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Interacting mast cells and myeloid derived suppressor cells in the regulation of tumor-specific immune response

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Abstract

Inflammation plays crucial roles at different stages of tumor development and may lead to the failure of immune surveillance and immunotherapy. Tumor-associated inflammatory microenvironment contains both innate and adaptive immune cells that communicate each other and engage a dynamic crosstalk with cancer cells (Grivennikov et al., 2010). Myeloid-derived suppressor cells (MDSC) are one of the major components of the immune-suppressive network that favors tumor growth (Gabrilovich and Nagaraj, 2009).

Due to their tissue distribution and their capability to release a large spectrum of mediators after stimulation, mast cells (MC) act as potential regulatory linkers between innate and acquired immunity in the control of immune system homeostasis (Frossi et al., 2004), but they are also implicated in the promotion of tumor growth (Maltby et al., 2009).

The aim of this work was to characterize the role of MCs in a colon cancer model and to investigate the possibility of MC interaction with MDSCs in the control of tumor-induced immune response. Obtained results demonstrated that MCs were recruited and had potential pro-tumoral functions in the growth of CT-26 colon cancer. At the same time, the tumor induced the accumulation of $CD11b^+$ $Gr\text{-}1^+$ immature MDSCs, which caused profound alteration of antitumor immune response. In the spleen, conditioned by the inflammatory response to tumor growth, MCs seemed to be able to directly contact accumulating MDSCs and direct their activation triggered by tumor specific IFN-γ-producing $CD4^+$ T cells. In fact, ex *vivo* MCs emerged for their ability to switch on the suppressive properties of spleen-derived monocytic-MDSCs, rendering them able to inhibit polyclonal antigen-non-specific activation through increased production of nitric oxide. In addiction, the CD40:CD40L cross talk between the two populations was responsible for the instauration of a pro-inflammatory microenvironment, with the exacerbation in the production of mediators that can further support MDSC mobilization and tumor growth.

List of abbreviations

AOM Azoxymethane
APC Antigen-Professional Cell
CAC Colitis-Associated Carcinoma
CD Cluster of Differentiation
CRC Colorectal Carcinoma
DC Dendritic Cell
DSS Dextran Sodium Sulphate
EAE Experimental autoimmune encephalomyelitis
FGF Fibroblast Growth Factor
GM-CSF Granulocyte Macrophage colony-stimulating factor
HIF Hypoxia Inducible Factor
IBD Inflammatory Bowel Disease
IFN Interferon
Ig Immunoglobulin
IL Interleukin
MC Mast Cell
MCP (1) Mast Cell Progenitor
MCP (2) Monocyte Chemoattractant Protein
M-CSF Macrophage Colony-Stimulating Factor
MDSC Myeloid Derived Suppressor Cell
M-MDSC Monocytic Myeloid Derived Suppressor Cell
MHC Major Histocompatibility Complex
MIP Macrophage Inflammatory Protein
MMP Matrix Metalloproteinases
NK Natural Killer
NO Nitric Oxide
NOS Nitric Oxide Synthase
PGE Prostaglandin E
PMN-MDSC Polymorphonuclear Myeloid Derived Suppressor Cell
RNS Reactive Nitrogen Species
ROS Reactive Oxygen Species
SCF Stem Cell Factor
TAM Tumor Associated Macrophage
TAN Tumor Associated Neutrophil
TCR T Cell Receptor
TGF Transforming Growth Factor
TIL Tumor-infiltrating lymphocytes
TLR Toll Like Receptor
TNF Tumor Necrosis Factor
TRAMP Transgenic Adenocarcinoma of the Mouse Prostate
TREG Regulatory T Cell
VEGF Vascular Endothelial Growth Factor

Chapter 1: INFLAMMATION AND CANCER

1.1 The "hallmarks" of cancer and the liaison with immune system

Tumors are more than masses of uncontrolled proliferating cancer cells: they are complex tissues composed of multiple different cell types in heterotypic reciprocal interaction. "Tumor microenvironment", constituted by tumor-associated stroma and inflammatory cells, actively contributes to the acquisition and expression of cancer hallmarks, that represent the biological capabilities typically acquired during the multistep development of cancer (reviewed in (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The acquired capabilities that allow survival, proliferation and dissemination of cancer cells are enabled by the prominent genetic instability, which generates the accumulation of random mutations. "Caretakers" of genome, involved in detection of DNA damage and repair machinery, are frequently inactivated through mutations or epigenetic repression leading to the accumulation of other mutations and being instrumental for cancer progression.

The most important trait of cancer cells involves their ability to sustain chronic proliferation, by deregulating growth-promoting signals that normally control cell growth and division cycle in homeostasis. For example, cancer cells acquire the ability to produce growth factor ligands themselves or to constitutively activate signaling pathways downstream receptors from growth factors, by-passing the need for ligand-receptor interaction.

Overcoming telomere shortening, which determines the limited replicative potential of normal cells, by activation of telomerase is the mayor mechanism related to the unlimited replicative potential of cancer cells.

In order to control the energy metabolism to fuel cell growth and division, most cancer cells exhibit increased glycolysis and use this metabolic pathway for generation of ATP as a main source of their energy supply. This phenomenon is known as the Warburg effect and is considered as one of the most fundamental metabolic alterations during malignant transformation. It consists in limiting energy production by glycolysis even in presence of oxygen, and it is the preferential metabolic choice of cancer cells, especially of hypoxic ones that depend on glucose as fuel and secrete lactate as waste. At this point, lactate could become the preferential fuel for better-oxygenated cancer cell subpopulations. Despite the relatively poor efficiency of generating ATP by glycolysis compared to mitochondrial oxidative phosphorylation, aerobic glycolysis is programmed by proliferation-inducing oncogenes and involves an increased glucose import into cancer cells. Biochemical and molecular studies

suggest several possible mechanisms by which this metabolic alteration may evolve during cancer development. These mechanisms include mitochondrial defects and dysfunctions, adaptation to hypoxic tumor microenvironment, oncogenic signaling, and abnormal expression of metabolic enzymes.

In addition to the capability of inducing and sustaining growth-stimulatory signals, cancer cells are able to evade growth suppressors known to limit cell growth and proliferation. Several tumor suppressor genes, such as the ones that encode the RB (retinoblastoma-associated) and TP53 proteins, are inactivated in different form of human cancer.

Resisting cell death by circumvent apoptosis is another hallmark of tumor cells. A variety of anti-apoptotic strategies are involved: loss of p53 tumor suppressor function, increase of anti-apoptotic regulators (i.e. Bcl-2, Bcl-x_L) and decrease of pro-apoptotic ones (Bax, Bim, Puma), or gain of pro-survival signals. Apparently similar to apoptosis, autophagy seems to represent another barrier that could limit cancer development. Instead, paradoxically, stressed cancer cells (for example during radiotherapy or chemotherapy) may present a state of autophagy-dependent reversible dormancy that enables the re-growth of tumor following potent treatment. Nevertheless, some degree of necrotic cell death, which in contrast to apoptosis and autophagy releases pro-inflammatory signals, is probably tolerated by growing tumors because the released factors may recruit tumor-promoting inflammatory cells that sustain tumor proliferation and/or invasiveness (see below).

Another requirement for the development of tumors is the possibility to activate an "angiogenic switch", which refers to the time-restricted event during tumor progression where the balance between pro- and anti-angiogenic factors tilts towards a pro-angiogenic outcome, resulting in the transition from dormant avascularized hyperplasia to outgrowing vascularized tumor and eventually to malignant tumor progression. This phenomenon causes the continuous sprout of new vessels that sustain tumor-request of nutrients and oxygen. Induction of VEGF-A, up-regulated by hypoxia and oncogene signaling, is the prototype marker of an on-going angiogenesis in the tumor microenvironment. Angiogenesis, which is sustained directly by cancer cells in collaboration with stromal and inflammatory cells, is crucial in the premalignant phase of neoplastic progression and become essential in growing macroscopic tumors. Tumor neovascularization is typically aberrant compared to physiological blood vessels architecture with capillary sprouting, excessive vessel branching, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis.

The multistep process of invasion and metastasis is a key feature in tumor biology, beginning with local invasion and ending with "colonization" of distant tissues and organs. The other steps

in invasion progression refer to intravasation by cancer cells into blood and lymphatic vessels with the transit in the circulatory system, followed by extravasation into the parenchyma of distant tissues and by formation of small micro-metastases that finally grow into macroscopic lesions. "Epithelial-mesenchymal transition" program refers as the acquired abilities of transformed epithelia cells to resist apoptosis, invade and disseminate. These properties are related to the loss of adherens junctions (with frequent loss of E-cadherin, a key cell-to-cell adhesion molecule), the acquisition of a fibroblastic morphology, the increased motility and the expression of matrix-degrading enzymes. The ability to acquire most of the steps involved in invasion-metastasis cascade may not be only dependent on additional mutation in cancer cells, but may be the result of stromal cells contribution. Mesenchymal stem cells and inflammatory cells, like tumor-associated macrophages, can facilitate invasion by production of matrix-degrading enzymes such as metalloproteinases or cysteine cathepsin proteases. Although metastasis has been described as the last step in carcinogenesis, nowadays it is emerging that cancer cells can disseminate relatively early during tumor development but often micro-metastases remain "dormant" and do not progress to macroscopic metastatic tumors.

It is important to underline that the biology of a tumor could not be understood without considering the tumor microenvironment in *toto*. In fact immune cells densely infiltrate tumors: they start to accumulate from the early stages of cancer and foster the development of initial tumors into large metastatic ones. Therefore, tumor-promoting inflammation can be considered as an enabling hallmark of cancer, being source of molecules, such as growth, survival or pro-angiogenic factors, which drive cancer cell proliferation, survival, angiogenesis and other hallmark-programs. The definition of tumor-promoting inflammation as a new emerging hallmark of cancer is only the final outcome of a *love-hate* relationship between cancer and immune system. In fact, an intriguing issue involves the effective role that immune system plays in resisting or promoting formation of incipient cancer. Cancer immunoediting (Dunn et al., 2002) is the concept that integrates the immunity's dual roles in cancer suppression and promotion. This process is the expanded and more realistic advancement of the cancer immunosurveillance hypothesis, which sustained that adaptive immunity was responsible for preventing developing cancer in immunocompetent hosts (Burnet, 1957). Nowadays, several preventing roles of immune system are recognized, such as protection against viral-induced tumors, prevention of inflammatory microenvironment establishment or direct elimination of tumor cells. In this context, innate and adaptive immune systems work to detect the presence and to prevent the formation of a developing tumor with the objective to destroy it. Probably highly immunogenic cancer cells are routinely eliminated in immunocompetent hosts. But this

elimination phase represents only the first sequential moment of the now well-accepted cancer immunoediting process. If elimination phase does not drive to tumor complete destruction, weakly immunogenic tumor cell variants may survive and enter in the equilibrium phase in which the immune system maintains residual tumor cells in a functional state of dormancy. Specifically, adaptive immunity is involved in the maintenance of the equilibrium and in the arrest of tumor proliferation, a situation that may persist in patients for decades before eventually restarting growth.

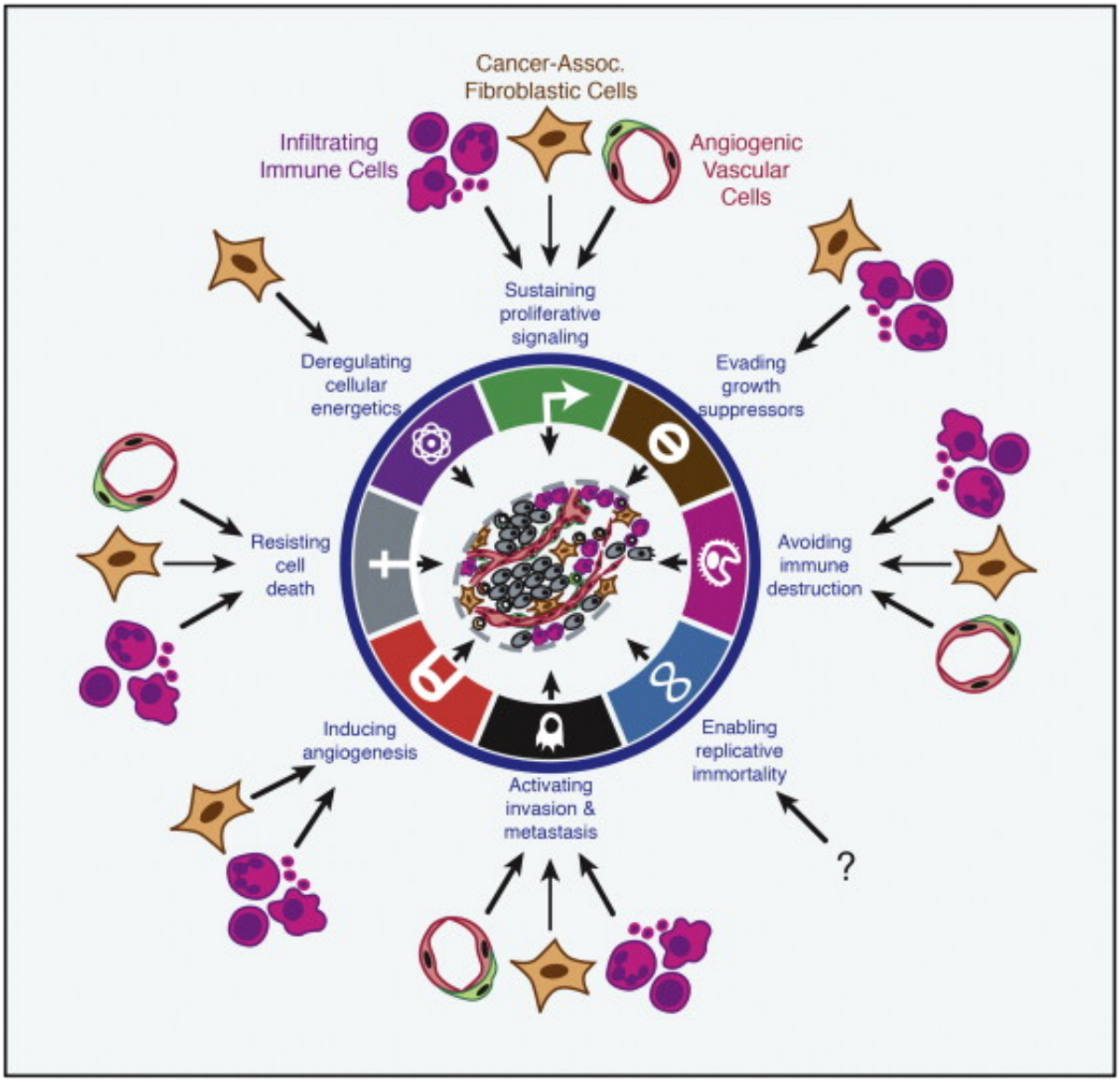

Figure 1.1: Hallmarks of cancer. The hallmarks of cancer (genome instability, sustaining proliferative signaling, enabling replicative immortality, deregulating cellular energetics, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion and metastasis, avoiding immune destruction and tumor-promoting inflammation) comprise the biological capabilities acquired during the multistep development of tumors and represent an organizing principle for rationalizing the complexities of neoplastic disease (Adapted from Hanahan and Weinberg, Cell 2011).

