 19 (2007) 289-295.
- [218] X. Zhang, D.M. Mosser, Macrophage activation by endogenous danger signals, *J Pathol* 214 (2008) 161-178.
- [219] K. Ogawa, M. Funaba, Y. Chen, M. Tsujimoto, Activin A functions as a Th2 cytokine in the promotion of the alternative activation of macrophages, *J Immunol* 177 (2006) 6787-6794.
- [220] J. Pesce, M. Kaviratne, T.R. Ramalingam, R.W. Thompson, J.F. Urban, A.W. Cheever, D.A. Young, M. Collins, M.J. Grusby, T.A. Wynn, The IL-21 receptor augments Th2 effector function and alternative macrophage activation, *J Clin Invest* 116 (2006) 2044-2055.
- [221] W. Noël, G. Raes, G. Hassanzadeh Ghassabeh, P. De Baetselier, A. Beschin, Alternatively activated macrophages during parasite infections, *Trends Parasitol* 20 (2004) 126-133.
- [222] T.A. Wynn, Fibrotic disease and the T(H)1/T(H)2 paradigm, *Nat Rev Immunol* 4 (2004) 583-594.
- [223] L. Barron, T.A. Wynn, Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages, *Am J Physiol Gastrointest Liver Physiol* 300 (2011) G723-728.
- [224] D.R. Herbert, T. Orekov, A. Roloson, M. Ilies, C. Perkins, W. O'Brien, S. Cederbaum, D.W. Christianson, N. Zimmermann, M.E. Rothenberg, F.D. Finkelman, Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis, *J Immunol* 184 (2010) 6438-6446.
- [225] J.T. Pesce, T.R. Ramalingam, M.S. Wilson, M.M. Mentink-Kane, R.W. Thompson, A.W. Cheever, J.F. Urban, T.A. Wynn, Retnla (relmalph/fizz1) suppresses helminth-induced Th2-type immunity, *PLoS Pathog* 5 (2009) e1000393.
- [226] L. Arnold, A. Henry, F. Poron, Y. Baba-Amer, N. van Rooijen, A. Plonquet, R.K. Gherardi, B. Chazaud, Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis, *J Exp Med* 204 (2007) 1057-1069.

- [227] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, *Trends Immunol* 25 (2004) 677-686.
- [228] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, *Nat Rev Immunol* 8 (2008) 958-969.
- [229] G. Natoli, Maintaining cell identity through global control of genomic organization, *Immunity* 33 (2010) 12-24.
- [230] J.E. Darnell, I.M. Kerr, G.R. Stark, Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins, *Science* 264 (1994) 1415-1421.
- [231] V. Toshchakov, B.W. Jones, P.Y. Perera, K. Thomas, M.J. Cody, S. Zhang, B.R. Williams, J. Major, T.A. Hamilton, M.J. Fenton, S.N. Vogel, TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages, *Nat Immunol* 3 (2002) 392-398.
- [232] K. Takeda, T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, S. Akira, Essential role of Stat6 in IL-4 signalling, *Nature* 380 (1996) 627-630.
- [233] M. Ricote, A.C. Li, T.M. Willson, C.J. Kelly, C.K. Glass, The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation, *Nature* 391 (1998) 79-82.
- [234] A. Szanto, B.L. Balint, Z.S. Nagy, E. Barta, B. Dezso, A. Pap, L. Szeles, S. Poliska, M. Oros, R.M. Evans, Y. Barak, J. Schwabe, L. Nagy, STAT6 transcription factor is a facilitator of the nuclear receptor PPAR γ -regulated gene expression in macrophages and dendritic cells, *Immunity* 33 (2010) 699-712.
- [235] K.C. El Kasmi, J.E. Qualls, J.T. Pesce, A.M. Smith, R.W. Thompson, M. Henao-Tamayo, R.J. Basaraba, T. König, U. Schleicher, M.S. Koo, G. Kaplan, K.A. Fitzgerald, E.I. Tuomanen, I.M. Orme, T.D. Kanneganti, C. Bogdan, T.A. Wynn, P.J. Murray, Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens, *Nat Immunol* 9 (2008) 1399-1406.
- [236] T. Bosschaerts, M. Guillemins, B. Stijlemans, Y. Morias, D. Engel, F. Tacke, M. Hérin, P. De Baetselier, A. Beschin, Tip-DC development during parasitic infection is regulated by IL-10 and requires CCL2/CCR2, IFN-gamma and MyD88 signaling, *PLoS Pathog* 6 (2010) e1001045.
- [237] S.J. Jenkins, D. Ruckerl, P.C. Cook, L.H. Jones, F.D. Finkelman, N. van Rooijen, A.S. MacDonald, J.E. Allen, Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation, *Science* 332 (2011) 1284-1288.
- [238] A. Sica, V. Bronte, Altered macrophage differentiation and immune dysfunction in tumor development, *J Clin Invest* 117 (2007) 1155-1166.

- [239] C. Murdoch, M. Muthana, S.B. Coffelt, C.E. Lewis, The role of myeloid cells in the promotion of tumour angiogenesis, *Nat Rev Cancer* 8 (2008) 618-631.
- [240] E.Y. Lin, J.F. Li, L. Gnatovskiy, Y. Deng, L. Zhu, D.A. Grzesik, H. Qian, X.N. Xue, J.W. Pollard, Macrophages regulate the angiogenic switch in a mouse model of breast cancer, *Cancer Res* 66 (2006) 11238-11246.
- [241] B. Qian, Y. Deng, J.H. Im, R.J. Muschel, Y. Zou, J. Li, R.A. Lang, J.W. Pollard, A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth, *PLoS One* 4 (2009) e6562.
- [242] Y. Zheng, Z. Cai, S. Wang, X. Zhang, J. Qian, S. Hong, H. Li, M. Wang, J. Yang, Q. Yi, Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis, *Blood* 114 (2009) 3625-3628.
- [243] A. Mantovani, A. Sica, P. Allavena, C. Garlanda, M. Locati, Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation, *Hum Immunol* 70 (2009) 325-330.
- [244] D.M. Kuang, Q. Zhao, C. Peng, J. Xu, J.P. Zhang, C. Wu, L. Zheng, Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1, *J Exp Med* 206 (2009) 1327-1337.
- [245] P.C. Rodriguez, D.G. Quiceno, J. Zabaleta, B. Ortiz, A.H. Zea, M.B. Piazuelo, A. Delgado, P. Correa, J. Brayer, E.M. Sotomayor, S. Antonia, J.B. Ochoa, A.C. Ochoa, Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses, *Cancer Res* 64 (2004) 5839-5849.
- [246] D.I. Gabrilovich, V. Bronte, S.H. Chen, M.P. Colombo, A. Ochoa, S. Ostrand-Rosenberg, H. Schreiber, The terminology issue for myeloid-derived suppressor cells, *Cancer Res* 67 (2007) 425; author reply 426.
- [247] D.I. Gabrilovich, S. Nagaraj, Myeloid-derived suppressor cells as regulators of the immune system, *Nat Rev Immunol* 9 (2009) 162-174.
- [248] E. Peranzoni, S. Zilio, I. Marigo, L. Dolcetti, P. Zanovello, S. Mandruzzato, V. Bronte, Myeloid-derived suppressor cell heterogeneity and subset definition, *Curr Opin Immunol* 22 (2010) 238-244.
- [249] L.A. Haile, J. Gamrekelashvili, M.P. Manns, F. Korangy, T.F. Greten, CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice, *J Immunol* 185 (2010) 203-210.
- [250] L. Dolcetti, E. Peranzoni, S. Ugel, I. Marigo, A. Fernandez Gomez, C. Mesa, M. Geilich, G. Winkels, E. Traggiai, A. Casati, F. Grassi, V. Bronte, Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF, *Eur J Immunol* 40 (2010) 22-35.