The ability to circumvent immune recognition and/or destruction emerges as tumors progressively grow. This escape phase can imply different mechanisms: from tumor cell reduced immune recognition (for example due to loss of strong immunogenic antigens or loss of MHC class I with lack of Ag presentation) to increased resistance to immune defensive response (such as over-expression of anti-apoptotic pathways) and the establishment of a immunosuppressive microenvironment (i.e. through generation and accumulation of regulatory T cells and/or myeloid derived suppressor cells). At this point, tumor-associated immune response emerges mainly for its tumor-promoting roles and immunoevasion can be viewed as one of the hallmarks of cancer. With emerging knowledge, an effective immunotherapy will have the properties to increase quality and quantity of immune effector cells, and/or eliminate cancer-induced immunosuppressive mechanisms (Schreiber et al., 2011).

1.2 Inflammatory microenvironment in tumorigenesis: general features and main actors

The inflammatory microenvironment is an essential component of all tumors and goes hand to hand with tumor development, conditioning every step of tumorigenesis, from initiation to metastatic program through promotion (reviewed in Grivennikov et al., 2010).

Many cancers are associated with environmental causes correlated to chronic infections. Tumorigenic pathogens, such as *H. pylori* and hepatitis B or C viruses (HBV, HCV) that are associated respectively with gastric and hepatocellular carcinoma, establish persistent infections associated with a stage of chronic inflammation that precedes cancer development. Instead inflammatory bowel disease (IBD), an important risk factor for colorectal cancer, is an example of chronic inflammation due to immune deregulation and autoimmunity that favors carcinogenesis. Inflammatory mediators, such as reactive oxygen and nitrogen species (ROS and RNS) or cytokines, are able to induce DNA damage and genomic instability, and participate in tumor initiation, which is the initial process of mutation acquisition and tumorigenic track of normal cells. In addition inflammation drives tumor promotion that represents the step of transformation from a single initiated cell into a growing autonomous tumor. The main inflammation-dependent mechanism involved in tumor-promotion is the production of cytokines (i.e. TNF-α, IL-6, IL-23 or IL-1β) that act in a paracrine manner to induce proliferation and survival signals in cancer cells, for example through activation of NF-κB and Stat-3 pathways. At the same time, continuous recruitment of inflammatory cells is essential for tumor promotion and is ensured by chemokines and cytokines.

Inflammatory cells may sense hypoxic signals from tumor microenvironment and in turn they produce chemokines and pro-angiogenic factors, such as VEGF, to sustain angiogenesis. In addition, inflammatory cells promote metastasis, being active sources of proteases that degrade extracellular matrix and cytokines that regulate and activate epithelial-mesenchymal transition (mainly TGF-β) or promote survival of circulating micrometastases (TNF-α, IL-6). Summing up, from initiation to metastatic progression, the building of a pro-inflammatory microenvironment is an intrinsic feature of emerging tumor mass.

In term of cellular response, in addition to cancer cells and stroma, the tumor microenvironment is composed by both innate (i.e. macrophages, dendritic cells, myeloid derived suppressor cells, mast cells, neutrophils, and natural killer cells) and adaptive immune cells (T and B lymphocytes).

Table 1. Roles of Different Subtypes of Immune and Inflammatory Cells in Antitumor Immunity and Tumor-Promoting Inflammation

Cell Types	Antitumor	Tumor-Promoting
Macrophages, dendritic cells, myeloid-derived suppressor cells	Antigen presentation; production of cytokines (IL-12 and type I IFN)	Immunosuppression; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors
Mast cells		Production of cytokines
B cells	Production of tumor-specific antibodies?	Production of cytokines and antibodies; activation of mast cells; immunosuppression
$CD8^+$ T cells	Direct lysis of cancer cells; production of cytotoxic cytokines	Production of cytokines?
$CD4^+$ Th2 cells		Education of macrophages; production of cytokines; B cell activation
$CD4^+$ Th1 cells	Help to cytotoxic T lymphocytes (CTLs) in tumor rejection; production of cytokines (IFNγ)	Production of cytokines
$CD4^+$ Th17 cells	Activation of CTLs	Production of cytokines
$CD4^+$ Treg cells	Suppression of inflammation (cytokines and other suppressive mechanisms)	Immunosuppression; production of cytokines
Natural killer cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Natural killer T cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Neutrophils	Direct cytotoxicity; regulation of CTL responses	Production of cytokines, proteases, and ROS

Figure 1.2: Immune cells in the cancer-associated inflammatory microenvironment: antitumor immunity against tumor-promoting inflammation (Adapted from Grivennikov *et al*., Cell 2010).

Tumor-associated macrophages (TAMs) are the most abundant infiltrating immune cells and high TAM density is mostly correlated with poor prognosis in patients. In response to different stimuli, plasticity is a main feature of macrophages: classically activated macrophages (M1 type with IL-12^{high} IL-10^{low} phenotype), following exposure to IFN-γ may have tumoricidal activity and elicit tissue destructive reactions, whereas in response to IL-4 or IL-13, macrophages undergo alternative activation (M2 type, IL-12^{low} IL-10^{high} phenotype) oriented to immunoregulation, tissue repair and remodeling. Dynamic changes of the tumor microenvironment that occur during the transition from early neoplastic phases toward advanced

tumor stages drive a M1 toward M2 switch of TAM functions. Although in the initial phases of tumor initiation and promotion infiltrating macrophages have inflammatory antitumor properties, the following switch to M2 phenotype directs non-resolving tumor-promoting inflammation (Mantovani and Sica, 2010). Within tumor, TAMs are significant for fostering tumor progression through modulation of angiogenesis, invasion, metastasis, and immunosuppression. In fact, TAMs regulate vascular programming in tumor microenvironment through production of VEGF-A, MMP-9 (that mediates VEGF-A bioavailability) and expression of Tie2, a receptor for angiopoietins (an axis important for TAM localization along the vasculature). Factors produced by CSF-1-recruited TAMs are crucial for enhancing invasion of malignant cells into ectopic tissue, in particular through epidermal growth factor (EGF)- and metalloproteinase-dependent remodeling of extracellular matrix. In addition TAMs express several genes with immunosuppressive potential to directly inhibit T cell proliferation and with the possibility to regulate other immune suppressive populations, such as regulatory T cells (reviewed in Ruffell et al., 2012).

The alteration status of tumor-associated DCs contributes to the failure of antitumor immune response and it is correlated with disease progression. The decreased uptake, processing and presentation of antigens, the lowered expression of co-stimulatory signals, the inefficient motility and migration, and the decrease production of IL-12 (with concomitant IL-10 and TGF-β up-regulation) are responsible for the inhibited ability of DCs to correctly stimulate an antitumor-specific T cell response. In addition, tumor associated-DC polarization is associated with the acquisition of immunosuppressive activities. Tumor-induced immature DCs induce tolerance determining abortive proliferation or anergy of antigen-specific T cells and through the generation of regulatory T cells and myeloid derived suppressor cells (Ma et al., 2012).

Within tumor microenvironment, several chemotactic substances, such as IL-8 and CXCL-2, are responsible for neutrophils recruitment and ensure the constant replenishment of these short-lived cells. The presence of tumor-associated neutrophils (TAN), clinically associated with poor prognosis, impacts tumor immune surveillance, metastasis, angiogenesis, and cellular proliferation. β2 integrin, granules-derived proteinases and elastase are the neutrophil-derived mediators involved respectively in metastasis promotion, in extracellular matrix remodeling and in tumor cell proliferation induction. In addition, TANs are an important source of pro-tumoral MMP-8 and MMP-9 that are associated with increased cell binding to laminin and collagen and metastasis reduction. In cancer, ROS production by TANs has been reported to exert genotoxic effects involved in tumor promotion and initiation but, conversely, also cytotoxic effects leading to tumor regression (Gregory and McGarry Houghton, 2011). Despite the relevance of TAN pro-

tumoral functions, emerging data indicate that neutrophils may be manipulated (i.e. through IFN-β or inhibition of TGF-β) to obtain an antitumor phenotype with cytotoxicity against tumors (Fridlender et al., 2009; Jablonska et al., 2010).

Conversely to the described tumor-promoting populations, Natural Killer (NK) cells play a role in tumor immune surveillance, through the direct lysis of tumor cells and the potential to induce a functional T cell-mediated antitumor immunity. Therapeutic approaches to stimulate NK activity, such as cytokine transfer (IL-2 or IL-15) to increase NK cytotoxic activity or tumor-targeted monoclonal antibodies to induce NK cell antibody-dependent cell-mediated cytotoxicity, are emerging in the field of antitumor immunotherapies (Langers et al., 2012).

Moving to adaptive immune cells, the aforementioned concept of cancer immunoediting and immune-surveillance assumes the presence of both tumor antigen specific-$CD4^+$ and $CD8^+$ T cells within cancer microenvironment. In fact the frequency of tumor infiltrating lymphocytes (TIL) is associated with an improved prognosis for a number of different tumor type, so that the type and density of lymphocytes infiltrating these cancers may be more powerful prognostic indicators than previous pathological criteria for tumor staging (Pagès et al., 2009). To exert maximal antitumor effects, a Th1-associated helper and cytotoxic response is most beneficial, and the expression of IFN-γ, granzyme B and perforin results in prolonged disease-free survival. Tumor immunity is usually mediated by activated cytotoxic T lymphocytes that require a Thl-type cytokine support, whereas in cancer a shift from Thl- to Th2-helper response may play an important role in tumor immune escape and progression. Remarkably, growing tumors adopt several other strategies to circumvent a functional antitumor response, such as generating ineffective antigen presenting cells and/or creating an immunosuppressive microenvironment. In this field, regulatory T cells (Treg) play an important dual role since they can both down-regulate inflammation correlated to tumor progression or, otherwise, they can suppress antitumor immunity, beneficial for cancer growth. The last consideration explains why Treg accumulation (and more relevant the decrease ratio of $CD8^+$ T cells to Treg cells) in cancer patients usually predicts poor outcome. This paradigm seems particularly appreciable for inflammation-associated cancer, such as colitis-associated colorectal carcinoma. In these models, the predisposing phase of chronic inflammation is associated with an accumulation of Tregs that act to limit tumor promotion and development; whereas in the presence of established tumor, Tregs participate in the elimination of antitumor functions of effectors cells, thus benefiting the tumor growth. Tumor-associated Tregs are stronger suppressive adaptive or inducible Treg (iTreg, Tr1) and differ from thymus-derived natural Tregs. In fact tumors may induce the generation of Tregs

by driving the differentiation of $CD4^+$ T cells reactive to tumor-associated antigens into iTregs (Facciabene et al., 2012; Savage et al., 2012).

It remains to be elucidated the important role of two other subsets of innate immune cells in carcinogenesis, that are MDSCs and MCs. Since the work of this thesis is focused on the interplay between these two populations, their biology and involvement in cancer development will be discussed in detail in the following paragraphs.

Chapter 2: MYELOID DERIVED SUPPRESSOR CELLS

Tumor-associated inflammatory response and tumor-induced immune suppression are likely to be an impairment to immunotherapy and immune-surveillance in patients and experimental animal models with malignant tumors. Multiple mechanisms are thought to facilitate tumor-induced immune suppression and myeloid-derived suppressor cells (MDSC) are one of the major components of the immune-suppressive network responsible for T cell defects in cancer and of the immune suppressive microenvironment that favors tumor growth. MDSCs, described for the first time more than 20 years ago in a patient with cancer, are a heterogeneous population with a very rapid turnover and they accumulate as the result of a partial block in immature myeloid cells differentiation in cancer and other pathological conditions, such as infectious diseases, sepsis, and trauma. In fact although most of the knowledge about MDSC biology and role in immune responses comes from works in the field of cancer research, recent evidences and accumulating data underline the regulating role of MDSCs during bacterial and parasitic infection, inflammation, trauma, sepsis and transplantation. In cancer setting, their activation results in the generation of a population with immune suppressive activity that regulates immune responses and non-immunological aspects, such as tumor angiogenesis and metastasis (reviewed in Gabrilovich and Nagaraj, 2009).

2.1 Origin and activation of MDSCs in cancer

MDSCs are an heterogeneous population of early myeloid progenitors, immature granulocytes, macrophages, and dendritic cells at different stages of maturation prevented from fully differentiation into mature cells (reviewed in Gabrilovich and Nagaraj, 2009). This mixture of myeloid cells, those have a granulocytic or monocytic morphology, lacks the expression of surface markers specifically expressed by mature myeloid cells. In mice MDSCs are broadly defined as double positive for the expression of myeloid markers Gr-1 and CD11b (Gabrilovich et al., 2007). The receptor Cd11b is a α_M-integrin expressed on monocytes/macrophages, dendritic cells, granulocytes and activated B- and T cells. The myeloid lineage differentiation antigen, Gr-1, is normally expressed on myeloid precursor cells, on mature granulocytes and transiently on monocytes. Since the antibody specific for Gr-1 recognizes a common epitope on the myeloid Ly6G and Ly6C antigens with varying levels of reactivity, this results in the identification at least of two major subsets of MDSCs, the so-termed granulocytic polymorphonuclear ($CD11b^+Ly6G^+Ly6C^{low}$, **PMN-MDSC**) and monocytic ($CD11b^+Ly6G^-$

Ly6C^{high}, **M-MDSC**) types (Movahedi et al., 2008). The two subsets may have different functions in cancer and other diseases and use different mechanisms to suppress T-cell function. The definition of MDSCs in human is complicated by the extreme heterogeneity of immature myeloid cells, most commonly defined as CD14^{-}CD11b^{+}, CD33^{+} cells, that lack the expression of mature specific markers of myeloid and lymphoid lineage (Almand et al., 2001). Overall, current data suggest that in humans MDSCs are better defined as a group of phenotypically heterogeneous myeloid cells with common biological activity that merely as a defined subset of cells.

MDSC systemic expansion was first described in tumor-bearing mice, both in transplantable and in spontaneous tumors developed in transgenic mice. Accumulation involves spleen (where MDSCs are 20-40% of nucleated splenocytes in contrast to 2-4% seen in normal mice), tumor tissue and lymph nodes of tumor-bearing mice, with granulocytic subset expansion higher than monocytic type (Youn et al., 2008). A recent report by Gabrilovich's group illustrates as PMN-MDSC predominant accumulation is partly due to the preferential differentiation of accumulating M-MDSC toward PMN-MDSCs in cancer (Youn et al., 2013).

In human, up to a tenfold increase in MDSCs was found in the blood of patients with cancer.

The expansion and activation of MDSCs is influenced by several different factors (i.e. cyclooxygenase 2, prostaglandins, stem-cell factor, IL-6, M-CSF, GM-CSF, and VEGF) produced primarily by tumor cells, with the contribution of stromal cells, T cells and other inflammatory cells, that stimulate myelopoiesis and prevent differentiation of immature myeloid cells. Many of these soluble factors are identified with chronic inflammation. Early studies demonstrated that the inflammation-associated molecules VEGF and GM-CSF were associated with the accumulation of MDSCs (reviewed in Ostrand-Rosenberg and Sinha, 2009). Subsequently the pro-inflammatory cytokines IL-1β (Bunt et al., 2006; Song et al., 2005) and IL-6 (Bunt et al., 2007) were shown to induce MDSCs. IL-1β-induced MDSCs have enhanced suppressive activity and are longer lived in *vivo*. In addition, stem-cell factor (SCF), expressed by various human and murine carcinomas, is involved in MDSC expansion and blocking SCF function can enhance immune-cancer therapy through prevention of tumor-specific T-cell anergy, Treg development, and tumor angiogenesis (Pan et al., 2007). Among the chemokines with tumor-promoting activities, CCL-2 is involved in efficient migration and in the promoting tumor role of MDSCs, which ubiquitously express CCR-2 (Huang et al., 2007).

PGE2, produced by tumor cells and by tumor milieu, is a known inducer of MDSCs (Sinha et al., 2007b) and, similarly, COX-2 inhibition blocks MDSC activation (via arginase 1) (Rodriguez et al., 2005). Proteins S100A8/A9 mediate MDSC accumulation and activation blocking the

differentiation of myeloid precursors into differentiated DC and macrophages through a Stat-3 dependent mechanism (Cheng et al., 2008) and exert chemoattractant effects on MDSCs toward tumor sites through a NF-κB-dependent pathway (Sinha et al., 2008). MDSCs also produce proteins S100A8/A9, with an autocrine positive feedback important for MDSC expansion (Cheng et al., 2008). The complement component C5a is another inflammatory factor that increases the migration of granulocytic MDSCs, but not monocytic subset, into solid tumors and peripheral lymphoid organs (Markiewski et al., 2008). In addition it has been proposed that, in murine models of cancer, IL-17 promotes tumor growth through recruitment and generation of functional suppressive MDSCs (He et al., 2010).

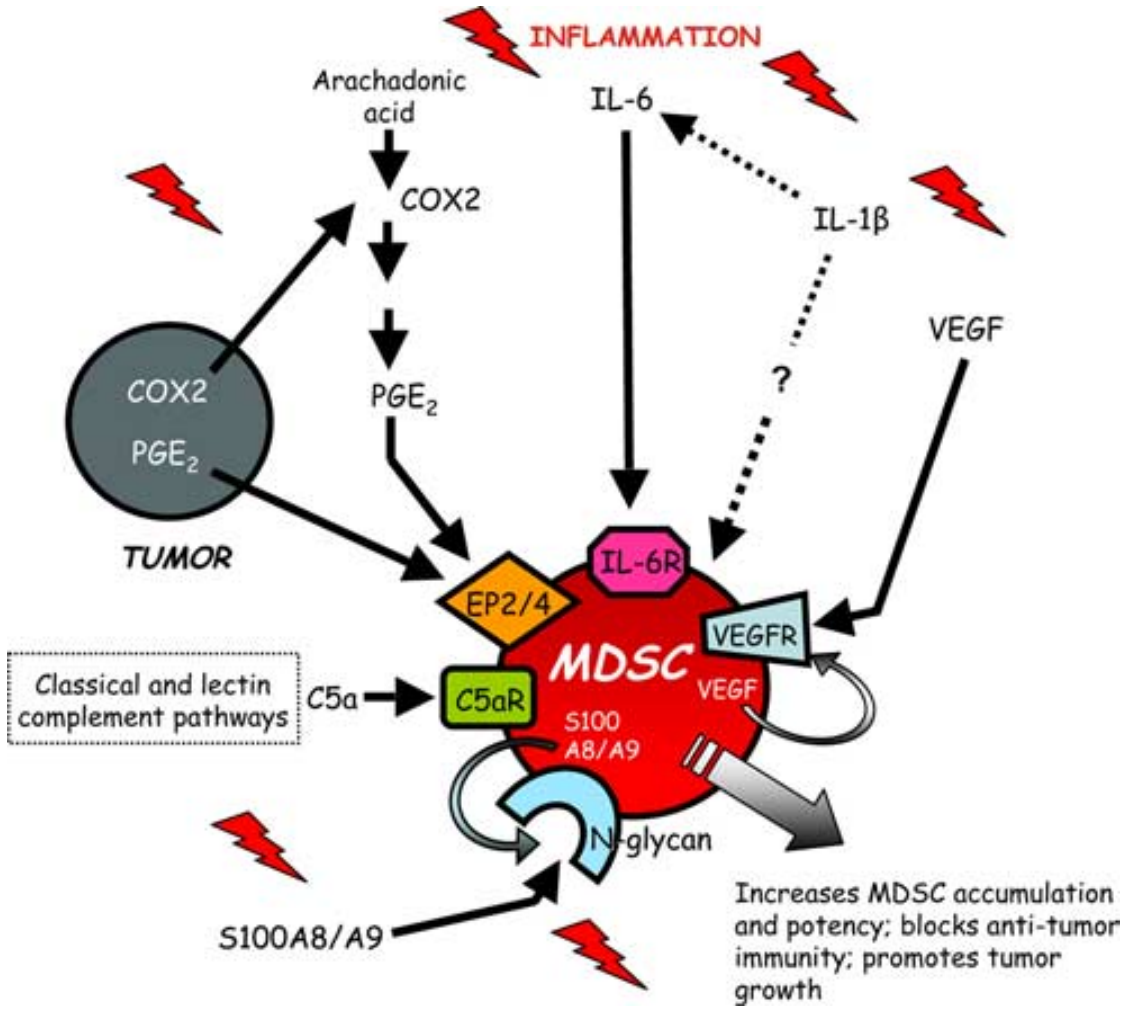

Figure 2.1: Induction and activation of MDSCs. MDSCs accumulate at tumor site and in the peripheral lymphoid organs in response to multiple pro-inflammatory molecules produced by tumor cells or by host cells in the tumor microenvironment (Adapted from Ostrand-Rosenberg and Sinha, J Immunol 2009).

Most of MDSC-recruiting factors trigger also an activation signaling in suppressor cells, with Stat-3 as the main transcription factor that regulates MDSC expansion and activation, as well as the survival and proliferation of myeloid progenitor cells (Nefedova et al., 2004). In myeloid

cells, Stat-3 signaling drives the expression of Bcl-xL, c-myc, cyclin D1 or survivin, which promote cell proliferation, preventing cell apoptosis and differentiation to mature cell types (Yu et al., 2009). Several pathways downstream of Stat-3 might be involved in the regulation of MDSC expansion and function. Activation of Stat-3 in hematopoietic progenitor cells (HPC) up-regulates S100A8 and S100A9 that in turn inhibit DC differentiation and promote MDSC accumulation (Cheng et al., 2008). Stat-3 dependent up-regulation of the transcription factor CCAAT-enhancer-binding protein beta (C/EBPβ) is also reported to play a crucial role in controlling the differentiation of myeloid precursors to functional MDSCs. In fact C/EBPβ induction by cytokines produced within microenvironment is involved in the regulation of MDSC numbers and function, regulating response to GM-CSF and G-CSF (Marigo et al., 2010).
To mention, in MDSCs from tumor-bearing mice there is a reduced expression of interferon regulatory 8 (IRF-8), which regulates late steps of granulocytes maturation. In addition reduced levels of IRF-8, which negatively controls the expression of several anti-apoptotic genes, can lead to resistance to apoptosis with accumulation of immature myeloid cells (Stewart et al., 2009a; 2009b).
The importance of TNF-α in MDSC biology has also emerged in the last years. The previously reported tumor rejection in mice deficient in both TNFRs (Wu et al., 2004) has been associated with impaired MDSC accumulation. In fact TNF-α signaling trough TNFR-2 protects MDSCs from apoptosis via c-FLIP, a caspase-8 inhibitor up-regulated after NF-κB activation, sustaining their accumulation and helping tumor cell evasion from immune system (Zhao et al., 2012).
Other factors, produced in cancer microenvironment such as IFN-γ, ligands for TLRs or IL-4, are crucial for the complete activation of MDSC suppressive potential and several signaling pathways and transcription factors (i.e. STAT-6, STAT-1, NF-κB) are involved (reviewed in Condamine and Gabrilovich, 2010). **STAT-1** is the main transcription factor activated by IFN-γ or IL-1β signaling and is particularly important for its implication in the regulation of inducible nitric oxide synthase (iNOS) and arginase activity (Kusmartsev and Gabrilovich, 2005; Movahedi et al., 2008). **STAT-6** activation in MDSCs occurs in response to the binding of IL-4 or IL-13 to the receptor CD124, responsible for the up-regulation of arginase activity and improvement of MDSC activity (Bronte et al., 2003; Sinha et al., 2005). The Toll-Like Receptor (TLR) family plays a prominent role in **NF-κB** activation in myeloid cells, primarily via the MyD88 adaptors, and could promote MDSC expansion. In fact *Myd88*$^{-/-}$ MDSCs have reduced ability to suppress T cell activity and to release cytokines compared to the wild type counterpart (Arora et al., 2010). TLR-NF-κB axis activation is consistent with MDSC accumulation and

activity during microbial and viral infections, as well as in trauma and sepsis. IL-1β is also able to activate MDSCs through NF-κB pathway (Tu et al., 2008).

2.2 Mechanisms of MDSC suppressive activity

The main feature of MDSCs is their ability to suppress T cell responses (reviewed in Ostrand-Rosenberg, 2010), but MDSCs suppress immunity also by perturbing both innate and adaptive immune responses and acting on different non-T cell-populations.

MDSCs suppress antitumor immunity through a variety of different mechanisms. Suppressive activity requires cell-to-cell contact and can be Ag-specific or non-specific depending on the MDSC subpopulation involved (reviewed in Ostrand-Rosenberg and Sinha, 2009).

The increased production of Reactive Oxygen Species (**ROS**) is one of the main features of MDSCs isolated from tumor-bearing mice and patients with cancer. Antigen-specific inhibition of $CD8^+$ T cell response in cancer is mediated by ROS, mainly in the form of H_2O_2, and requires the presence of Ag and the direct contact between MHC-I^{pos} MDSCs and Ag-specific $CD8^+$ T cells. In this setting, the ligation of integrins expressed by MDSCs up-regulates ROS production by MDSCs (Kusmartsev et al., 2004). The increased ROS production by MDSCs is mediated by the up-regulated activity of NADPH oxidase (NOX2), which expression is controlled by the STAT-3 transcription factor. In fact in the absence of NOX2 activity, MDSCs lose their ability to suppress T cell responses and they differentiate into mature macrophages and dendritic cells (Corzo et al., 2009).

The metabolism of L-arginine, which serves as a substrate for iNOS (which generate nitric oxide) and arginase 1 (that generate urea and L-ornithine), has been associated with the suppressive activity of MDSCs. Increased **arginase 1** levels enhance L-arginine catabolism, depleting this amino acid essential for protein synthesis from microenvironment. Loss of L-arginine is involved in T-cell decrease of CD3 receptor (Rodriguez et al., 2005) and in prevention of cell cycle regulators cyclin D3 and cyclin-dependent kinase 4 up-regulation with the arrest in the G_o–G_1 phase of the cell cycle (Rodriguez et al., 2007).

Different mechanisms are involved in **nitric oxide** (**NO**)-dependent suppression of T-cell function. Experiments with iNOS knockout mice demonstrated that the inhibition of T cell proliferation by $CD11b^+Gr\text{-}1^+$ cells is dependent upon NO production (Mazzoni et al., 2002). In addition NO mediates a marked reduction of tyrosine phosphorylation of Jak-3, STAT-5, Akt and extracellular signal-regulated kinases in T cells, rendered them unable to proliferate (Bingisser et al., 1998; Mazzoni et al., 2002). NO attenuates MHC class II induction through negative effects on gene expression and induces T cell apoptosis (Harari and Liao, 2004).