- [251] J.I. Youn, M. Collazo, I.N. Shalova, S.K. Biswas, D.I. Gabrilovich, Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice, *J Leukoc Biol* 91 (2012) 167-181.
- [252] V. Bronte, P. Zanovello, Regulation of immune responses by L-arginine metabolism, *Nat Rev Immunol* 5 (2005) 641-654.
- [253] Y. Narita, D. Wakita, T. Ohkur, K. Chamoto, T. Nishimura, Potential differentiation of tumor bearing mouse CD11b+Gr-1+ immature myeloid cells into both suppressor macrophages and immunostimulatory dendritic cells, *Biomed Res* 30 (2009) 7-15.
- [254] C.A. Corzo, T. Condamine, L. Lu, M.J. Cotter, J.I. Youn, P. Cheng, H.I. Cho, E. Celis, D.G. Quiceno, T. Padhya, T.V. McCaffrey, J.C. McCaffrey, D.I. Gabrilovich, HIF-1 α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment, *J Exp Med* 207 (2010) 2439-2453.
- [255] A. Mazzoni, V. Bronte, A. Visintin, J.H. Spitzer, E. Apolloni, P. Serafini, P. Zanovello, D.M. Segal, Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism, *J Immunol* 168 (2002) 689-695.
- [256] E.M. Hanson, V.K. Clements, P. Sinha, D. Ilkovitch, S. Ostrand-Rosenberg, Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells, *J Immunol* 183 (2009) 937-944.
- [257] P.Y. Pan, G. Ma, K.J. Weber, J. Ozao-Choy, G. Wang, B. Yin, C.M. Divino, S.H. Chen, Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer, *Cancer Res* 70 (2010) 99-108.
- [258] G. Gallina, L. Dolcetti, P. Serafini, C. De Santo, I. Marigo, M.P. Colombo, G. Basso, F. Brombacher, I. Borrello, P. Zanovello, S. Bicchato, V. Bronte, Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells, *J Clin Invest* 116 (2006) 2777-2790.
- [259] I. Marigo, E. Bosio, S. Solito, C. Mesa, A. Fernandez, L. Dolcetti, S. Ugel, N. Sonda, S. Bicchato, E. Falisi, F. Calabrese, G. Basso, P. Zanovello, E. Cozzi, S. Mandruzzato, V. Bronte, Tumor-induced tolerance and immune suppression depend on the C/EBP β transcription factor, *Immunity* 32 (2010) 790-802.
- [260] A. Ludin, T. Itkin, S. Gur-Cohen, A. Mildner, E. Shezen, K. Golan, O. Kollet, A. Kalinkovich, Z. Porat, G. D'Uva, A. Schajnovitz, E. Voronov, D.A. Brenner, R.N. Apte, S. Jung, T. Lapidot, Monocytes-macrophages that express α -smooth muscle actin preserve primitive hematopoietic cells in the bone marrow, *Nat Immunol* 13 (2012) 1072-1082.
- [261] I.G. Winkler, N.A. Sims, A.R. Pettit, V. Barbier, B. Nowlan, F. Helwani, I.J. Poulton, N. van Rooijen, K.A. Alexander, L.J. Raggatt, J.P. Lévesque, Bone marrow macrophages maintain

- hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs, *Blood* 116 (2010) 4815-4828.
- [262] A. Chow, D. Lucas, A. Hidalgo, S. Méndez-Ferrer, D. Hashimoto, C. Scheiermann, M. Battista, M. Leboeuf, C. Prophete, N. van Rooijen, M. Tanaka, M. Merad, P.S. Frenette, Bone marrow CD169⁺ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche, *J Exp Med* 208 (2011) 261-271.
 - [263] M.J. Christopher, M. Rao, F. Liu, J.R. Woloszynek, D.C. Link, Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice, *J Exp Med* 208 (2011) 251-260.
 - [264] P. Singh, P. Hu, J. Hoggatt, A. Moh, L.M. Pelus, Expansion of bone marrow neutrophils following G-CSF administration in mice results in osteolineage cell apoptosis and mobilization of hematopoietic stem and progenitor cells, *Leukemia* 26 (2012) 2375-2383.
 - [265] A. Mansour, G. Abou-Ezzi, E. Sitnicka, S.E. Jacobsen, A. Wakkach, C. Blin-Wakkach, Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow, *J Exp Med* 209 (2012) 537-549.
 - [266] S. Lymperi, A. Ersek, F. Ferraro, F. Dazzi, N.J. Horwood, Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo, *Blood* 117 (2011) 1540-1549.
 - [267] O. Kollet, A. Dar, S. Shivtiel, A. Kalinkovich, K. Lapid, Y. Sztainberg, M. Tesio, R.M. Samstein, P. Goichberg, A. Spiegel, A. Elson, T. Lapidot, Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells, *Nat Med* 12 (2006) 657-664.
 - [268] E. Peranzoni, I. Marigo, L. Dolcetti, S. Ugel, N. Sonda, E. Taschin, B. Mantelli, V. Bronte, P. Zanovello, Role of arginine metabolism in immunity and immunopathology, *Immunobiology* 212 (2007) 795-812.
 - [269] C. Bogdan, Nitric oxide and the immune response, *Nat Immunol* 2 (2001) 907-916.
 - [270] J. Shinoda, K.E. McLaughlin, H.S. Bell, G.R. Swaroop, S. Yamaguchi, M.C. Holmes, I.R. Whittle, Molecular mechanisms underlying dexamethasone inhibition of iNOS expression and activity in C6 glioma cells, *Glia* 42 (2003) 68-76.
 - [271] D.A. Wink, Y. Vodovotz, J. Laval, F. Laval, M.W. Dewhirst, J.B. Mitchell, The multifaceted roles of nitric oxide in cancer, *Carcinogenesis* 19 (1998) 711-721.
 - [272] S. Ambs, W.P. Bennett, W.G. Merriam, M.O. Ogunfusika, S.M. Oser, M.A. Khan, R.T. Jones, C.C. Harris, Vascular endothelial growth factor and nitric oxide synthase expression in human lung cancer and the relation to p53, *Br J Cancer* 78 (1998) 233-239.
 - [273] B. Hinz, K. Brune, A. Pahl, Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages, *Biochem Biophys Res Commun* 271 (2000) 353-357.

- [274] Q.W. Xie, Y. Kashiwabara, C. Nathan, Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase, *J Biol Chem* 269 (1994) 4705-4708.
- [275] G. Melillo, T. Musso, A. Sica, L.S. Taylor, G.W. Cox, L. Varesio, A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter, *J Exp Med* 182 (1995) 1683-1693.
- [276] R.W. Ganster, B.S. Taylor, L. Shao, D.A. Geller, Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa B, *Proc Natl Acad Sci U S A* 98 (2001) 8638-8643.
- [277] A. Pautz, J. Art, S. Hahn, S. Nowag, C. Voss, H. Kleinert, Regulation of the expression of inducible nitric oxide synthase, *Nitric Oxide* 23 (2010) 75-93.
- [278] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, *Am J Physiol* 271 (1996) C1424-1437.
- [279] N.S. Bryan, K. Bian, F. Murad, Discovery of the nitric oxide signaling pathway and targets for drug development, *Front Biosci* 14 (2009) 1-18.
- [280] F. Murad, Shattuck Lecture. Nitric oxide and cyclic GMP in cell signaling and drug development, *N Engl J Med* 355 (2006) 2003-2011.
- [281] L. Leon, J.F. Jeannin, A. Bettaieb, Post-translational modifications induced by nitric oxide (NO): implication in cancer cells apoptosis, *Nitric Oxide* 19 (2008) 77-83.
- [282] S. Singh, A.K. Gupta, Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies, *Cancer Chemother Pharmacol* 67 (2011) 1211-1224.
- [283] J. MacMicking, Q.W. Xie, C. Nathan, Nitric oxide and macrophage function, *Annu Rev Immunol* 15 (1997) 323-350.
- [284] C. Bogdan, M. Rollinghoff, A. Diefenbach, Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity, *Curr Opin Immunol* 12 (2000) 64-76.
- [285] S. Moncada, R.M. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, *Pharmacol Rev* 43 (1991) 109-142.
- [286] D.I. Gabrilovich, S. Ostrand-Rosenberg, V. Bronte, Coordinated regulation of myeloid cells by tumours, *Nat Rev Immunol* 12 (2012) 253-268.
- [287] J.B. Weinberg, Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review, *Mol Med* 4 (1998) 557-591.
- [288] H. Kolb, V. Kolb-Bachofen, Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator?, *Immunol Today* 19 (1998) 556-561.
- [289] T.A. Fischer, A. Palmethofer, S. Gambaryan, E. Butt, C. Jassoy, U. Walter, S. Sopfer, S.M. Lohmann, Activation of cGMP-dependent protein kinase I β inhibits interleukin 2 release and proliferation of T cell receptor-stimulated human peripheral T cells, *J Biol Chem* 276 (2001) 5967-5974.