The chemical reaction between NO and superoxide anion generates **peroxynitrites**, powerful oxidants and new emerging mediators of MDSC suppression function. Peroxynitrites promote the impairment of tyrosine phosphorylation and apoptotic death, inhibiting T lymphocyte activation and proliferation (Brito et al., 1999). High levels of peroxynitrites are associated with tumor progression and inhibition of antitumor T-cell immune response. In human prostate adenocarcinomas, nitrotyrosines are highly expressed in unresponsive $CD8^+$ T cells suggesting peroxynitrites production in tumor microenvironment. After inhibition of the activity of arginase 1 and iNOS, T-cell responsiveness is restored and nitration tyrosine levels are reduced (Bronte et al., 2005). Furthermore, MDSCs disrupt the binding of specific peptide–MHC dimers to $CD8^+$ T cells through nitration of tyrosines in a T-cell receptor (TCR)-CD8 complex. Nitration state of TCR-CD8 is induced by MDSCs through hyperproduction of ROS and peroxynitrite during direct cell-to-cell contact and a peroxynitrite scavenger completely eliminates MDSC-induced T-cell tolerance. This process makes $CD8^+$ T cells unable to respond to the specific peptide, although they retain their ability to respond to non-specific stimuli (Nagaraj et al., 2007). MDSCs, distributed between various lymphatic organs, also suppress T-cell proliferation by down-regulation of TCR-associated ζ-chain, rendering T cells unable to transmit activation signals (Ezernitchi et al., 2006).

MDSCs, likely through plasma membrane expression of ADAM17, down-regulate T cell levels of L-selectin (CD62L), a molecule necessary for migration of naïve T cells to lymph node. In this way, activation of T cells by tumor antigens is prevented (Hanson et al., 2009).

Moreover, MDSCs inhibit T cell activation by **cysteine deprivation**, an amino acid essential for T cell during antigen presentation, activation and proliferation. In fact, during antigen presentation and T-cell activation, T cells, which are unable to produce their own cysteine because they lack cystathionase (required to convert intracellular methionine to cysteine) and the xc−transporter (to import disulfide-bonded cystine from the extracellular environment), import cysteine through their ASC neutral amino acid transporter typically from macrophages and dendritic cells. Instead MDSCs express the xc−transporter and are able to import cystine but they do not express the ASC transporter and subsequently they do not export cysteine. This means that, when MDSCs are present at high concentrations, they can consume and compete for extracellular cystine, thereby depriving T cells of the cysteine they require for activation and function (Srivastava et al., 2010).

Recent data showed that the two major described populations of MDSCs might be discriminate on the basis of subset specific suppressive mechanism. In fact granulocytic MDSCs have increased levels of ROS and undetectable level of NO; conversely monocytic MDSCs have

increased levels of NO and undetectable level of ROS. Notwithstanding, total suppressive activity is comparable (Youn et al., 2008).

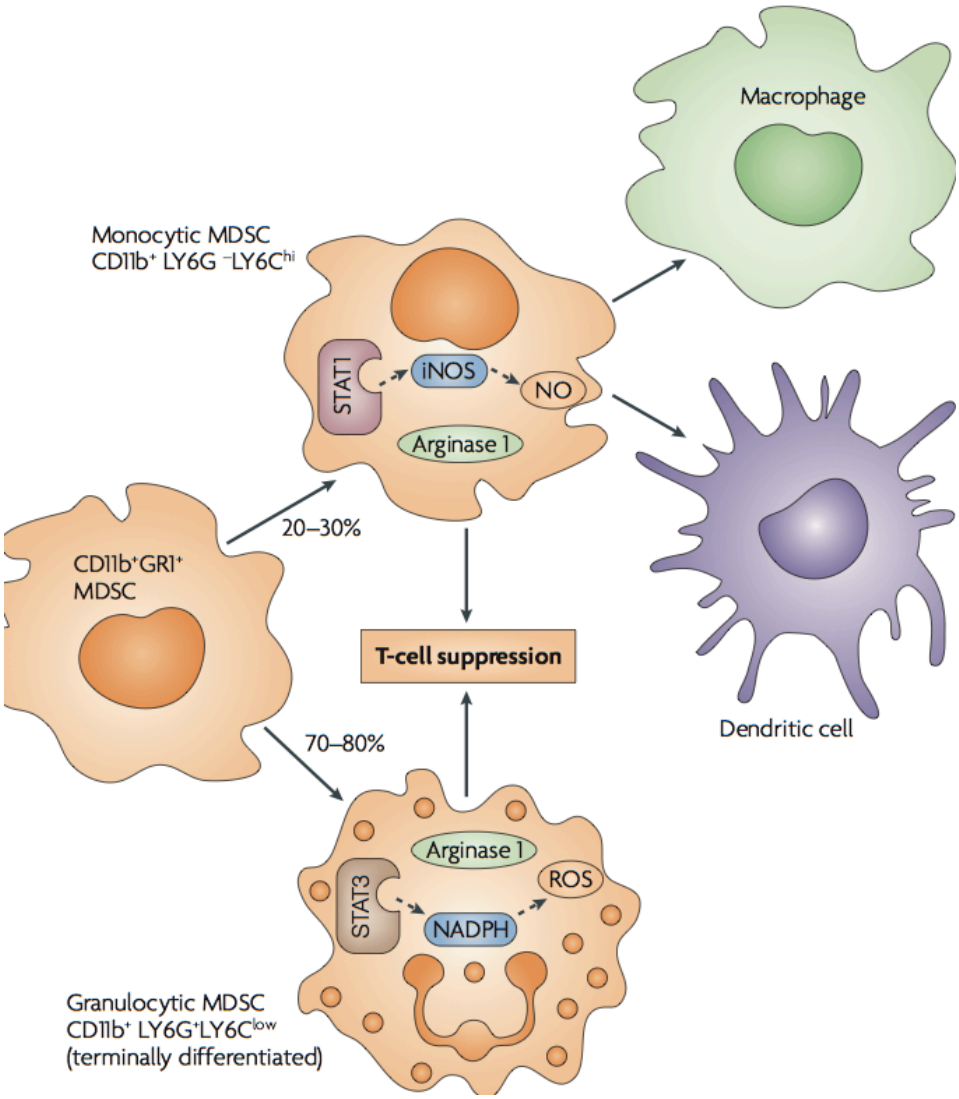

Figure 2.2: Suppressive mechanisms mediated by different types of MDSCs. MDSCs consist of two main subsets that differ for the preferential suppressive mechanism: monocytic MDSCs (M-MDSC), which have a $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ phenotype, and granulocytic MDSCs (PMN-MDSC), which have a $CD11b^{+}Ly6G^{+}Ly6C^{low}$ phenotype. PMN-MDSCs have increased high levels of ROS but low NO production. M-MDSC subset has up-regulated expression of STAT1 and iNOS, with increased levels of NO but low ROS production. Only M-MDSCs can differentiate into mature dendritic cells and macrophages in *vitro* (Adapted from Gabrilovich and Nagaraj, Nat Rev Immunol 2009).

It's important to clarify the issue of antigen-specific nature of MDSC effects on T cells. In the peripheral lymphoid organs, suppression of $CD8^{+}$ T cells requires three factors: activated antigen-specific $CD8^{+}$ T cells, tumor-associated antigen and MDSCs. The outcome of this direct cell-to-cell interaction (depending on MHC class I : Ag : TCR recognition phase) results in

antigen specific T cell tolerance, mainly mediated by PMN-granulocytic MDSCs that are prevalent in lymphoid organs in tumor-bearing hosts and contain high levels of ROS and peroxynitrites. These T cells maintain the capability to respond to other non-specific stimuli, such as anti-CD3/CD28 antibody, co-stimulatory molecules or IL-2 (reviewed in Nagaraj and Gabrilovich, 2012). On the other side, $CD4^+$ T cell inhibition by MDSCs requires the expression of MHC class II by MDSC and is dependent on the presence of specific peptide. Furthermore, the direct interaction between $CD4^+$ T cell and MDSCs can result in the conversion of MDSCs to non-specific suppressor cells, via up-regulation of Cox-2 and PGE-2 (Nagaraj et al., 2012). So, in the tumor microenvironment, MDSCs inhibit T cell function, and at the same time, activated $CD4^+$ T cells convert MDSC to non-specific suppressors, amplifying their inhibitory potential. In addition, recent data pointed out that inflammation-dependent functional regulation of MDSCs exert a critical contribute to the nature of MDSC "phenotype" and explain the profound immune suppression observed within the tumor microenvironment. It is widely demonstrated that in *vitro* MDSCs isolated and/or purified from multiple tissue sites (i.e. spleen, lymph nodes, tumor) have suppressive activity, but recently attention was focused on the relative capacity of MDSCs in *vivo*. Haverkamp and co-workers demonstrated that, in a model of prostate inflammation and cancer, MDSCs from inflammatory sites or within tumor possess intrinsic capacity to suppress T cells, whereas those isolated from peripheral tissues (such as spleens and liver) are not suppressive without activation of iNOS by exposure to IFN-γ. These data suggest that only MDSCs present in the tumor microenvironment possess the immediate capacity to regulate T-cell activation (Haverkamp et al., 2011).

Similarly it has also been demonstrate that although MDSCs from peripheral lymphoid organs and at the tumor site share similar phenotype and morphology with a similar mixed granulocytic and monocytic subset distribution, they display profound functional differences. In fact MDSCs from peripheral lymphoid organs (i.e. spleen) suppress the proliferation and activation of antigen-specific stimulated T cells but are not able to interfere with non-specific T cell activation. In contrast, tumor-derived MDSCs suppress both antigen-specific and non-specific T cell activation and proliferation. Highly suppressive MDSCs from tumor site show up-regulation of arginase I and iNOS, accompanied by down-regulation of ROS, which conversely is the preferential suppressive mechanism of peripheral MDSCs. Hypoxia, via HIF-1α signaling, was found to be responsible for MDSC differentiation in tumor microenvironment and to produce the conversion of splenic MDSCs to non-specific suppressors and their preferential differentiation to macrophages (Corzo et al., 2010).

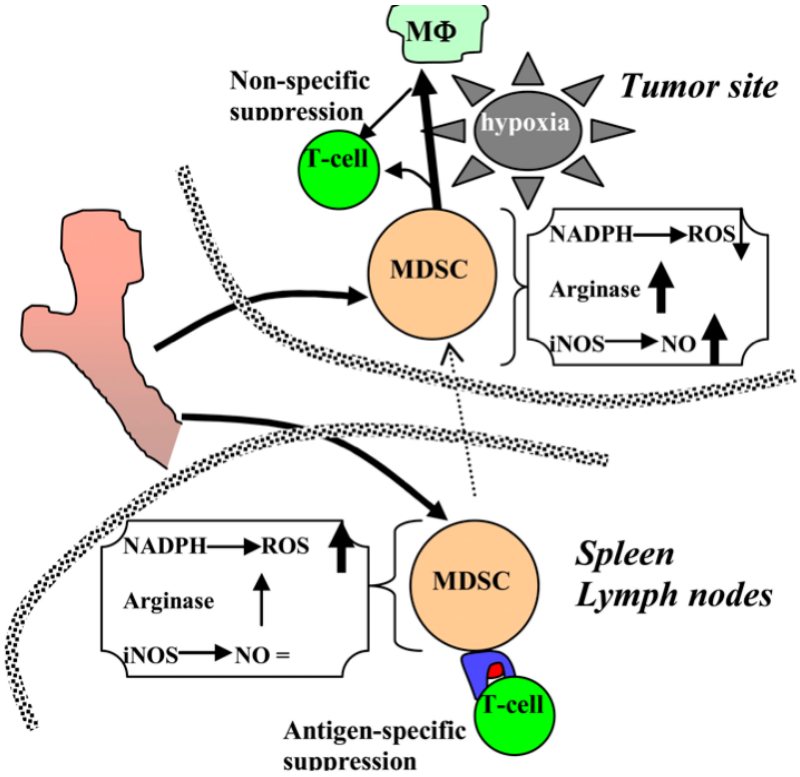

Figure 2.3: MDSC function and differentiation in cancer. Tumor microenvironment and the associated hypoxia cause the conversion of peripheral MDSCs from ROS-dependent T-cell antigen specific suppressors to nonspecific suppressors characterized by up-regulated levels of arginase I and iNOS in the tumor site (Adapted from Corzo *et al*., J.Exp.Med 2010).

2.3 MDSC cross talk with other immune cells in tumor microenvironment

In addition to the described specific mechanisms of T-cell proliferation inhibition, MDSC suppression in tumor microenvironment is exerted also by indirect effects, such as influence of **regulatory T cells** (Treg) biology. MDSCs can induce the development of suppressive Tregs in *vivo*. This was found to require antigen-associated activation of tumor-specific T cells and to depend on the presence of IFN-γ and IL-10 (Huang et al., 2006). In a murine lymphoma model, MDSCs are described as tolerogenic APCs capable of antigen uptake and presentation to tumor-specific Tregs, mediating Treg induction through a mechanism involving arginase 1 (Serafini et al., 2008). In another recent study, Pan and co-workers demonstrated that the expression of the immune stimulatory receptor CD40 on MDSCs is required to induce T-cell tolerance and Treg accumulation in *vivo* (Pan et al., 2010).

Bidirectional cross talk between MDSCs and **tumor-associated macrophages** (TAM) exacerbates immune suppression. Cell contact between MDSCs and TAMs, in fact, results in IL-10-dependent-MDSC mediated down-regulation of IL-12 production by macrophages. M2-like

TAMs exacerbate their polarization because they stimulate MDSCs to produce more IL-10. This increased production of IL-10 and the reduction of IL-12 level favor the development of Th2 cells, with a decrease in NK cell activation, counteracting cytotoxic $CD8^+$ T cell development and enhancing immune suppression (Sinha et al., 2007a). Inflammatory milieu, for example under condition of high IL-1β levels, increases IL-10 production by MDSCs, that is further amplified by the presence of IL-6-producing macrophages (Bunt et al., 2009). MDSC-macrophage cross talk experiments were performed in presence of LPS and/or with TRL4-deficient mice, underlining that the increase in IL-10 is mediated by signaling through TLR4 in both cell subsets (Bunt et al., 2009). In addition, MDSCs render macrophages less effective antigen presenting cells by alteration of MHC-II molecules in a cell-to-cell contact and IL-10 dependent mechanism, further diminishing T cell activation (Clements and Ostrand-Rosenberg, unpublished results; reviewed in Ostrand-Rosenberg et al., 2012). Although an inflammatory microenvironment is crucial for their accumulation and the production of pro-inflammatory mediators, IL-10 production by MDSCs implicates a condition of inflammation reduction. This contradictory situation is explained by the requirement of chronic inflammation to promote the development of MDSCs and TAMs, whereas anti-inflammatory IL-10 is important to switch off acute inflammation that have the potential to mediate T cell activation and tumor rejection (discussed in Ostrand-Rosenberg et al., 2012).

The expansion of MDSCs during tumor growth is inversely correlated with **natural killer** (NK) activation. MDSCs were shown to suppress NK cell cytotoxicity by inhibition of perforin, but not granzyme B production, and by Stat-5 activation in IL-2 stimulated NK cells. The inhibition of NK cell cytotoxicity is cell-to-cell contact dependent (Liu et al., 2007). Inflammation, via IL-1β, is able to induce a subset of highly suppressive $Ly6C^{neg}$ MDSCs that block NK cell differentiation and functions in *vitro* and in *vivo* (Elkabets et al., 2010). Of note, an unexpected activating role of MDSCs on NK cells has been reported: in this paper, M-MDSCs do not suppress but activate NK cells to produce high amounts of IFN-γ and, in addition, after activation NK cells kill the same MDSCs (Nausch et al., 2008). This study reveals that MDSCs do not necessarily suppress all aspects of antitumor immune responses.

Tumor microenvironment is characterized by accumulation of immature **dendritic cells** (DC) and apoptotic DCs, that can present antigens but promote Th-2 responses, secrete TGF-β to induce regulatory T cells and induce anergy favoring tumor evasion (Bennaceur et al., 2008). Reduction in DC number in cancer patients is associated with the preferential accumulation of MDSCs instead of DCs, due to common MDSC/DC progenitor. In addition MDSCs induce a decrease in DC maturation, in their ability to take up antigen, to migrate and induce full

competent T cells (Greifenberg et al., 2009; Poschke et al., 2011).

Summarizing, in addition to their intrinsic immune suppressive functions on T cell populations, MDSCs amplify the immune suppressive microenvironment through cross talk with macrophages, NK cells and dendritic cells.

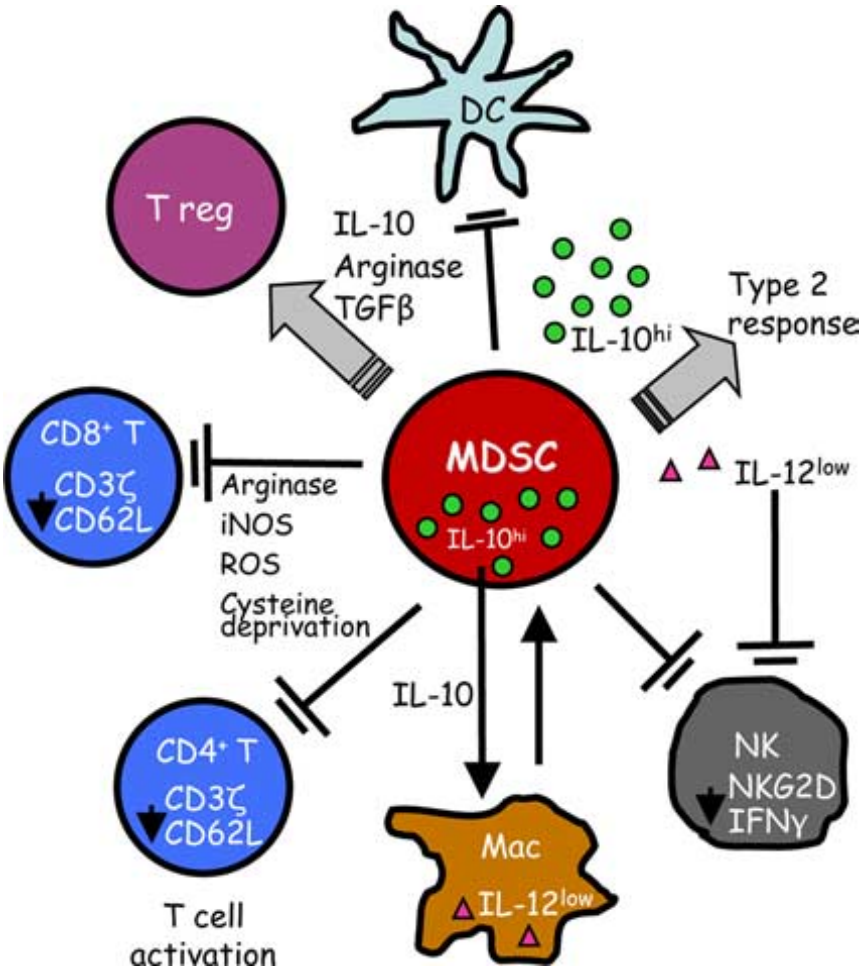

Figure 2.4: MDSC suppression of antitumor immunity. MDSCs use a diversity of mechanisms to suppress antitumor response: T cell activation is directly suppressed by the production of arginase, nitric oxide, ROS and through cysteine deprivation, with concomitant induction of Tregs. MDSC cross talk with macrophages, dendritic cells and NK cells impairs innate immunity and drives the generation of an immunosuppressive microenvironment (Adapted from Ostrand-Rosenberg and Sinha, J Immunol 2009).

2.4 Non-immune suppressive functions of MDSCs: angiogenesis and tumor invasion promotion

MDSCs modulate tumor microenvironment through their **pro-angiogenic** functions and tissue remodeling properties. In fact, tumor-infiltrating MDSCs promote tumor vascularization, growth, and metastasis through modulation of VEGF bioavailability and protease activity in the tumor microenvironment. Increased vascular density, vascular maturation, and decreased

necrosis of tumor is linked with accumulation of MMP9-producing MDSCs, found to directly incorporate into tumor endothelium and acquire endothelial cell properties with expression of both VEGFR2 and VE-Cadherin (Yang et al., 2004). They can also directly secrete other pro-angiogenesis factors, such VEGF and bFGF to promote tumor angiogenesis, via Stat-3 signaling activation (Kujawski et al., 2008). The pro-angiogenic function of suppressive myeloid cells is sufficient to confer tumor refractoriness to anti-VEGF treatment, an essential regulator of blood vessel growth (Shojaei et al., 2007). Regulator of G protein signaling-2 (Rgs2) is a critical regulator of the pro-angiogenic function of MDSCs in the tumor microenvironment, since $Rgs2^{-/-}$ MDSCs produce less CCL-2 leading to decreased angiogenesis (Boelte et al., 2011).

Furthermore, MDSCs are closely related to tumor **invasion** and **metastasis** by increasing production of MMPs and chemoattractants as well as creating a pre-metastatic environment niche. In this context MDSCs, recruited in the invasive front of tumor tissue (by different chemokines axis, such as SDF-1/CXCR4 and CXCL5/CXCR2), promote tumor invasion and metastasis through production of multiple MMPs (i.e. MMP-2, -13, -14), which play an essential role in matrix degradation. At the same time, secreted cytokines and growth factors produced by hypoxic primary tumor cells (such as CCL-2) are involved in creating a permissive niche for metastasis, which is populated by a specific subset of MDSCs (that represent the granulocytic $CD11b^{+}Ly6c^{med}Ly6G^{+}$ population). Myeloid cell accumulation is correlated with a reduced antitumor activity of NK cells: in this way, hypoxia-induced microenvironment is further able to support metastatic growth of disseminated tumor cells (Sceneay et al., 2012). Another hypothesis relies on the cancer cell fusion with migratory bone marrow-derived cells to explain metastasis: MDSC could be able to make hybrid with the cancer cell, generating a metastatic phenotype cell (Pawelek and Chakraborty, 2008).

2.5 IFN-γ signaling in MDSC biology

As previously described, MDSCs could be induced by different factors (including GM-CSF, IL-1β, IL-6, COX-2 and PGE-2, S100A8/A9 and VEGF), but there is not a single molecule essential for their generation. In contrast, IFN-γ has been reported as necessary for the development and the nitric oxide (NO)-mediated suppressive activity of M-MDSCs.

First reports demonstrated that NO is the major mediator of bone marrow-derived natural suppressors, a subset of strong inhibitors of lymphoproliferative responses. In this context, NO is produced through an IFN-γ-dependent inducible-Nitric Oxide Synthase (iNOS) induction (Angulo et al., 1995). This signaling is triggered in early myeloid cells by activated T cells that are the initial source of IFN-γ, in a mechanism involving CD40-CD40L axis and the production

of TNF-α and/or IL-1α in the system (Angulo et al., 2000).

Subsequent works confirmed that $CD11b^{+}Gr\text{-}1^{+}$ MDSCs derived from tumor-bearing mice mediate suppression through NO, which is secreted in response to signals from activated T cells, including IFN-γ and a contact-dependent stimulus (Mazzoni et al., 2002).

Despite these observations, recent findings indicate that, although MDSCs express functional IFN-γ receptor, in *vivo* IFN-γ does not impact the phenotype (comparable levels of CD11b, Gr-1, Ly6C, Ly6G, arginase, iNOS, or ROS, between tumor bearing-wild type, IFN-$\gamma^{-/-}$, and IFN-$\gamma R^{-/-}$ mice), accumulation, or T-cell suppressive functions of MDSCs (Sinha et al., 2012), assuming the existence of alternative and redundant pathways in regulation of MDSC suppressive function in *vivo*.

Nevertheless it's likely that IFN-γ delivering signals are crucial in the triggering phase of MDSC accumulation and activation, as shown in *vitro* for lack of MDSC-mediated suppressive function in presence of effector IFN-$\gamma^{-/-}$ T cells (Gallina et al., 2006). Therefore T cells activated with proliferative signals release IFN-γ that, possibly in conjunction with others signals, activates peripheral MDSCs. In a second phase, pre-activated suppressor cells begin to independently release IFN-γ, maintaining in an "autocrine circuit" the prolonged expression of iNOS and generation of NO to mediate inhibition of T cells proliferation (discussed in Gallina et al., 2006).

As a final outcome, nitric oxide produced by activated MDSCs leads to reduced IFN-γ responsiveness in T cells and NK cells, due to increased nitration of STAT-1 on tyrosine residues (Mundy-Bosse et al., 2011). The ability of MDSCs to make faulty levels of IFN-γ responsiveness represents another mechanism of tumor-induced immune suppression, since overexpression of IFN-γ in tumor microenvironment results in a marked delay of tumor growth concomitant with reduction of myeloid-derived suppressor cells (and Tregs) accumulation (Carrio et al., 2011).

Cyclophosphamide (CTX), a DNA alkylating agent, has been used as a chemotherapeutic drug, but its efficacy is complicated by MDSC increase in blood and lymphoid organs. In fact, recently, it has been demonstrated that, after CTX treatment, T cells undergo expansion and activation before the increase in MDSC population and they are involved in MDSC accumulation and survival through IFN-γ production (Guo et al., 2012).

Summing up, these results reveal a paradoxical finding: the expansion of IFN-γ-producing T cells, expected to mount a strong antitumor immune response, correlates with the increase and activation of MDSCs, one of the main cell populations that contributes to the negative regulation of antitumor response.

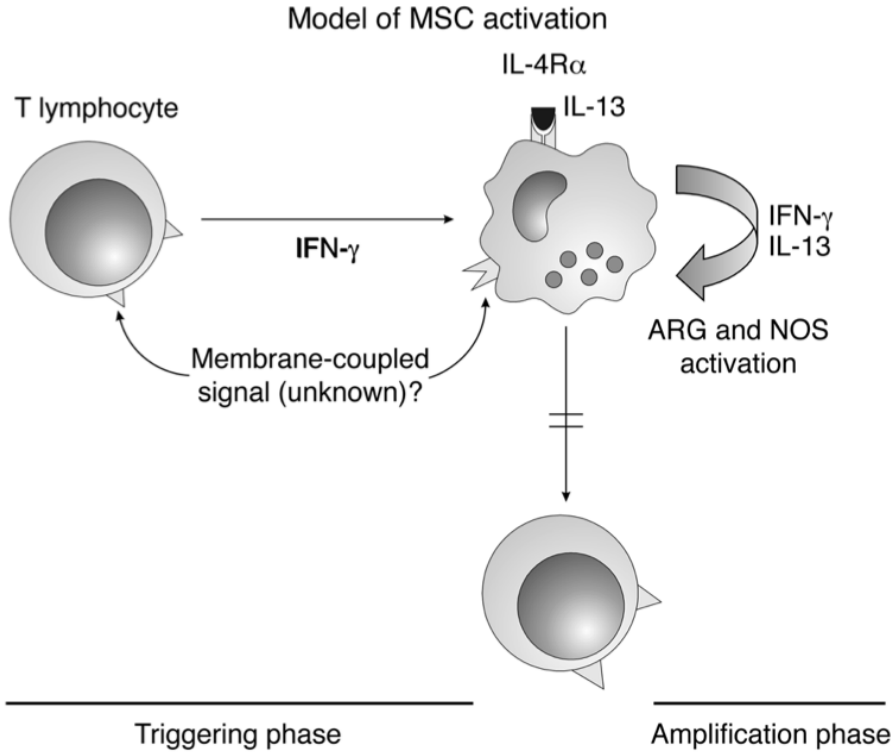

Figure 2.5: IFN-γ produced by T cells activates MDSCs. MDSCs require IFN-γ stimulation to become fully suppressive. In an initial "triggering phase" proliferating T cells release IFN-γ that activates peripheral MDSCs. Then, MDSCs begin their own IFN-γ production via an autocrine circuit and are able to maintain a prolonged activation of the enzymes iNOS and Arg I, which ultimately originate the immunosuppressive mediators acting on T cells ("amplification phase"). This model, proposed by Gallina and colleagues, explains the behaviour of spleen-derived MDSC, whereas tumor-infiltrating MDSCs present constitutively activation of iNOS and Arg I, which account for the prompt immunosuppression provided by these cells (Adapted from Gallina et al., 2006 J.Clin.Invest.).