- [290] R.J. Duhe, G.A. Evans, R.A. Erwin, R.A. Kirken, G.W. Cox, W.L. Farrar, Nitric oxide and thiol redox regulation of Janus kinase activity, *Proc Natl Acad Sci U S A* 95 (1998) 126-131.
- [291] R. Bingisser, P. Tilbrook, P. Holt, U. Kees, Macrophage-derived nitric oxide regulates T-cell activation via reversible disruption of the Jak3/STAT5 signaling pathway., *J. Immunol.* 160 (1998) 5729-5734.
- [292] J.P. Jayasekera, C.G. Vinuesa, G. Karupiah, N.J. King, Enhanced antiviral antibody secretion and attenuated immunopathology during influenza virus infection in nitric oxide synthase-2-deficient mice, *J Gen Virol* 87 (2006) 3361-3371.
- [293] A. Diefenbach, H. Schindler, M. Rollinghoff, W.M. Yokoyama, C. Bogdan, Requirement for type 2 NO synthase for IL-12 signaling in innate immunity, *Science* 284 (1999) 951-955.
- [294] W. Niedbala, X.Q. Wei, D. Piedrafita, D. Xu, F.Y. Liew, Effects of nitric oxide on the induction and differentiation of Th1 cells, *Eur J Immunol* 29 (1999) 2498-2505.
- [295] H. Bauer, T. Jung, D. Tsikas, D.O. Stichtenoth, J.C. Frolich, C. Neumann, Nitric oxide inhibits the secretion of T-helper 1- and T-helper 2-associated cytokines in activated human T cells, *Immunology* 90 (1997) 205-211.
- [296] P. Vincendeau, A.P. Gobert, S. Daulouede, D. Moynet, M. Djavad Mossalayi, Arginases in parasitic diseases, *Trends Parasitol* 19 (2003) 9-12.
- [297] L.I. Terrazas, K.L. Walsh, D. Piskorska, E. McGuire, D.A. Harn, Jr., The schistosome oligosaccharide lacto-N-neotetraose expands Gr1(+) cells that secrete anti-inflammatory cytokines and inhibit proliferation of naive CD4(+) cells: a potential mechanism for immune polarization in helminth infections, *J Immunol* 167 (2001) 5294-5303.
- [298] O. Goni, P. Alcaide, M. Fresno, Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G (Gr1(+))CD11b(+) immature myeloid suppressor cells, *Int Immunol* 14 (2002) 1125-1134.
- [299] A. Mencacci, C. Montagnoli, A. Bacci, E. Cenci, L. Pitzurra, A. Spreca, M. Kopf, A. Sharpe, L. Romani, CD80+Gr-1+ myeloid cells inhibit development of antifungal Th1 immunity in mice with candidiasis, *J Immunol* 169 (2002) 3180-3190.
- [300] B. Ahn, H. Ohshima, Suppression of intestinal polyposis in *Apc(Min/+)* mice by inhibiting nitric oxide production, *Cancer Res* 61 (2001) 8357-8360.
- [301] K.T. Nam, S.Y. Oh, B. Ahn, Y.B. Kim, D.D. Jang, K.H. Yang, K.B. Hahm, D.Y. Kim, Decreased *Helicobacter pylori* associated gastric carcinogenesis in mice lacking inducible nitric oxide synthase, *Gut* 53 (2004) 1250-1255.
- [302] W.C. Sessa, eNOS at a glance, *J Cell Sci* 117 (2004) 2427-2429.
- [303] B. Brune, J. Zhou, The role of nitric oxide (NO) in stability regulation of hypoxia inducible factor-1alpha (HIF-1alpha), *Curr Med Chem* 10 (2003) 845-855.
- [304] C.A. Corzo, T. Condamine, L. Lu, M.J. Cotter, J.I. Youn, P. Cheng, H.I. Cho, E. Celis, D.G. Quiceno, T. Padhya, T.V. McCaffrey, J.C. McCaffrey, D.I. Gabrilovich, HIF-1alpha regulates

- function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment, *J Exp Med* 207 (2010) 2439-2453.
- [305] D.J. Scott, M.A. Hull, E.J. Cartwright, W.K. Lam, A. Tisbury, R. Poulson, A.F. Markham, C. Bonifer, P.L. Coletta, Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the Apc(Min/+) mouse, *Gastroenterology* 121 (2001) 889-899.
 - [306] D. Wei, E.L. Richardson, K. Zhu, L. Wang, X. Le, Y. He, S. Huang, K. Xie, Direct demonstration of negative regulation of tumor growth and metastasis by host-inducible nitric oxide synthase, *Cancer Res* 63 (2003) 3855-3859.
 - [307] T.E. Konopka, J.E. Barker, T.L. Bamford, E. Guida, R.L. Anderson, A.G. Stewart, Nitric oxide synthase II gene disruption: implications for tumor growth and vascular endothelial growth factor production, *Cancer Res* 61 (2001) 3182-3187.
 - [308] K.M. Fries, T. Blieden, R.J. Looney, G.D. Sempowski, M.R. Silvera, R.A. Willis, R.P. Phipps, Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis, *Clin Immunol Immunopathol* 72 (1994) 283-292.
 - [309] R.E. Mebius, Organogenesis of lymphoid tissues, *Nat Rev Immunol* 3 (2003) 292-303.
 - [310] G. Parsonage, A.D. Filer, O. Haworth, G.B. Nash, G.E. Rainger, M. Salmon, C.D. Buckley, A stromal address code defined by fibroblasts, *Trends Immunol* 26 (2005) 150-156.
 - [311] C.N. Serhan, S.D. Brain, C.D. Buckley, D.W. Gilroy, C. Haslett, L.A. O'Neill, M. Perretti, A.G. Rossi, J.L. Wallace, Resolution of inflammation: state of the art, definitions and terms, *FASEB J* 21 (2007) 325-332.
 - [312] R.A. Reilkoff, R. Bucala, E.L. Herzog, Fibrocytes: emerging effector cells in chronic inflammation, *Nat Rev Immunol* 11 (2011) 427-435.
 - [313] R. Abe, S.C. Donnelly, T. Peng, R. Bucala, C.N. Metz, Peripheral blood fibrocytes: differentiation pathway and migration to wound sites, *J Immunol* 166 (2001) 7556-7562.
 - [314] R. Bucala, L.A. Spiegel, J. Chesney, M. Hogan, A. Cerami, Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair, *Mol Med* 1 (1994) 71-81.
 - [315] S. Lorenzini, T.G. Bird, L. Boulter, C. Bellamy, K. Samuel, R. Aucott, E. Clayton, P. Andreone, M. Bernardi, M. Golding, M.R. Alison, J.P. Iredale, S.J. Forbes, Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver, *Gut* 59 (2010) 645-654.
 - [316] R. Fischer, A. Cariers, R. Reinehr, D. Häussinger, Caspase 9-dependent killing of hepatic stellate cells by activated Kupffer cells, *Gastroenterology* 123 (2002) 845-861.
 - [317] J.S. Duffield, S.J. Forbes, C.M. Constandinou, S. Clay, M. Partolina, S. Vuthoori, S. Wu, R. Lang, J.P. Iredale, Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair, *J Clin Invest* 115 (2005) 56-65.

- [318] J.S. Duffield, P.G. Tipping, T. Kipari, J.F. Cailhier, S. Clay, R. Lang, J.V. Bonventre, J. Hughes, Conditional ablation of macrophages halts progression of crescentic glomerulonephritis, *Am J Pathol* 167 (2005) 1207-1219.
- [319] S.L. Pull, J.M. Doherty, J.C. Mills, J.I. Gordon, T.S. Stappenbeck, Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury, *Proc Natl Acad Sci U S A* 102 (2005) 99-104.
- [320] P. Schaerli, K. Willmann, L.M. Ebert, A. Walz, B. Moser, Cutaneous CXCL14 targets blood precursors to epidermal niches for Langerhans cell differentiation, *Immunity* 23 (2005) 331-342.
- [321] J.W. Kim, C. Evans, A. Weidemann, N. Takeda, Y.S. Lee, C. Stockmann, C. Branco-Price, F. Brandberg, G. Leone, M.C. Ostrowski, R.S. Johnson, Loss of fibroblast HIF-1 α accelerates tumorigenesis, *Cancer Res* 72 (2012) 3187-3195.
- [322] D. Gao, N. Joshi, H. Choi, S. Ryu, M. Hahn, R. Catena, H. Sadik, P. Argani, P. Wagner, L.T. Vahdat, J.L. Port, B. Stiles, S. Sukumar, N.K. Altorki, S. Rafii, V. Mittal, Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition, *Cancer Res* 72 (2012) 1384-1394.
- [323] F. Guilloton, G. Caron, C. Ménard, C. Pangault, P. Amé-Thomas, J. Dulong, J. De Vos, D. Rossille, C. Henry, T. Lamy, O. Fouquet, T. Fest, K. Tarte, Mesenchymal stromal cells orchestrate follicular lymphoma cell niche through the CCL2-dependent recruitment and polarization of monocytes, *Blood* 119 (2012) 2556-2567.
- [324] F. Arai, A. Hirao, M. Ohmura, H. Sato, S. Matsuoka, K. Takubo, K. Ito, G.Y. Koh, T. Suda, Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche, *Cell* 118 (2004) 149-161.
- [325] Y. Katayama, M. Battista, W.M. Kao, A. Hidalgo, A.J. Peired, S.A. Thomas, P.S. Frenette, Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow, *Cell* 124 (2006) 407-421.
- [326] A. Spiegel, S. Shivtiel, A. Kalinkovich, A. Ludin, N. Netzer, P. Goichberg, Y. Azaria, I. Resnick, I. Hardan, H. Ben-Hur, A. Nagler, M. Rubinstein, T. Lapidot, Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34⁺ cells through Wnt signaling, *Nat Immunol* 8 (2007) 1123-1131.
- [327] I. Visigalli, S. Ungari, S. Martino, H. Park, M. Cesani, B. Gentner, L. Sergi Sergi, A. Orlicchio, L. Naldini, A. Biffi, The galactocerebrosidase enzyme contributes to the maintenance of a functional hematopoietic stem cell niche, *Blood* 116 (2010) 1857-1866.
- [328] K. Golan, Y. Vagima, A. Ludin, T. Itkin, S. Cohen-Gur, A. Kalinkovich, O. Kollet, C. Kim, A. Schajnovitz, Y. Ovadya, K. Lapid, S. Shivtiel, A.J. Morris, M.Z. Ratajczak, T. Lapidot, S1P promotes murine progenitor cell egress and mobilization via S1P1-mediated ROS signaling and SDF-1 release, *Blood* 119 (2012) 2478-2488.