2.6 Role of MDSCs in non-tumorigenic setting

In addition to their recognized role in tumor tolerance and their negative impact on host survival, MDSCs exert a role in the induction and maintenance of transplant tolerance. Due to their recruitment, accumulation and ability to inhibit allogeneic T cell responses, in murine models MDSCs have been described for their involvement in graft-versus-host-disease inhibition and prevention, in long-term survival of skin allograft and in tolerance of pancreatic islet and cardiac transplant. iNOS machinery activation, Arg1 up-regulation, expression by myeloid cells of regulatory transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) and the critical role of IFN-γ are the proposed mechanisms involved in MDSC-induced transplant tolerance (reviewed in Dilek et al., 2012). Elevated frequencies of circulating $CD14^-$ and $CD14^+$ MDSCs have recently been recorded in patients recipients of renal transplants, with potential relevance in transplant tolerance (Hock et al., 2012).

Similarly, in contrast to the proposed deleterious role in cancer, MDSCs have the potential to suppress the immune response, thereby limiting tissue injury, in the context of autoimmunity. Adoptive transfer of exogenous MDSCs in mouse model of inflammatory bowel diseases, alopecia areata or type 1 diabetes results protective and capable to suppress autoimmune injury. On the contrary, in *vivo*, such as in EAE models of multiple sclerosis, endogenous MDSCs seem to be not effective in mitigating autoimmune diseases and they potentially contribute to pathology. An intriguing hypothesis is that the dysfunction of endogenous MDSCs may be a factor driving autoimmune inflammatory pathology: MDSCs accumulate in response to inflammation but are not able to limit autoimmunity (Cripps and Gorham, 2011).

During acute infections or endogenous inflammation, the observed accumulation of MDSCs could be considered as a condition of "emergency myelopoiesis". These MDSCs retain the potential to further differentiate and mature, but during inflammation, the environmental milieu traps these cells in an immature and immunosuppressive phenotype. Studies of MDSCs during inflammatory responses, including trauma or burn injury and sepsis, suggest they can protect or damage the host. Attenuated peritoneal cytokine production, increased bacterial clearance and improved survival rate are the protective effects of MDSCs during sepsis (Derive et al., 2012). Conversely, another recent work demonstrated an heterogeneity of MDSC state and function as the sepsis inflammatory process progresses: they shift to a more immature state and from being pro-inflammatory to anti-inflammatory, with outcome from increased early mortality to long-term survival (Brudecki et al., 2012).

Parasitic infections are notoriously associated with the suppression of immune responses and MDSC recruitment and accumulation. In this regard, efficient strategies to increase myeloid cell generation have been developed by several parasites species, such as *Leishmania* and *Plasmodium* sp.; host factors and parasites-induced production of myelopoietic growth factors are involved in MDSC induction. During adaptive T-cell response against protozoa, IFN-γ produced by Th-1 oriented T cells is involved in the activation of iNOS machinery in recruited MDSCs, with the outcome of protective Th-1 immunity inhibition and switch to a Th-2 response. MDSC-dependent suppression of immune responses can play a critical role in the long-term persistence of parasites in the host, but at the same time it reduces parasite-induced morbidity, limiting the significant tissue damage that can arise as a consequence of an excessive effector response. Immunosuppressive activity makes MDSC potential targets for therapeutic intervention but their dual role may be considered and evaluated before contemplating therapies (Van Ginderachter et al., 2010).

Summing up, the potential protective roles of MDSCs in transplant tolerance or autoimmunity elucidate that these myeloid populations may act as a double edge sword and that they are not exclusively detrimental as in cancer microenvironment. In other terms, emerging reports show that MDSC accumulation must be considered more a normal reaction of the inflammatory response rather than simply a pathological response to a growing tumor.

Chapter 3: MAST CELLS

Mast cells (MC) are important effector and immunoregulatory cells derived from hematopoietic stem cells, described for the first time in 1878 by Paul Ehrlich that observed their "protoplasmic deposits" and named them "mastzellen" for well-fed, fat cells (Ehrlich, 1878). Although MCs were initially studied for their role in the pathogenesis of allergy (initially described as the cells that make humans "sneeze and wheeze") and other inflammatory diseases, they are increasingly viewed as being able to perform a spectrum of positive and negative immunoregulatory functions that can either exacerbate disease or help to sustain health.

3.1 Mast cell biology

MCs derive from multipotent hematopoietic stem cells in the bone marrow before entering in the circulation as committed MC precursors (MCPs) that reach peripheral tissues, where they complete differentiation and reside as mature cells. In particular MCs are derived from a Sca-1^{lo}-Common Myeloid Progenitor (SL-CMP), but not from the SL-Granulocytic Myeloid Progenitor (SL-GMP), suggesting that MC development is most closely associated with the megakaryocyte/erythrocyte lineage (Franco et al., 2010). The interaction of c-kit (CD117), a tyrosine-kinase receptor expressed in all stages of MC maturation, with its ligand Stem Cell Factor (SCF) is crucial for the appropriate differentiation, migration, accumulation and maturation of mast cells in periphery tissues (Okayama and Kawakami, 2006). MCs are widely distributed throughout all vascularized tissues, in close proximity to blood vessels, nerves, smooth muscle cells, mucus-producing glands, hair follicles and they are present in anatomical sites that are directly exposed to the external environment, such as skin, airways, and gastrointestinal tract.

Terminal differentiation driven by selective growth factors within different tissues gives rise to specific subsets of MCs with characteristic profiles of intracellular mediators, proteases, proteoglycans, and characteristic content of the granules in term of cytokines and lipid mediators. Classically, two main populations of MCs are described in mice: the first "connective tissue MC" (CTMC) population is characterized by safranin-positive granules and resides in connective tissue (present in the cute and in peritoneal cavity; they contain the proteoglycan heparin and large amounts of histamine and carboxypeptidase A); the second "mucosal MC" (MMC) population is safranine-negative and resides mainly in the mucosal epithelial surface of the intestine (their granules contain chondroitin sulfate as the major proteoglycan and relatively less levels of histamine and carboxypeptidase A).

Instead human mast cells are classified on the basis of proteases content in MC_{TC}, which expresses tryptase and chymase in its citoplasmatic granules (present in intestinal and nasal mucosa, lung alveolar wall), and in MC_T, which contains exclusively tryptase (mainly present in intestinal submucosa and skin) (Metcalfe et al., 1997).

A relevant characteristic of all MCs subsets is their ability to release a large amount of diverse biologically active mediators that can be divided in three groups: (i) preformed mediators, such as histamine, proteoglycans, neutral proteases, and certain cytokines (such as TNF-α), that are stored within the cytoplasmic MC granules and are released immediately after activation; (ii) lipid mediators that are newly synthesized following activation (for example prostaglandins, leukotrienes, or platelet-activating factor); (iii) de *novo* synthetized cytokines, chemokines, growth and angiogenic factors. The mechanism of activation and the strength of the signal influence the spectrum of mediators secretion: all categories (e.g. in IgE-mediated MC degranulation) or distinct subsets of mediators with specific functions can be involved (Metz and Maurer, 2007).

Histamine is the most abundant vasoactive amine stored in MC granules and it is the main responsible for the early effects of asthma and other allergic responses. It induces contraction and vasospasm in airway smooth muscle cells and in gastrointestinal cells through binding to specific histamine receptors (H receptors). The major proteic products of MC granules are proteases (chymase, tryptase and carboxypeptidase A are the most represented) that exert important biological functions and are involved in protection against parasites and venoms, but they also allow tissue remodeling and induction of persistent influx of neutrophils with long lasting inflammation (reviewed in (Metz and Maurer, 2007). Bioactivity of MCs is also dependent on the huge amount of cytokines (pro-inflammatory IL-6 and TNF-α; both Th-1 type, such as IFN-γ an IL-8, and Th-2 ones, like IL-4, IL-10 or IL-13) and chemokines (for example MIP-1α, MIP-1β, and MCP-1) that they may express and secrete.

Class	Mediators	Physiological effects
PREFORMED		
Biogenic amines	Histamine	Vasodilatation
	5-hydroxytryptamine	Leukocyte regulation, pain, vasoconstriction
Proteoglycans	Heparin, heparin sulfate	Angiogenesis, coagulation
	Chondroitin sulfate	Tissue remodeling
Proteases	Tryptase	Inflammation, pain, tissue damage, PAR activation
	Chymase	Inflammation, pain, tissue damage
	MC-CPA/Carboxypeptidase A	Enzyme degradation
	CathepsinB, C, D, E, G, L, S[b]	Pathogen killing, tissue remodeling
	MCP5/6	Pathogenesis of asthma and other allergic disorders
Lysosomial enzymes	β-hexosaminidase, β-glucuronidase, β-galactosidase, arylsulfataseA	ECM remodeling
Others	Nitric oxide synthase	NO production
	Endothelin	Sepsis
	Kinins	Inflammation, pain, vasodilatation
		Anti-inflammatory effects
NEWLY SYNTHESIZED		
Lipid-derived	LTB_4, LTC_4, PGD_2, PAF	Inflammation, leukocyte recruitment, endothelial adhesion, *smooth* muscle cells contraction, vascular permeability
Cytokines	IL-1α[a], IL-1β[a], IL-2[b], IL-3, IL-4, IL-5, IL-6, IL8[a], IL-9, IL-10, IL-11[a], IL-12, IL-13, IL-14[a], IL-15[a], IL-16, IL-17, IL-18[a], IL-22[b], IL-25[b], IL-33[b], MIF, TNFα, IFNα, IFNβ[b], IFNγ[b]	Inflammation, leukocyte proliferation and activation immunoregulation
Chemokines	CCL1, CCL2, CCL3[a,b], CCL4[a] CCL5[a], CCL7[a,b], CCL8[a], CCL11[a], CCL13[a], CCL16[a], CCL17, CCL19[a], CCL20[a], CCL22a,[b], CCL25[b] CXCL1[a], CXCL2, CXCL3[a], CXCL4, CXCL5, CXCL8[a], CXCL10[a], CX3CL	Leukocyte chemotaxis
Growth factors	TGFβ, SCF[a], G-CSF, M-CSF, GM-CSF, VEGF, NGFβ, LIF[a], bFGF	Growth of various cell types
Antimicrobic species	Antimicrobial peptides, NO, superoxide, ROS	Pathogen killing

Figure 3.1: Mast cell mediators can mediate different pro-inflammatory, anti-inflammatory and/or immunoregulatory effects. Mast cells can be activated to release preformed and/or de novo-generated products. The secretion of mast cell products can have numerous effects and improve innate and/or acquired immune responses. Some mediators have been detected only in studies on human[a] or murine [b] MCs; where not indicated molecules are expressed in both species (Adapted from Gri *et al*., Front Immunol 2012).

3.2 Mast cell activation

Mast cells are armed with a large repertoire of receptors that allow them to sense pathogens and environmental toxins and to selectively respond to a great variety of signals. MC activation has been classically distinguished into IgE-dependent activation and IgE-independent activation.

The canonical pathway that leads to MC activation is via IgE and its receptor FcεRI. The FcεRI is a constitutively expressed tetrameric receptor that comprises the IgE-binding α chain, the membrane-tetraspanning β chain, and a disulfide-linked homodimer of the γ chains with high affinity for IgE.

The interaction of specific antigen with IgE already bound to FcεRI (IgE-presensitized MC), on the cell surface, leads to the aggregation of FcεRI and initiates a series of biochemical events resulting in MC-degranulation and in the subsequent release of biologically active mediators. This IgE–dependent MC response results in rapid release (in minutes) of preformed

inflammatory mediators and in the production, with a prolonged kinetics, of many cytokines, chemokines and growth factors (Siraganian, 2003).

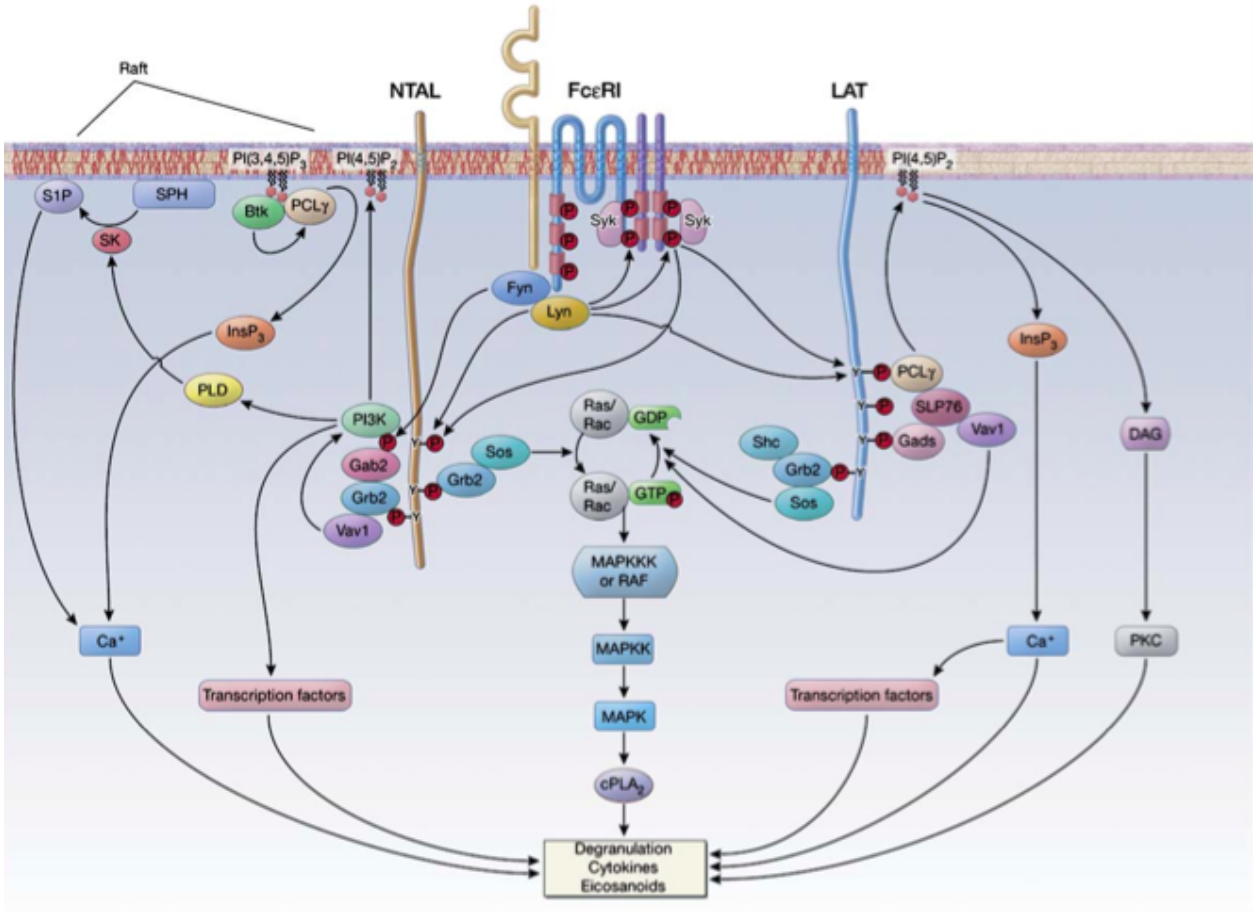

Figure 3.2: Molecular regulation of mast cell activation. Simplified scheme of FcεRI signaling in mast cells. Engagement of FcεRI results in its inclusion in lipid rafts, in the phosphorylation (P) of receptor ITAMs (both β- and γ-chains contain ITAMs) by Lyn kinase, and in the activation of Syk kinase through ITAM binding. Fyn kinase is also activated and is important for phosphorylation of the adapter known as Gab2, involved in activation of PI3K activity. Lyn and Syk phosphorylate several adaptor proteins that regulate MC activation, such as LAT and NTAL. LAT primarily regulates the activation of PLC-γ that cleaves phosphatidylinositol-4,5-bisphosphate in two important second messengers, inositol-1,4,5-trisphosphate (IP3), and membrane-bound 1,2-diacylglycerol (DAG). IP3 binds to its receptors in the ER membrane and gives rise to the first Ca^{2+} wave through MC cytoplasm. Ca^{2+} flux are involved in activation of Ca^{2+}-dependent kinases (such as protein kinase C (PKC)), that precede MC degranulation, and activation of NF-κB and NFAT transcription factors necessary for regulation of gene expression. DAG is a key molecule for early activation of PKC and MC cytokine production and degranulation. MAP kinase and transcription factor activation is dependent on LAT leading to MC cytokine production and eicosanoid production through cPLA2 activation. PI3K activity is also required for activation of PLD and SphK1. SphK generates S1P from sphingosine (Sph), which influences calcium mobilization and other MC effector responses (Adapted from Rivera and Gilfillan, J Allergy Clin Immunol 2006)

MC activation in host defense to pathogens is dependent on IgE-independent triggers. MCs express many Toll like Receptors (TLR), including TLR1, TLR2, TLR3, TLR4, TLR6 and

TLR9. In *vivo* responses to the bacterial wall component peptidoglycan, to lipopolysaccharide and to viral double-stranded RNA respectively through TLR2, TLR4 and TLR3 ligation have been demonstrated (Rao and Brown, 2008). Activation mediated by CD48, which binds to the fimbrial protein FimH on Gram-negative bacteria, is an additional mechanism of MC-pathogen direct interaction (Malaviya and Abraham, 2001).

MCs also express complement receptors (CRs). MCs could be recruited and activated to the site of complement activation by complement products such as C3a and C5a (Nilsson et al., 1996). Accordingly, complement has been demonstrated to be essential for host defense in a MC-dependent model of bacterial infection (Prodeus et al., 1997). Also the integrin α2β1 expressed on MCs was found to function as a receptor for the complement protein C1q and to be critical for MC-mediated host defense to peritonitis (Edelson et al., 2006).

In addition, MC exocytosis is stimulated by a family of polybasic compounds (including compound 48/80, mastoparan, polymyxin B), by a number of peptides (peptides corresponding to the IgE C_H4 domain, neuropeptides and rab3a ones are the main groups of histamine-releasing peptides), and cytokines (IL-1, IL-3, GM-CSF, IL-8, PF4, as well as SCF). Furthermore, MCs express at basal level several Fc receptors on their surface specific for IgG. FcγRI is the high affinity receptor, while FcγRII and FcγRIII are low affinity receptors of IgG. Activation through FcγRI or FcγRIII plays a role in IgG-dependent allergic reactions (type III hypersensitivity reactions) and in host defense to pathogens that produce superantigens (reviewed in Metcalfe et al., 1997).

3.3 Mast cell in health and diseases

MCs have been defined as an "antenna of the microenvironment" (Frossi et al., 2004) since their ability to respond to several biological stimuli and to extensively release a great variety of potent mediators: due to these capacities, they are involved in physiological processes and in the maintenance of homeostasis, in addition to playing a critical role in host defenses. In this paragraph a short overview of MC roles in various biological setting will be given, while their implication in cancer will be deepened treated separately (3.5) because relevant for the work of this thesis.

A complex picture of mast cell functions has emerged over the past decade, extending beyond their role in mediating allergy and asthma (reviewed in Rao and Brown, 2008). In fact, classically MCs have been studied for their role in IgE-mediated allergic inflammation (type I hypersensitivity). After Ag-specific IgE triggering, secretion of MC mediators gives rise to the so called "immediate hypersensitivity" and is responsible for the majority of the pathological

symptoms associated with allergy (anaphylaxis is the most severe manifestation), including vascular permeabilization, smooth muscle contraction, and induction of mucous secretion. In late phase response MC products continue to sustain the emerging chronic inflammatory response (reviewed in Metz and Maurer, 2007).

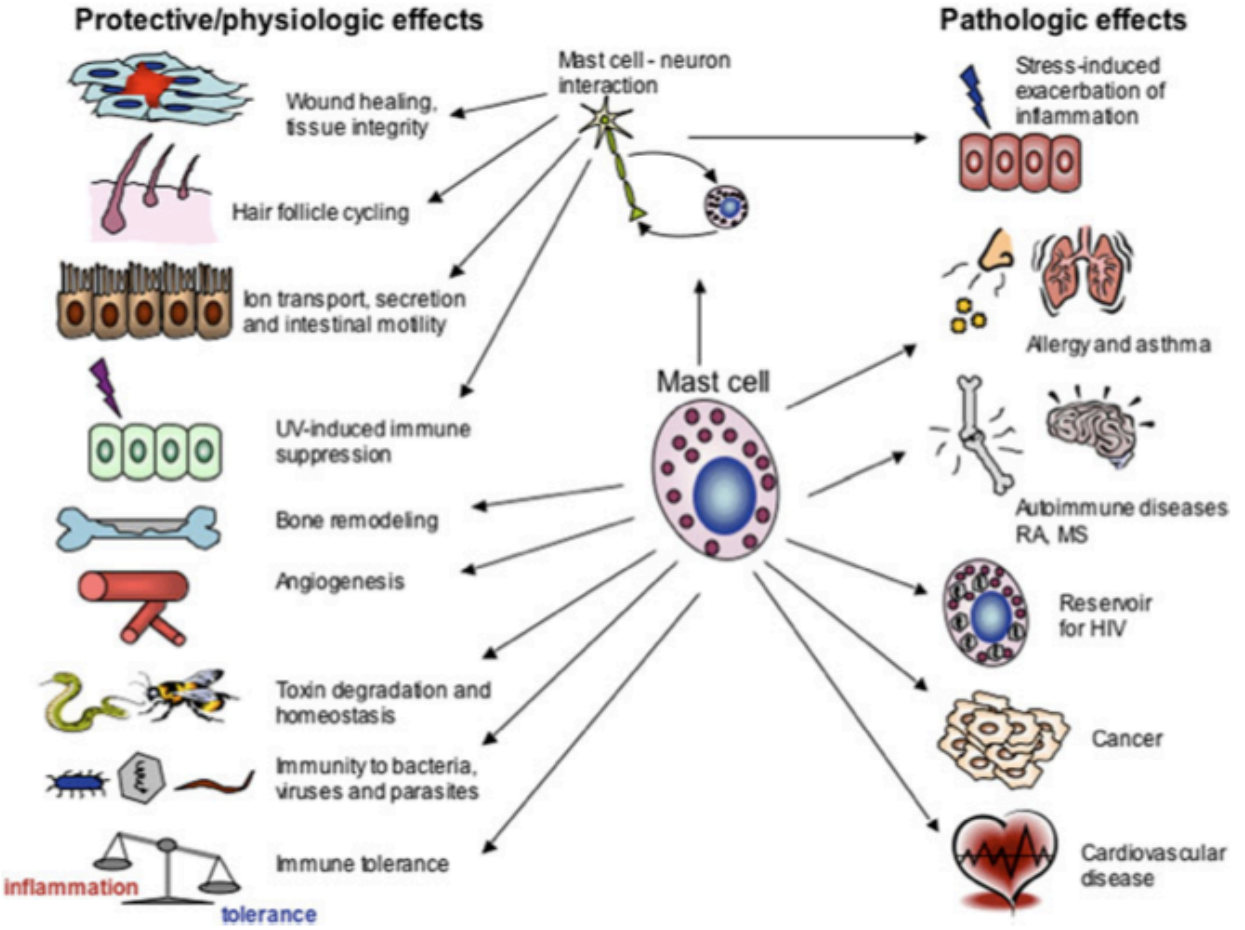

Figure 3.3: Mast cell roles in health and disease: A complex picture of mast cell functions has emerged extending beyond their role in mediating allergy and asthma. Nowadays MCs are known as multifaceted immune cells with several protective and physiological roles, but also with potential involvement in the pathogenesis of a large spectrum of diseases, such as autoimmune disorders, cardiovascular diseases, and cancer (Adapted from Rao and Brown, Ann. N.Y. Acad. Sci. 2008).

In term of physiological responses, the bidirectional interaction of mast cells with the nervous system regulates various processes involving tissue repair and remodeling such as wound healing, hair follicle cycling, and bone remodeling. In the gastrointestinal tract, mast cell–neuron interaction regulates functions such as ion transport, secretory activity of mucosal epithelial cells, and intestinal motility. This dynamic interaction is also responsible for UV-induced immune suppression.

In term of protective effects, MCs are crucial in the defense from harmful toxins such as snake and honeybee venom by degrading them. They are critical mediators of host defense to bacteria, viruses, and parasites and they are important in the regulation of the balance between inflammation and immune tolerance (reviewed in Urb and Sheppard, 2012).
On the contrary, in addition to their involvement in asthma and other allergic diseases, improper functioning of MC could be central in the pathogenesis of autoimmune diseases (reviewed in Walker et al., 2012) such as rheumatoid arthritis or multiple sclerosis, and of inflammatory diseases, like cardiovascular diseases or obesity (reviewed in Theoharides et al., 2011).

3.4 Mouse models for analyzing mast cell roles in vivo

The knowledge about the implication of mast cells in *vivo* in a variety of biological processes is the result of different experimental approaches.
A first possibility to understand MC involvement in a particular setting is the employ of drugs or antibodies that selectively inhibit the function of MCs in *vivo.* Cromolyn (sodium cromoglycate) is the best-known "MC stabilizer" and is currently used for the treatment of mast cell-associated diseases, such as allergic rhinitis and mastocytosis, although the mechanism of action that inhibits the release of mediators from MCs has never been clearly clarified (stabilization of membrane and block of the calcium uptake are two proposed models of action). Several studies explore the possibility that cromolyn may limit only some mast cell functions (such as secretion of proteases) but not others (for example, IgE-dependent activation of skin MCs). In confirmation of controversial results about cromolyn utilization in the study of MC biology, further doubts emerge from a recent work that systematically explores cromolyn effects on mouse mast cells, questioning the effectiveness and selectivity of cromolyn as a MC stabilizer in mice (Oka et al., 2012).
In addition, the lack of selective and unique markers to discriminate MC population limits the possibility to deplete MC by other conventional techniques, such the injection of depleting antibodies. For these reasons, nowadays, genetically MC-deficient mice represent the best way to assess the real contribute of mast cells in *vivo*. From an historically point of view, MC-deficient mice could be classified as older models, with abnormalities affecting KIT, or newer models, in which specific MC deficiency is KIT-independent, with their known or potential limitations. In this field the main problem is the apparent discordancy about some MC functions, proposed on evidences obtained in older models but not confirmed with newer ones. Mice with abnormalities affecting KIT, the receptor for MC essential growth factor SCF, are deficient in MC population, but also in melanocytes and present other phenotypic and hematopoietic

abnormalities. For example, WBB6F-Kit$^{W/W-v}$ mice (with mutations causing the production of a truncated KIT that is not expressed in membrane or that has a reduced kinase activity) are anemic and sterile with reduced number of basophils and neutrophils; by contrast, C57BL/6-Kit$^{W-sh/Wsh}$ mice (that present an inversion mutation affecting the transcriptional regulation of c-kit) present increased number of basophils and neutrophils but are not sterile or anemic. When studying differences between wild type and kit-dependent-MC deficient mice, to overcome roles of not-MC-abnormalities in the final results, MC knock-in mice (in which MC deficiency is repaired by intravenous, intraperitoneal or intradermal transfer of in *vitro* derived MCs) have been extensively used to assess the effective contribute of MCs. However, MC reconstitution does not always realistically reproduce the numbers and anatomically distribution of the corresponding wild type animals. In addition in kit mutant MCs may play more roles than in standard animals because transferred MCs could compensate abnormalities in other kit dependent-not MC lineage due to the redundancy of biological responses. Newer mouse models try to overcome complexities of kit-deficient mice by generation of mice the lack MC-not associated abnormalities, but they are not yet well characterized. A first approach is the generation of mice in which Cre-recombinase is expressed under the control of MC-specific promoters resulting in a constitutive deficiency in MC subsets (Mcpt5-Cre, R-DTA (Dudeck et al., 2011); Cre-Master Cpa3$^{Cre/+}$(Feyerabend et al., 2011); Hello Kitty Cpa3-Cre Mcl-1 $^{fl/fl}$ (Lilla et al., 2011)). The potential limit of these constitutive models of MC deficiency (shared with older ones) is that the biological response may vary compared to a situation of MC ablation just before or during immunological response. Inducible and selective MC ablation (Mcpt5-Cre, iDTR mice (Dudeck et al., 2011) and Mas-TRECK mice (Otsuka et al., 2011)) by injection of diphtheria toxin into transgenic mice bearing diphtheria receptor under the control of MC-selective genes, or the generation of mutant with deletion of MC-associated products (by target the specific deletion of floxed genes in MC-specific Cre mice (Dudeck et al., 2011; Gerbaulet et al., 2011) represent the newest steps for in *vivo* studies of MC functions. The obvious criticism of these methods is that Cre activity and Cre-mediated gene inactivation must be efficient only in MCs (but often impairment of basophil population is described).