- [329] J. Hoggatt, P. Singh, J. Sampath, L.M. Pelus, Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation, *Blood* 113 (2009) 5444-5455.
- [330] E. Bruno, S.D. Luikart, M.W. Long, R. Hoffman, Marrow-derived heparan sulfate proteoglycan mediates the adhesion of hematopoietic progenitor cells to cytokines, *Exp Hematol* 23 (1995) 1212-1217.
- [331] M. Siczkowski, D. Clarke, M.Y. Gordon, Binding of primitive hematopoietic progenitor cells to marrow stromal cells involves heparan sulfate, *Blood* 80 (1992) 912-919.
- [332] C. Mazzon, A. Anselmo, J. Cibella, C. Soldani, A. Destro, N. Kim, M. Roncalli, S.J. Burden, M.L. Dustin, A. Sarukhan, A. Viola, The critical role of agrin in the hematopoietic stem cell niche, *Blood* 118 (2011) 2733-2742.
- [333] N. Kim, A.L. Stiegler, T.O. Cameron, P.T. Hallock, A.M. Gomez, J.H. Huang, S.R. Hubbard, M.L. Dustin, S.J. Burden, Lrp4 is a receptor for Agrin and forms a complex with MuSK, *Cell* 135 (2008) 334-342.
- [334] J. Zhang, Y. Wang, Y. Chu, L. Su, Y. Gong, R. Zhang, S. Xiong, Agrin is involved in lymphocytes activation that is mediated by alpha-dystroglycan, *FASEB J* 20 (2006) 50-58.
- [335] R.A. Williamson, M.D. Henry, K.J. Daniels, R.F. Hrstka, J.C. Lee, Y. Sunada, O. Ibraghimov-Beskrovnaya, K.P. Campbell, Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice, *Hum Mol Genet* 6 (1997) 831-841.
- [336] C. Mazzon, A. Anselmo, C. Soldani, J. Cibella, C. Ploia, F. Moalli, S.J. Burden, M.L. Dustin, A. Sarukhan, A. Viola, Agrin is required for survival and function of monocytic cells, *Blood* 119 (2012) 5502-5511.
- [337] Y. Ueda, D.W. Cain, M. Kuraoka, M. Kondo, G. Kelsoe, IL-1R type I-dependent hemopoietic stem cell proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia, *J Immunol* 182 (2009) 6477-6484.
- [338] D.J. Rossi, J. Seita, A. Czechowicz, D. Bhattacharya, D. Bryder, I.L. Weissman, Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging, *Cell Cycle* 6 (2007) 2371-2376.
- [339] H. Takizawa, R.R. Regoes, C.S. Boddupalli, S. Bonhoeffer, M.G. Manz, Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation, *J Exp Med* 208 (2011) 273-284.
- [340] Y. Nagai, K.P. Garrett, S. Ohta, U. Bahrn, T. Kouro, S. Akira, K. Takatsu, P.W. Kincade, Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment, *Immunity* 24 (2006) 801-812.
- [341] X. Zhao, G. Ren, L. Liang, P.Z. Ai, B. Zheng, J.A. Tischfield, Y. Shi, C. Shao, Brief report: interferon-gamma induces expansion of Lin(-)Sca-1(+)C-Kit(+) Cells, *Stem Cells* 28 (2010) 122-126.

- [342] M.A. Essers, S. Offner, W.E. Blanco-Bose, Z. Waibler, U. Kalinke, M.A. Duchosal, A. Trumpp, IFN α activates dormant haematopoietic stem cells in vivo, *Nature* 458 (2009) 904-908.
- [343] T. Sato, N. Onai, H. Yoshihara, F. Arai, T. Suda, T. Ohteki, Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion, *Nat Med* 15 (2009) 696-700.
- [344] C.J. Pronk, O.P. Veiby, D. Bryder, S.E. Jacobsen, Tumor necrosis factor restricts hematopoietic stem cell activity in mice: involvement of two distinct receptors, *J Exp Med* 208 (2011) 1563-1570.
- [345] M.T. Baldrige, K.Y. King, N.C. Boles, D.C. Weksberg, M.A. Goodell, Quiescent haematopoietic stem cells are activated by IFN- γ in response to chronic infection, *Nature* 465 (2010) 793-797.
- [346] V.I. Rebel, S. Hartnett, G.R. Hill, S.B. Lazo-Kallanian, J.L. Ferrara, C.A. Sieff, Essential role for the p55 tumor necrosis factor receptor in regulating hematopoiesis at a stem cell level, *J Exp Med* 190 (1999) 1493-1504.
- [347] F. Rezzoug, Y. Huang, M.K. Tanner, M. Wysoczynski, C.L. Schanie, P.M. Chilton, M.Z. Ratajczak, I.J. Fugier-Vivier, S.T. Ildstad, TNF- α is critical to facilitate hemopoietic stem cell engraftment and function, *J Immunol* 180 (2008) 49-57.
- [348] S. Hayashi, T. Yamada, M. Tsuneto, T. Yamane, M. Takahashi, L.D. Shultz, H. Yamazaki, Distinct osteoclast precursors in the bone marrow and extramedullary organs characterized by responsiveness to Toll-like receptor ligands and TNF- α , *J Immunol* 171 (2003) 5130-5139.
- [349] N. Sato, N. Takahashi, K. Suda, M. Nakamura, M. Yamaki, T. Ninomiya, Y. Kobayashi, H. Takada, K. Shibata, M. Yamamoto, K. Takeda, S. Akira, T. Noguchi, N. Udagawa, MyD88 but not TRIF is essential for osteoclastogenesis induced by lipopolysaccharide, diacyl lipopeptide, and IL-1 α , *J Exp Med* 200 (2004) 601-611.
- [350] D. Ricklin, G. Hajishengallis, K. Yang, J.D. Lambris, Complement: a key system for immune surveillance and homeostasis, *Nat Immunol* 11 (2010) 785-797.
- [351] H.M. Lee, W. Wu, M. Wysoczynski, R. Liu, E.K. Zuba-Surma, M. Kucia, J. Ratajczak, M.Z. Ratajczak, Impaired mobilization of hematopoietic stem/progenitor cells in C5-deficient mice supports the pivotal involvement of innate immunity in this process and reveals novel promobilization effects of granulocytes, *Leukemia* 23 (2009) 2052-2062.
- [352] J.P. Lévesque, J. Hendy, Y. Takamatsu, P.J. Simmons, L.J. Bendall, Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by G-CSF or cyclophosphamide, *J Clin Invest* 111 (2003) 187-196.
- [353] H.M. Lee, M. Wysoczynski, R. Liu, D.M. Shin, M. Kucia, M. Botto, J. Ratajczak, M.Z. Ratajczak, Mobilization studies in complement-deficient mice reveal that optimal AMD3100

- mobilization of hematopoietic stem cells depends on complement cascade activation by AMD3100-stimulated granulocytes, *Leukemia* 24 (2010) 573-582.
- [354] M.Z. Ratajczak, R. Reca, M. Wysoczynski, M. Kucia, J.T. Baran, D.J. Allendorf, J. Ratajczak, G.D. Ross, Transplantation studies in C3-deficient animals reveal a novel role of the third complement component (C3) in engraftment of bone marrow cells, *Leukemia* 18 (2004) 1482-1490.
- [355] C.H. Kim, W. Wu, M. Wysoczynski, A. Abdel-Latif, M. Sunkara, A. Morris, M. Kucia, J. Ratajczak, M.Z. Ratajczak, Conditioning for hematopoietic transplantation activates the complement cascade and induces a proteolytic environment in bone marrow: a novel role for bioactive lipids and soluble C5b-C9 as homing factors, *Leukemia* 26 (2012) 106-116.
- [356] C. Delaney, M.Z. Ratajczak, M.J. Laughlin, Strategies to enhance umbilical cord blood stem cell engraftment in adult patients, *Expert Rev Hematol* 3 (2010) 273-283.
- [357] Y. Kubota, K. Takubo, T. Suda, Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche, *Biochem Biophys Res Commun* 366 (2008) 335-339.
- [358] K. Parmar, P. Mauch, J.A. Vergilio, R. Sackstein, J.D. Down, Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia, *Proc Natl Acad Sci U S A* 104 (2007) 5431-5436.
- [359] R.D. Unwin, D.L. Smith, D. Blinco, C.L. Wilson, C.J. Miller, C.A. Evans, E. Jaworska, S.A. Baldwin, K. Barnes, A. Pierce, E. Spooncer, A.D. Whetton, Quantitative proteomics reveals posttranslational control as a regulatory factor in primary hematopoietic stem cells, *Blood* 107 (2006) 4687-4694.
- [360] G.L. Semenza, S.T. Koury, M.K. Nejfelt, J.D. Gearhart, S.E. Antonarakis, Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice, *Proc Natl Acad Sci U S A* 88 (1991) 8725-8729.
- [361] A.P. Levy, N.S. Levy, S. Wegner, M.A. Goldberg, Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia, *J Biol Chem* 270 (1995) 13333-13340.
- [362] S.P. Liu, S.D. Lee, H.T. Lee, D.D. Liu, H.J. Wang, R.S. Liu, S.Z. Lin, C.Y. Su, H. Li, W.C. Shyu, Granulocyte colony-stimulating factor activating HIF-1alpha acts synergistically with erythropoietin to promote tissue plasticity, *PLoS One* 5 (2010) e10093.
- [363] D.J. Ceradini, A.R. Kulkarni, M.J. Callaghan, O.M. Tepper, N. Bastidas, M.E. Kleinman, J.M. Capla, R.D. Galiano, J.P. Levine, G.C. Gurtner, Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1, *Nat Med* 10 (2004) 858-864.
- [364] P. Staller, J. Sulitkova, J. Lisztwan, H. Moch, E.J. Oakeley, W. Krek, Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL, *Nature* 425 (2003) 307-311.

- [365] S.A. Gharib, E.A. Dayyat, A. Khalyfa, J. Kim, H.B. Clair, M. Kucia, D. Gozal, Intermittent hypoxia mobilizes bone marrow-derived very small embryonic-like stem cells and activates developmental transcriptional programs in mice, *Sleep* 33 (2010) 1439-1446.
- [366] S.M. Cabarcas, L.A. Mathews, W.L. Farrar, The cancer stem cell niche--there goes the neighborhood?, *Int J Cancer* 129 (2011) 2315-2327.
- [367] Q. Li, M. Michaud, W. Stewart, M. Schwartz, J.A. Madri, Modeling the neurovascular niche: murine strain differences mimic the range of responses to chronic hypoxia in the premature newborn, *J Neurosci Res* 86 (2008) 1227-1242.
- [368] A. Bøyum, H.B. Fjerdingsstad, V.A. Tennfjord, H.B. Benestad, D. Løvhaug, Specific antibodies to mouse Sca-1- (Ly-6A/E) or Thy-1-positive haematopoietic progenitor cells induce formation of nitric oxide which inhibits subsequent colony formation, *Eur J Haematol* 73 (2004) 427-430.
- [369] T. Michurina, P. Krasnov, A. Balazs, N. Nakaya, T. Vasilieva, B. Kuzin, N. Khrushchov, R.C. Mulligan, G. Enikolopov, Nitric oxide is a regulator of hematopoietic stem cell activity, *Mol Ther* 10 (2004) 241-248.
- [370] P. Krasnov, T. Michurina, M.A. Packer, Y. Stasiv, N. Nakaya, K.A. Moore, K.E. Drazan, G. Enikolopov, Neuronal nitric oxide synthase contributes to the regulation of hematopoiesis, *Mol Med* 14 (2008) 141-149.
- [371] A. Aicher, C. Heeschen, C. Mildner-Rihm, C. Urbich, C. Ihling, K. Technau-Ihling, A.M. Zeiher, S. Dimmeler, Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells, *Nat Med* 9 (2003) 1370-1376.
- [372] S.M. Guthrie, L.M. Curtis, R.N. Mames, G.G. Simon, M.B. Grant, E.W. Scott, The nitric oxide pathway modulates hemangioblast activity of adult hematopoietic stem cells, *Blood* 105 (2005) 1916-1922.
- [373] M.A. Packer, Y. Stasiv, A. Benraiss, E. Chmielnicki, A. Grinberg, H. Westphal, S.A. Goldman, G. Enikolopov, Nitric oxide negatively regulates mammalian adult neurogenesis, *Proc Natl Acad Sci U S A* 100 (2003) 9566-9571.
- [374] N. Kim, S.J. Burden, MuSK controls where motor axons grow and form synapses, *Nat Neurosci* 11 (2008) 19-27.
- [375] M.E. Groh, B. Maitra, E. Szekely, O.N. Koc, Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells, *Exp Hematol* 33 (2005) 928-934.
- [376] J.M. Austyn, S. Gordon, F4/80, a monoclonal antibody directed specifically against the mouse macrophage, *Eur J Immunol* 11 (1981) 805-815.
- [377] V. Bronte, P. Serafini, C. De Santo, I. Marigo, V. Tosello, A. Mazzoni, D.M. Segal, C. Staib, M. Lowel, G. Sutter, M.P. Colombo, P. Zanollo, IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice, *J Immunol* 170 (2003) 270-278.

- [378] S.P. Cobbold, E. Adams, C.A. Farquhar, K.F. Nolan, D. Howie, K.O. Lui, P.J. Fairchild, A.L. Mellor, D. Ron, H. Waldmann, Infectious tolerance via the consumption of essential amino acids and mTOR signaling, *Proc Natl Acad Sci U S A* 106 (2009) 12055-12060.
- [379] X.D. Yang, W. Ai, S. Asfaha, G. Bhagat, R.A. Friedman, G. Jin, H. Park, B. Shykind, T.G. Diacovo, A. Falus, T.C. Wang, Histamine deficiency promotes inflammation-associated carcinogenesis through reduced myeloid maturation and accumulation of CD11b+Ly6G+ immature myeloid cells, *Nat Med* 17 (2011) 87-95.
- [380] M.L. Boulland, J. Marquet, V. Molinier-Frenkel, P. Möller, C. Guiter, F. Lasoudris, C. Copie-Bergman, M. Baia, P. Gaulard, K. Leroy, F. Castellano, Human IL4I1 is a secreted L-phenylalanine oxidase expressed by mature dendritic cells that inhibits T-lymphocyte proliferation, *Blood* 110 (2007) 220-227.
- [381] P.J. Shami, J.B. Weinberg, Differential effects of nitric oxide on erythroid and myeloid colony growth from CD34+ human bone marrow cells, *Blood* 87 (1996) 977-982.
- [382] M.M. Markiewski, R.A. DeAngelis, F. Benencia, S.K. Ricklin-Lichtsteiner, A. Koutoulaki, C. Gerard, G. Coukos, J.D. Lambris, Modulation of the antitumor immune response by complement, *Nat Immunol* 9 (2008) 1225-1235.
- [383] R.C. Schwarte, E.W. Godfrey, Nitric oxide synthase activity is required for postsynaptic differentiation of the embryonic neuromuscular junction, *Dev Biol* 273 (2004) 276-284.
- [384] E.W. Godfrey, R.C. Schwarte, The role of nitric oxide signaling in the formation of the neuromuscular junction, *J Neurocytol* 32 (2003) 591-602.
- [385] I. Dybedal, D. Bryder, A. Fossum, L.S. Rusten, S.E. Jacobsen, Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells, *Blood* 98 (2001) 1782-1791.
- [386] L.S. Rusten, F.W. Jacobsen, W. Lesslauer, H. Loetscher, E.B. Smeland, S.E. Jacobsen, Bifunctional effects of tumor necrosis factor alpha (TNF alpha) on the growth of mature and primitive human hematopoietic progenitor cells: involvement of p55 and p75 TNF receptors, *Blood* 83 (1994) 3152-3159.
- [387] R. Reca, D. Mastellos, M. Majka, L. Marquez, J. Ratajczak, S. Franchini, A. Glodek, M. Honczarenko, L.A. Spruce, A. Janowska-Wieczorek, J.D. Lambris, M.Z. Ratajczak, Functional receptor for C3a anaphylatoxin is expressed by normal hematopoietic stem/progenitor cells, and C3a enhances their homing-related responses to SDF-1, *Blood* 101 (2003) 3784-3793.
- [388] J. Ratajczak, R. Reca, M. Kucia, M. Majka, D.J. Allendorf, J.T. Baran, A. Janowska-Wieczorek, R.A. Wetsel, G.D. Ross, M.Z. Ratajczak, Mobilization studies in mice deficient in either C3 or C3a receptor (C3aR) reveal a novel role for complement in retention of hematopoietic stem/progenitor cells in bone marrow, *Blood* 103 (2004) 2071-2078.

- [389] W. Wagner, C. Roderburg, F. Wein, A. Diehlmann, M. Frankhauser, R. Schubert, V. Eckstein, A.D. Ho, Molecular and secretory profiles of human mesenchymal stromal cells and their abilities to maintain primitive hematopoietic progenitors, *Stem Cells* 25 (2007) 2638-2647.
- [390] S. Gottschling, R. Saffrich, A. Seckinger, U. Krause, K. Horsch, K. Miesala, A.D. Ho, Human mesenchymal stromal cells regulate initial self-renewing divisions of hematopoietic progenitor cells by a beta1-integrin-dependent mechanism, *Stem Cells* 25 (2007) 798-806.
- [391] D. Schlondorff, The glomerular mesangial cell: an expanding role for a specialized pericyte, *Faseb j* 1 (1987) 272-281.
- [392] L. da Silva Meirelles, P.C. Chagastelles, N.B. Nardi, Mesenchymal stem cells reside in virtually all post-natal organs and tissues, *J Cell Sci* 119 (2006) 2204-2213.
- [393] I. Marigo, F. Dazzi, The immunomodulatory properties of mesenchymal stem cells, *Semin Immunopathol* 33 (2011) 593-602.
- [394] J. Fujisaki, J. Wu, A.L. Carlson, L. Silberstein, P. Putheti, R. Larocca, W. Gao, T.I. Saito, C. Lo Celso, H. Tsuyuzaki, T. Sato, D. Côté, M. Sykes, T.B. Strom, D.T. Scadden, C.P. Lin, In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche, *Nature* 474 (2011) 216-219.
- [395] S. Gurumurthy, S.Z. Xie, B. Alagesan, J. Kim, R.Z. Yusuf, B. Saez, A. Tzatsos, F. Oszolak, P. Milos, F. Ferrari, P.J. Park, O.S. Shirihai, D.T. Scadden, N. Bardeesy, The Lkb1 metabolic sensor maintains haematopoietic stem cell survival, *Nature* 468 (2010) 659-663.
- [396] B. Angelin, T.E. Larsson, M. Rudling, Circulating fibroblast growth factors as metabolic regulators--a critical appraisal, *Cell Metab* 16 (2012) 693-705.

APPENDIX

C. Trento, F. Dazzi, **Mesenchymal stem cells and innate tolerance: biology and clinical applications**, Swiss Med Wkly. 2010;140:w13121

Mesenchymal stem cells and innate tolerance: biology and clinical applications

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Summary

The properties of mesenchymal stem cells (MSC) have been widely investigated during the last decade, from their differentiation capacity to their immunosuppressive effect on any type of immune cell. These properties have been successfully harnessed for the treatment of inflammatory diseases such as graft versus host disease (GvHD). Different mechanisms have been proposed for their immunosuppressive properties, although it seems likely that they are used in concert. The inflammatory environment to which MSC are exposed plays a pivotal role in activating their functions.

Conversely, the interplay of MSC with the immunoregulatory networks recruited during inflammation is fundamental to the delivery of immunosuppression. Since other types of terminally differentiated stromal cells share these properties, it is plausible that stemness is not a required feature. Therefore these functions may be involved in the physiological control of acute inflammation in various tissues. These notions highlight the importance of investigating the role of stromal cells as modulators of immune responses.

Mesenchymal stem cells (MSC) are multipotent progenitor cells of stromal origin, originally isolated from adult bone marrow and subsequently from other tissues including in both adult and foetal life [1–9]. Even though MSC are defined according to their ability to differentiate into various tissues of mesodermal origin (osteocytes, chondrocytes, adipocytes), there are unconfirmed reports that they can also differentiate into endothelial cells [10], as well as neural cells [11], and cells of endodermal origin [12].

The identification of MSC with the use of specific markers remains elusive. They are commonly described

as expressing CD73, CD105, CD90 and negative for the haematopoietic (CD45) and vascular (CD31) markers [13]. In mouse MSC markers of embryonic origin such as SSCA-1 [14] and SSCA-4 [15] have been identified, but more recently the co-expression of PDGFR α and Sca-1 [16] appears to be particularly effective at selectively identifying MSC because the vast majority of cells with progenitor activity resides in this subset. The efforts at detecting markers of human MSC have not delivered consistent results, but have indicated that they may preferentially express markers of neuronal lineage like low-affinity nerve growth factor receptor-1 (LNFR1) [17] and ganglioside GD2 [18]. Although these markers have not been entirely confirmed, the notion of the neuroepithelial origin of MSC has recently been supported by an elegant study showing that Sox1⁺ neuroepithelial cells supply the earliest wave of MSC differentiation during embryogenesis [19].

Pre-MSC type cells with characteristics of pluripotency have been isolated in the bone marrow or in foetal/perinatal tissues. Good examples are multipotent adult progenitor cells (MAPC), which differentiate into various lineages *in vitro* using defined cytokine combinations, and when transplanted they directly contribute to haematopoiesis *in vivo* and generate long-term repopulating haematopoietic stem cells and the full repertoire of haematopoietic progenitors [20].

The relative ease with which MSC can be isolated from adult tissues and the lack of ethical concern have probably been the main reason for their popularity. MSC have been successfully tested for their ability to protect from a variety of tissue injuries both in experimental [21–23] and clinical [24] settings.

Key words: *Mesenchymal; stem cells; immune responses; immunoregulation; graft-versus-host disease; autoimmune diseases*

The immunosuppressive activity of MSC

A further aspect that makes MSC of particular interest is the finding that they exert immunoregulatory activities. MSC from various species (humans, rodents and primates) can suppress the response of T cells to mitogenic and poly-

clonal stimuli [25, 26] and to their cognate peptide [27]. Such an effect is not cognate dependent because it can still be observed using MSC from third-party donors fully mismatched for the MHC haplotype of the responder T cells [28] or MSC which are constitutively negative for MHC molecule expression [27]. MSC-induced unresponsiveness lacks any selectivity, as it similarly affects memory and naïve T cells [27] as well as CD4⁺ and CD8⁺ subsets [29].

The characterisation of MSC-induced anergic T cells showed that the inhibitory effect of MSC is directed mainly at the level of T cell proliferation. T cells stimulated in the presence of MSC are arrested at the G1 phase as a result of cyclin D2 downregulation. The expression of CD25 and CD69 markers of T cell activation is completely unaffected by MSC co-culture, and inhibition of T cell effector functions can be reversed by MSC removal [29]. Whilst MSC induce an unresponsive T cell profile, they can prevent the apoptosis of activated T cells [30], indicating that MSC-mediated immunosuppression results from an induced division arrest anergy.

The effects of MSC on immune responses are not confined to T cells. Although they are susceptible to recognition and lysis by IL-2 activated cells and natural killer (NK) cells *in vitro*, due to their low expression of HLA class I [31, 32], MSC have been demonstrated to be capable of inhibiting the proliferation of interleukin-2 (IL-2) or IL-15 stimulated NK cells [31–33]. Whilst there is agreement on the immunosuppressive ability of MSC on NK cells, their influence on NK cell-mediated cytotoxicity remains controversial. Initial data suggested that MSC could inhibit the cytolytic activity of IL-2 activated NK cells [33], but more recent studies have shown that lysis of HLA I positive allogeneic targets by freshly isolated NK cells is not inhibited by MSC [31]. NK cells' cytokine production is also influenced by MSC, which are able to induce the release of IFN- γ [32, 34] and TNF- α [34].

The effect of MSC on B cell proliferation remains controversial. Studies in the mouse [29] and humans [35] showed that MSC inhibit B cell proliferation, inducing a block in G0/G1 phase of the cell cycle. MSC have also been shown to inhibit the differentiation of B cells to antibody secreting cells [35, 36] as well as downregulating CXCR4, CXCR5 and CCR7 chemokine receptors [35]. In contrast, other studies have suggested that human MSC promote the proliferation and differentiation of B cells from healthy donors and patients with systemic lupus erythematosus [37]. Although apparently in contradiction, the opposing results of these studies can be reconciled by the different conditions in which B cells have been stimulated. As a result of different B cell stimulation, the secreted cytokines could in fact polarise MSC towards a proinflammatory phenotype. This concept is well established for other cell types with regulatory functions, such as monocytes/macrophages [38].

The immunosuppressive properties of MSC can also target antigen-presenting cells (APC). The same effects exerted on cell cycle progression in T cells have been documented to affect monocytes [39]. MSC inhibit the differentiation of monocytes or CD34⁺ haematopoietic progenitors into mature dendritic cells (DC) [40]. DC precursors exposed to MSC lose their ability to stimulate alloresponses

and acquire regulatory features producing large amounts of interleukin-10 [41].

Besides their effect on immune cells, MSC exhibit an antiproliferative activity *in vitro* on different tumour cell lines, whereas *in vivo* they facilitate tumour engraftment and growth [42]. Since MSC contribute to the stem cell niche, these findings indicate that MSC can also provide the niche for cancer stem cells [43] and may influence the course of malignant diseases not only by creating an immunosuppressive environment within the tumour but also protecting tumour cells from apoptosis and facilitating its spread [44].

Mechanisms involved in the immunosuppressive effect

The mechanisms by which MSC exert their antiproliferative effect have still to be fully elucidated, although several candidate molecules have been proposed that are likely to act in concert and/or in alternate fashion depending on the environmental conditions to which MSC are exposed. Studies in both animal and human systems have shown that, although the effect requires an initial cell contact phase, the ultimate signal is mediated by several factors, which include transforming growth factor β -1 (TGF- β 1) [26], indoleamine 2,3-dioxygenase (IDO) [45], prostaglandin E₂ (PGE₂) [41], nitric oxide (NO) [46], heme oxygenase-1 (HO-1) [47], and insulin-like growth factor-binding proteins [48].

The role of these molecules is different in the mouse and in humans, as in human MSC the effect of IDO is prominent, whereas in murine MSC NO seems to play a major role.

Transforming growth factor β -1 (TGF- β 1) and hepatocyte growth factor (HGF) were the first molecules to be described as mediators of the immunosuppressive properties of MSC [26]. Recently it has been proposed that TGF- β gene expression is modulated in a contact-dependent mechanism by MSC [49].

Indoleamine 2,3-dioxygenase (IDO) is one of the immunosuppressive mechanisms believed to control T cell responses to autoantigens and alloantigens [50, 51], because its activity causes tryptophan depletion and kynurenine synthesis, capable of inhibiting the growth and function of immune cells by depleting nutrients and/or direct toxic activity of their catabolites [52]. IDO has been observed to be produced by MSC under inflammatory conditions such as exposure to IFN γ , and has been implicated in the inhibition of T-cell [45], NK-cell [32] and activated B-cell [33] proliferation.

Another mediator with immunosuppressive potential secreted by MSC is prostaglandin E₂ (PGE₂). Inhibitors of PGE₂ synthesis mitigate the overall human MSC suppressive effects [32, 41], with IDO as a synergistic partner [32]. It has been shown that MSC-derived PGE₂ is involved in skewing an inflammatory environment into an antiinflammatory environment, altering the cytokine secretion profile of dendritic cell subsets (DC1 and DC2) and T-cell subsets (Th1, Th2, or T_{regs}) [41].

Nitric oxide (NO) is synthesised by the inducible isoform of the NO synthase (iNOS), which is induced in MSC

by contact with activated CD4⁺ or CD8⁺ lymphocytes [46]. The proliferation of T cells is inhibited by NO-mediated suppression of phosphorylation of Stat5, a transcription factor crucial for T cell activation and proliferation [53]. Sato *et al.* have shown that iNOS^{-/-} MSC are less effective in suppressing T cell proliferation than wild type MSC, and this is demonstrated also by the blocking effect of *N*-nitro-L-arginine methyl ester, a specific inhibitor of the iNOS, on MSC immunomodulatory potential [46].

It has also been shown that HLA-G, a nonclassical MHC class I, is involved in immunomodulation by MSC [54]. Dendritic cells, NK, and T cells present inhibitory receptors that interact with both the membrane-bound and the soluble isoform of HLA-G, which are expressed by human MSC. The soluble isoform HLA-G5, secreted by MSC after IL-10 stimulation, can inhibit the cytotoxic activity of NK and CD8⁺ T cells, shift the T cell response to a Th2 cytokine profile and induce the expansion of regulatory T cells [55]. Taken together these data suggest that all these factors may be involved, depending on the environmental conditions to which MSC are exposed, and are crucial for their antiproliferative activity.

Stem/progenitor cells exhibit a particularly active transcriptional activity that might account for the several properties described in undifferentiated cells, including immunosuppression [56]. However, it has been shown that the immunosuppressive effect of MSC is not a property confined to mesenchymal progenitor cells, but is rather a function exerted by most mesenchymal cells including those terminally differentiated. This has been demonstrated on primary articular human chondrocytes [57] and fibroblasts from synovial joints, lung and skin [30, 58].

All the immunosuppressive activities described so far are not a constitutive property of MSC. For the MSC to inhibit immune responses, they need to be “licensed”. In fact, Jones and colleagues showed that only the supernatants obtained from co-cultures of stromal cells and activated T cells displayed an immunosuppressive effect when added to secondary cultures of proliferating T cells [30]. The immunosuppressive function of MSC is elicited by IFN- γ [33] and other proinflammatory cytokines such as TNF- α , IL-1 α , or IL-1 β [59].

MSC-mediated immunosuppression is not exclusively the result of a direct inhibitory effect but involves the recruitment of other regulatory networks. MSC act in concert with monocytes because the magnitude of the effect seems to be proportional to the number of monocytes in culture [60, 61]. Furthermore, MSC can also activate and expand regulatory T cells (T_{regs}) [62], although T_{regs} themselves are not required as a unique component to effect MSC immunosuppressive activity [27].

These findings suggest a crosstalk between MSC and the environment whereby first inflammatory monocytes ‘license’ MSC to acquire their immunosuppressive properties, and in turn MSC skew the inflammatory environment into an antiinflammatory environment both directly and through the effect on immunoregulatory circuits involving monocytes and T_{regs}.

Clinical applications

The tissue repair function and the inhibitory effect on the cell cycle on immune and nonimmune cells are properties that lend themselves to therapeutic exploitation, and there are a variety of disorders for which the use of MSC has been proposed. One of the first preclinical studies in baboons transplanted with allogeneic skin grafts showed that the *in vivo* administration of donor MSC to MHC-mismatched recipient prolonged the survival of third-party skin grafts [25]. On the same lines are the findings in islet transplantation. The use of MSC has been tested in a rat model of streptozotocin-induced diabetes with a view to producing haematopoietic chimerism in concomitance with allogeneic islet transplantation [63].

Further preclinical studies have shown that MSC could be successfully exploited in autoimmune diseases. MSC can ameliorate experimental autoimmune encephalomyelitis (EAE), reducing central nervous system inflammation and demyelination through the induction of peripheral T cell tolerance [64], and reduce the relapse rate of EAE impairing pathogenic T and B cell responses directed against the immunising antigen [65]. Whilst a first report on cell therapy for collagen induced arthritis (CIA) using MSC showed that their use was not beneficial in curing arthritis, suggesting that activation of the TNF α inflammatory pathway in the injured tissues might reverse their immunomodulatory effect [66], it has recently been demonstrated that a single injection of allogeneic MSC in a CIA model can prevent the occurrence of irreversible damage to bone and cartilage [67]. On the basis of their ability to skew the inflammatory environment to an antiinflammatory environment, MSC have also been investigated in sepsis [68] and colitis [69]. Activated MSC can induce *in vivo* the production of higher amounts of IL-10 from macrophages [68, 69] by releasing PGE₂, thus preventing neutrophils from migrating into tissues and causing oxidative damage [68].

The most studied therapeutic application for MSC is graft-versus-host disease (GvHD), a severe condition that develops after allogeneic haematopoietic stem cell transplantation (HSCT). The clinical efficacy of MSC in GvHD was initially observed in a 9-year-old boy suffering from steroid-resistant grade IV acute GvHD who received haploidentical third-party MSC [70]. Subsequently a phase II trial involving 55 patients with the same condition demonstrated that the infusion of MSC could significantly improve overall survival [71]. A previous multicentre phase I/II clinical trial in which MSC were given at the time of HSCT before any sign of GvHD produced a different outcome, no difference being observed in the incidence of GvHD between the group receiving MSC and the controls [72]. The discrepancies might be explained by the findings of the preclinical studies. Initial studies reported that a single infusion of MSC at the time of the transplant did not prevent the development of GvHD in MHC-mismatched donor-recipient pairs [73]. The work of Tisato and colleagues, although confirming the ineffectual activity of MSC injected in a single dose at the beginning of HSCT, showed that GvHD could be totally prevented by multiple doses [74]. Polchert *et al.* observed that MSC could significantly increase the survival rate of recipient mice only

when given at day +2 or +20 when IFN- γ levels are at their peak, MSC efficacy being dependent on the presence of IFN- γ in the environment [75]. Timing is therefore essential for MSC to exert their inhibitory effect, due to the need for the appropriate inflammatory environment to 'licence' the MSC. The role of IFN- γ [33, 59] and of the inflammatory environment [30] in activating MSC had also been previously described *in vitro*, as already discussed. Another suggested role for the inflammation derived from *in vivo* studies [23, 74, 76] is the recruitment of MSC. IFN- γ might be able to cause the accumulation of antigen-specific T cells at the site of inflammation by inducing MHC molecule expression on the endothelium [77]. IFN- γ could therefore promote accumulation of MSC and recruitment of antigen-specific T cells at the same site, retaining suppressive and effector cells in the same anatomical compartment [78].

Besides their use to modulate immune responses, MSC have been employed to promote tissue repair. It is likely that the antiproliferative and antiapoptotic activity of MSC on parenchymal cells [42] effect a cytoprotective action that preserves residual stem cells from further destruction thus favouring their recovery and spontaneous tissue repair. The anti-inflammatory activity also favours the generation of anti-inflammatory macrophages, which are crucial for promotion of tissue repair [79]. MSC has therefore been used to promote the expansion and development of islet β -cells in diabetes therapy. Repeated transplantation of human MSC-induced repair of pancreatic islets and renal glomeruli in NOD/*scid* mice suffering from STZ-induced diabetes [80]. By coinjecting sex mismatched bone marrow cells (BMC) and syngeneic or allogeneic MSC it was possible to demonstrate that tissue repair was not the result of trans-differentiation but rather the consequence of an endogenous repair process initiated by the graft and the suppression of T cell-mediated immune response against newly formed β -cells by donor MSC [81]. A recent field of investigation is represented by the therapeutic potential of MSC in acute renal failure [82] in which a paracrine activity mediated by MSC appears to play the main role in tissue repair [23]. Similarly, in a model of bleomycin-induced pulmonary fibrosis, inflammation and collagen deposition were significantly reduced after MSC administration via a mechanism involving IL-1 receptor antagonist [22].

Conclusions

The immunosuppressive properties of MSC have aroused keen interest in the last few years. Current data indicate that MSC utilise a number of synergistic mechanisms to non-specifically control immune responses and activate further immunosuppressive circuits to boost MSC action. *In vitro* and *in vivo* studies have suggested that the inflammatory environment is crucial to enable MSC to exert their antiproliferative and antiapoptotic effects, thus highlighting the importance of dissecting the molecular features of the microenvironment to maximise the therapeutic impact. This notion is also important in improving our understanding of the function of tissue stroma as effector of innate tolerance to rapidly modulate immune response and, with overlapping mechanisms, to protect tissue progenitors from dying,

activate their self-renewal programme and contribute to tissue repair.

Funding / potential competing interests

This work was supported by the leukaemia lymphoma research and leuka.

References

- Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood*. 2001;98(8):2396–402.
- Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol*. 2000;109(1):235–42.
- Igura K, Zhang X, Takahashi K, Mitsuru A, Yamaguchi S, Takashi TA. Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy*. 2004;6(6):543–53.
- in 't Anker PS, Noort WA, Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, et al. Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica*. 2003;88(8):845–52.
- O'Donoghue K, Chan J, de la Fuente J, Kennea N, Sandison A, Anderson JR, et al. Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. *Lancet*. 2004;364(9429):179–82.
- O'Donoghue K, Choolani M, Chan J, de la Fuente J, Kumar S, Campagnoli C, et al. Identification of fetal mesenchymal stem cells in maternal blood: implications for non-invasive prenatal diagnosis. *Mol Hum Reprod*. 2003;9(8):497–502.
- Tsai MS, Lee JL, Chang YJ, Hwang SM. Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum Reprod*. 2004;19(6):1450–6.
- Villaron EM, Almeida J, Lopez-Holgado N, Alcoceba M, Sanchez-Abarca LI, Sanchez-Guijo FM, et al. Mesenchymal stem cells are present in peripheral blood and can engraft after allogeneic hematopoietic stem cell transplantation. *Haematologica*. 2004;89(12):1421–7.
- Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, et al. Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res*. 2000;2(6):477–88.
- Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest*. 2002;109(3):337–46.
- Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res*. 2000;61(4):364–70.
- Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, et al. Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. *Blood*. 2005;106(2):756–63.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8(4):315–7.
- Anjos-Afonso F, Bonnet D. Nonhematopoietic/endothelial SSEA-1+ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. *Blood*. 2007;109(3):1298–306.
- Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood*. 2007;109(4):1743–51.
- Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, Hiratsu E, et al. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J Exp Med*. 2009;206(11):2483–96.

- 17 Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol.* 2002;30(7):783–91.
- 18 Martinez C, Hofmann TJ, Marino R, Dominici M, Horwitz EM. Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. *Blood.* 2007;109(10):4245–8.
- 19 Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, et al. Neuroepithelial cells supply an initial transient wave of MSC differentiation. *Cell.* 2007;129(7):1377–88.
- 20 Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.* 2002;418(6893):41–9.
- 21 Li TZ, Kim JH, Cho HH, Lee HS, Kim KS, Lee SW, et al. Therapeutic Potential of Bone-Marrow-Derived Mesenchymal Stem Cells Differentiated with Growth-Factor-Free Coculture Method in Liver-Injured Rats. *Tissue Eng Part A.* 2010.
- 22 Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci. USA.* 2007;104(26):11002–7.
- 23 Togel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol.* 2005;289(1):F31–42.
- 24 Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med.* 1999;5(3):309–13.
- 25 Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol.* 2002;30(1):42–8.
- 26 Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood.* 2002;99(10):3838–43.
- 27 Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood.* 2003;101(9):3722–9.
- 28 Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol.* 2003;31(10):890–6.
- 29 Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood.* 2005;105(7):2821–7.
- 30 Jones S, Horwood N, Cope A, Dazzi F. The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells. *J Immunol.* 2007;179(5):2824–31.
- 31 Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevas CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells.* 2006;24(1):74–85.
- 32 Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood.* 2006;107(4):1484–90.
- 33 Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells.* 2006;24(2):386–98.
- 34 Poggi A, Prevosto C, Massaro AM, Negrini S, Urbani S, Pierri I, et al. Interaction between human NK cells and bone marrow stromal cells induces NK cell triggering: role of NKp30 and NKG2D receptors. *J Immunol.* 2005;175(10):6352–60.
- 35 Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. *Blood.* 2006;107(1):367–72.
- 36 Deng W, Han Q, Liao L, You S, Deng H, Zhao RC. Effects of allogeneic bone marrow-derived mesenchymal stem cells on T and B lymphocytes from BXS mice. *DNA Cell Biol.* 2005;24(7):458–63.
- 37 Traggiai E, Volpi S, Schena F, Gattorno M, Ferlito F, Moretta L, et al. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells.* 2008;26(2):562–9.
- 38 Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004;25(12):677–86.
- 39 Ramasamy R, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F. Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation.* 2007;83(1):71–6.
- 40 Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+ derived and monocyte-derived dendritic cells. *J Immunol.* 2006;177(4):2080–7.
- 41 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood.* 2005;105(4):1815–22.
- 42 Ramasamy R, Lam EW, Soeiro I, Tisato V, Bonnet D, Dazzi F. Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth. *Leukemia.* 2007;21(2):304–10.
- 43 Orimo A, Weinberg RA. Stromal fibroblasts in cancer: a novel tumor-promoting cell type. *Cell Cycle.* 2006;5(15):1597–601.
- 44 Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature.* 2007;449(7162):557–63.
- 45 Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood.* 2004;103(12):4619–21.
- 46 Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood.* 2007;109(1):228–34.
- 47 Chabannes D, Hill M, Merieau E, Rossignol J, Brion R, Souillou JP, et al. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood.* 2007;110(10):3691–4.
- 48 Giesecke F, Schutt B, Viebahn S, Koscielniak E, Friedrich W, Handgretinger R, et al. Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFN γ and IDO expression. *Blood.* 2007;110(6):2197–200.
- 49 Nasef A, Chapel A, Mazurier C, Bouchet S, Lopez M, Mathieu N, et al. Identification of IL-10 and TGF- β transcripts involved in the inhibition of T-lymphocyte proliferation during cell contact with human mesenchymal stem cells. *Gene Expr.* 2007;13(4-5):217–26.
- 50 Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol.* 2000;164(7):3596–9.
- 51 Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science.* 1998;281(5380):1191–3.
- 52 Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J Exp Med.* 2002;196(4):459–68.
- 53 Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J Immunol.* 1998;160(12):5729–34.
- 54 Nasef A, Mathieu N, Chapel A, Frick J, Francois S, Mazurier C, et al. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation.* 2007;84(2):231–7.
- 55 Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. *Stem Cells.* 2008;26(1):212–22.

- 56 Li L, Baroja ML, Majumdar A, Chadwick K, Rouleau A, Gallacher L, et al. Human embryonic stem cells possess immune-privileged properties. *Stem Cells*. 2004;22(4):448–56.
- 57 Bocelli-Tyndall C, Barbero A, Candrian C, Ceredig R, Tyndall A, Martin I. Human articular chondrocytes suppress in vitro proliferation of anti-CD3 activated peripheral blood mononuclear cells. *J Cell Physiol*. 2006;209(3):732–4.
- 58 Haniffa MA, Wang XN, Holtick U, Rae M, Isaacs JD, Dickinson AM, et al. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. *J Immunol*. 2007;179(3):1595–604.
- 59 Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell*. 2008;2(2):141–50.
- 60 Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood*. 2005;105(5):2214–9.
- 61 Groh ME, Maitra B, Szekely E, Koc ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp Hematol*. 2005;33(8):928–34.
- 62 Prevosto C, Zancolli M, Canevali P, Zocchi MR, Poggi A. Generation of CD4+ or CD8+ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. *Haematologica*. 2007;92(7):881–8.
- 63 Itakura S, Asari S, Rawson J, Ito T, Todorov I, Liu CP, et al. Mesenchymal stem cells facilitate the induction of mixed hematopoietic chimerism and islet allograft tolerance without GVHD in the rat. *Am J Transplant*. 2007;7(2):336–46.
- 64 Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood*. 2005;106(5):1755–61.
- 65 Gerdoni E, Gallo B, Casazza S, Musio S, Bonanni I, Pedemonte E, et al. Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. *Ann Neurol*. 2007;61(3):219–27.
- 66 Djouad F, Fritz V, Apparailly F, Louis-Plence P, Bony C, Sany J, et al. Reversal of the immunosuppressive properties of mesenchymal stem cells by tumor necrosis factor alpha in collagen-induced arthritis. *Arthritis Rheum*. 2005;52(5):1595–603.
- 67 Augello A, Tasso R, Negrini SM, Cancedda R, Pennesi G. Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum*. 2007;56(4):1175–86.
- 68 Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*. 2009;15(1):42–9.
- 69 Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut*. 2009;58(7):929–39.
- 70 Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet*. 2004;363(9419):1439–41.
- 71 Le Blanc K, Frasson F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet*. 2008;371(9624):1579–86.
- 72 Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant*. 2005;11(5):389–98.
- 73 Sudres M, Norol F, Trenado A, Gregoire S, Charlotte F, Levacher B, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol*. 2006;176(12):7761–7.
- 74 Tisato V, Nares K, Girdlestone J, Navarrete C, Dazzi F. Mesenchymal stem cells of cord blood origin are effective at preventing but not treating graft-versus-host disease. *Leukemia*. 2007;21(9):1992–9.
- 75 Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur J Immunol*. 2008;38(6):1745–55.
- 76 Marinova-Mutafchieva L, Williams RO, Funa K, Maini RN, Zvaifler NJ. Inflammation is preceded by tumor necrosis factor-dependent infiltration of mesenchymal cells in experimental arthritis. *Arthritis Rheum*. 2002;46(2):507–13.
- 77 Marelli-Berg FM, James MJ, Dangerfield J, Dyson J, Millrain M, Scott D, et al. Cognate recognition of the endothelium induces HY-specific CD8+ T-lymphocyte transendothelial migration (diapedesis) in vivo. *Blood*. 2004;103(8):3111–6.
- 78 Dazzi F, Marelli-Berg FM. Mesenchymal stem cells for graft-versus-host disease: close encounters with T cells. *Eur J Immunol*. 2008;38(6):1479–82.
- 79 Ruffell D, Mourkioti F, Gambardella A, Kirstetter P, Lopez RG, Rosenthal N, et al. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc Natl Acad Sci USA*. 2009;106(41):17475–80.
- 80 Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci USA*. 2006;103(46):17438–43.
- 81 Urban VS, Kiss J, Kovacs J, Gocza E, Vas V, Monostori E, et al. Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes. *Stem Cells*. 2008;26(1):244–53.
- 82 Lange C, Togel F, Itrich H, Clayton F, Nolte-Ernsting C, Zander AR, et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. *Kidney Int*. 2005;68(4):1613–7.