In term of results, concordant outcome in different models justifies a consistent role of MC in IgE-dependent passive anaphylaxis and in resistance to venoms or their components. Instead, different mutant mice, but also differences in experimental procedures in the same animal (such as doses of antigen or adjuvant used for sensitization or duration of protocol), may lead to discrepant conclusion and underline the involvement of other immune cells in addition to MCs in a particular setting, such as in asthma, in antibody-dependent arthritis (role for neutrophils), in

experimental allergic encephalomyelitis or in contact hypersensibility (reviewed in Reber et al., 2012). In conclusion, it seems that the combination of multiple experimental models, with their potentials and their limitations, is crucial to obtain robust conclusions about MC roles in health and disease.

3.5 Mast cells and cancer

Mast cells (MC) were first described to infiltrate the interface between developing tumors and normal tissue in 1891 by Westphal, a student of P. Ehrlich, the "talent scout" of MCs (Westphal, 1891). Since that time, MCs have been found around and within many types of solid and hematopoietic cancers, both in human patients and in animal model of tumor, with no conclusive or univocal role regard their involvement since they may shift the balance in favor or against neoplastic growth. In the majority of human cancers, higher MC numbers are associated with a worse prognosis and reduced patient survival. Notwithstanding, several studies demonstrate a correlation between high MC number and improved prognosis, such as in breast cancer, reflecting the ability of MC to mediate direct tumor killing (Dabiri et al., 2004).

During tumorigenesis it is not completely clear the nature of MC origin and recruitment: it may occur from resident MC migrating from healthy tissue, or from de novo recruitment of mast cell progenitors via healthy circulation, or both. Proliferation of mature MCs is another option to sustain focal mastocytosis seen in tumors. MCs migrate toward supernatants of numerous tumorigenic cell lines with different mediators involved. In some cases recruitment and activation of MCs in tumor infiltrates are mediated by tumor-derived SCF and its receptor c-kit on MCs (Huang et al., 2008). MC accumulation may depend by local expansion of the MC population, as detected in a mouse model of glioblastoma, involving strong expression of SCF and CXCL12/CXCR4 axis (Põlajeva et al., 2011). MCs are enriched both within and at the invasive front of human thyroid carcinoma. Thyroid cancer cell-derived VEGF is involved in MC migration and a complex mixture of mediators in MC activation that promotes proliferation, survival and invasive ability of cancer cells (via histamine, CXCL1, CXCL10), contributing to carcinoma growth and invasiveness (Melillo et al., 2010). Other immune cells, such as dendritic cells and T cells may be involved in MC and their progenitor recruitment.

MC effects can be divided into direct activities on survival or killing of tumor cells, or into indirect effects in the tumor microenvironment. In fact MCs are able to secrete a range of mediators promoting remodeling of local tissue and angiogenesis, but also recruitment of other immune effector cells. This is particularly relevant as MCs are an early and persistent cell type in tumors, and their accumulation could become significant before tumor growth and angiogenesis

have occurred (Maltby et al., 2009). Degranulation pattern is frequently observed in MC infiltrating area of chronic inflammation and tumors and this mechanism is responsible for MC release of proteases, anticoagulants, cytokines and lipid mediators.

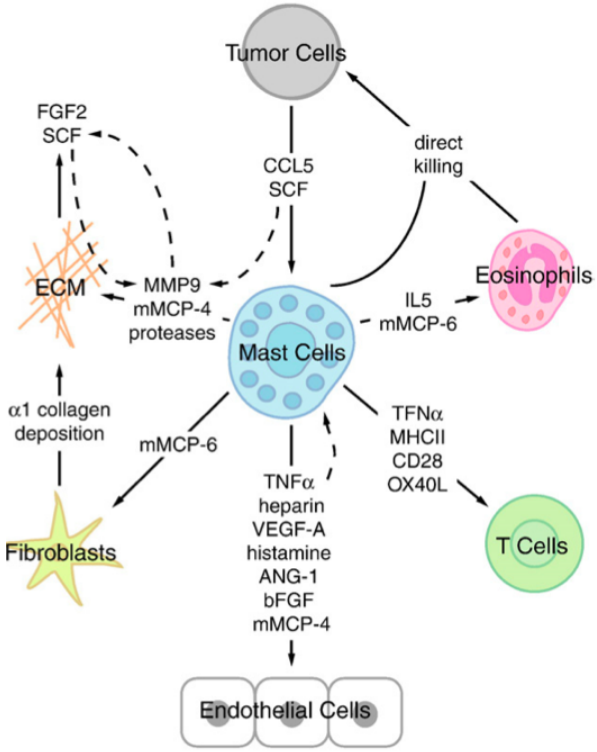

Figure 3.4: Interactions between mast cells and other cells within the tumor microenvironment. After recruitment in the tumor-associated inflammatory microenvironment, MCs create a complex network of interaction with tumor cells and other immune cells. Developing tumor cells secrete a number of molecules promoting mast cell recruitment. MCs release a range of mediators affecting tumor cell survival, as well as remodeling of local tissues and recruitment of immune effector cells (Adapted from Maltby *et al*., Biochimica and Biophysica Acta 2009).

3.5.1 Mast cell role in tumor promotion

Correlations between the presence of MCs and tumor development have been documented in several studies. Increase MC number is a predictor of poor outcome in different models of human cancer, such as prostate cancer, follicular and Hodgkins lymphoma, Merkel cell carcinomas or neurofibroma. High MC density and angiogenesis are associated with unfavorable prognosis in patients with colorectal, lung or pancreatic cancer (reviewed in Khazaie et al., 2011).

A number of mouse models of cancer has demonstrated the essential role of MCs in tumor progression and has been useful for the identification of MC-dependent mechanisms involved in tumor promotion.

Angiogenesis is a crucial feature of tumor development required for its growth. MCs play a critical role in the "angiogenic switch" during carcinogenesis. As documented in a model of squamous epithelial carcinogenesis, during premalignant progression from hyperplasia to dysplasia, early infiltrating MCs by secreting several mediators (e.g. FGF, VEFG, heparin, histamine, chymase and tryptase) act to start and sustain angiogenesis, until, in the cancer core, tumor cells become self-sufficient at sustaining neo-angiogenesis (Coussens et al., 1999). Similarly, mast cells are recruited with a mechanism involving CCL-5 to the periphery of Myc-induced pancreatic islet tumors and they are crucial for angiogenesis and macroscopic expansion of β-cell cancers. In fact inhibition of MC degranulation results in hypoxic environment and tumor growth regression (Soucek et al., 2007). In a mouse model of multiple myeloma, MC-derived angiopoietin 1, an endothelial growth factor, plays an important role in promoting angiogenesis and increasing tumors size (Nakayama, 2004). Other than promoting angiogenesis, MCs are able to modulate haemostasis and blood perfusion in tumors: MC-derived anticoagulant heparin is required to inhibit selective thrombosis of blood vessels within transplanted tumors (Samoszuk and Corwin, 2003).

MC capacity to promote tumor proliferation is partly related to secretion of proteases. For example, tryptase production is implicated in promoting tumor proliferation of human colon adenocarcinomas by activating protease-activated receptor-2, which activation may be considered as an oncogenic event in colon cancer. Chymases also have mitogenic properties and co-operate with heparin and TNF-α in tumor-promotion (Yoshii et al., 2005).

The specialized accumulation of MCs at the invasive front of tumors is explained by their role in degrading the extracellular matrix in preparations for tumor invasion and in facilitating dissemination. MC-derived chymases, tryptases, collagenases, MMP-9/gelatinases, and cysteinyl cathepsins are possible mediators contributing to tissue remodeling through proteolysis of matrix proteins, activation of other metalloproteinases and modulation of angiogenesis.

Tumor promoting potential of MCs could be augmented through stimulation with SCF and ligand for TLR4, or molecules released from damaged tumor cells, that induce IL-10 and angiogenic factors production (Wei et al., 2011).

When considering MC role in developing tumor, it's important to consider the effects of MC-mediated immune modulation. Within cancer microenvironment, MC-mediators are involved in recruitment and activation of others immune effector cells, creating a complex set of interactions. For instance, MCs recruit eosinophils and modulate their ability to kill tumor cells (Simson et al., 2007). In a mouse model of squamous carcinogenesis, B cells foster cancer development by activating Fcgamma receptors (FcgammaRs) on resident and recruited myeloid

cells. In this way, IgG-stimulated MCs, in combination with similarly activated macrophages, enhance tumor development and angiogenesis (Andreu et al., 2010).

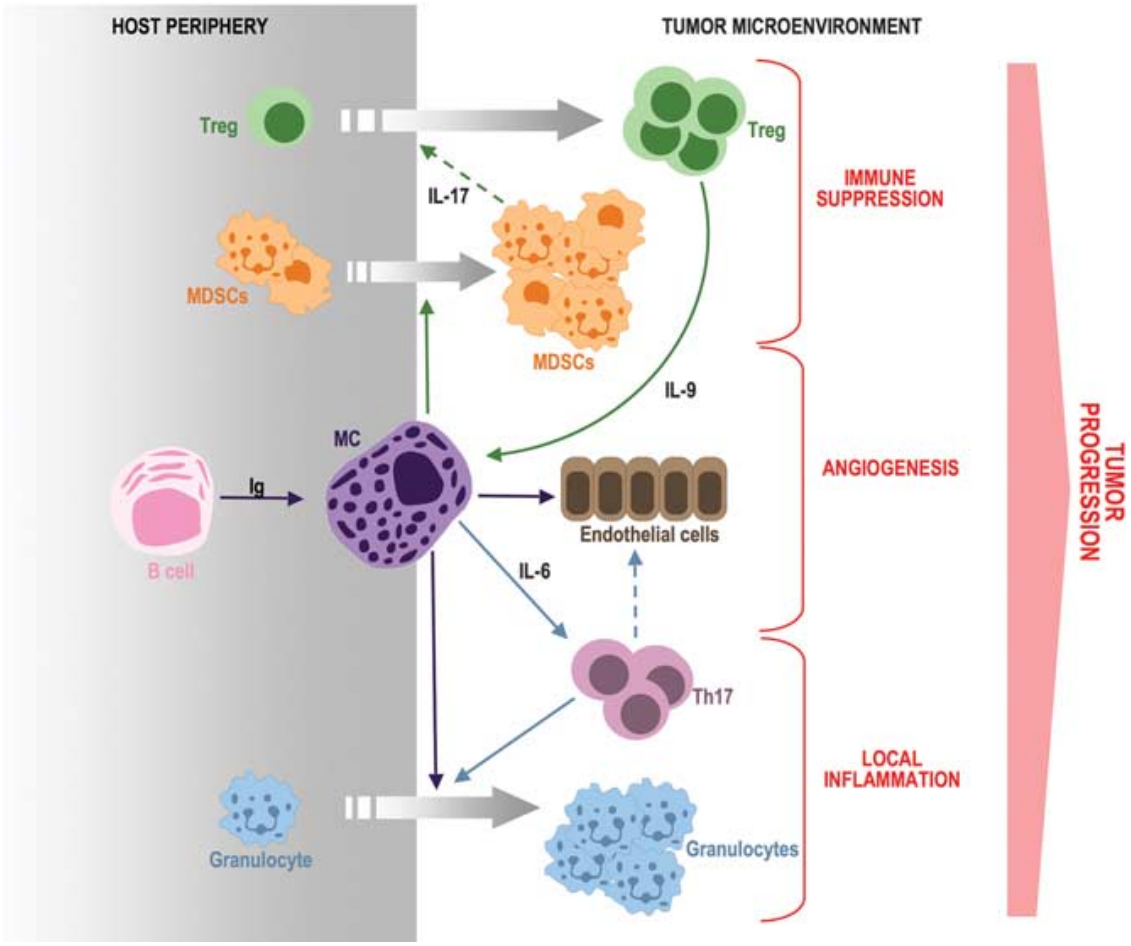

Figure 3.5: Mast cells foster tumor progression. MC interactions at the tumor-host interface sustain immunosuppression, angiogenesis and local inflammation, thus promoting tumor growth (Adapted from Pittoni *et al*., Oncogene 2010).

In addition, MCs also activate and modulate T cell function, but most of the research in this field results from studies in non-tumor microenvironment. Despite this consideration, an emerging aspect of tumor microenvironment is the close relationship between MCs and regulatory T cells: their reciprocal interaction drives the levels of cancer-associated inflammation and can influence the outcome of disease. In a physiological context, Tregs suppress MC progenitors expansion and differentiation and inhibit their degranulation (Gri et al., 2008), whereas MC engagement with Tregs abolishes Treg-suppressive capability and generates Th17-producing cells (Piconese et al., 2009), creating a crucial network responsible for the control of allergic responses severity. In fact the correct balance between anti-inflammatory IL-10-producing and pro-inflammatory IL-17-producing Tregs regulates the outcome of immune response. Within tumor microenvironment, MC interaction with Tregs generates pro-inflammatory IL-17-positive-Tregs

that maintain their T-cell-suppressive properties contributing to tumor-specific immune tolerance. At the same time, these cells lose their capability to inhibit MC degranulation: in this context MC-dependent perturbation of Treg equilibrium helps tumor growth (Gounaris et al., 2009). Concluding, within tumor microenvironment, MCs are able to alter Treg properties and promote differentiation of tumor-promoting Tregs that determine the fate of cancer development (Colombo and Piconese, 2009).

Furthermore, mast cell infiltration and Tregs accumulation are linked with the suppression of cytotoxic T- and natural killer (NK) cell-mediated antitumor immune responses (Huang et al., 2008).

3.5.2 Protective role of mast cells in cancer

Despite the predominant notion of MC as a tumor-promoting cell, there are studies sustaining a protective role of MC both in human and in mouse models of cancer. High numbers of MCs have been associated with good survival in a multivariate analysis of colorectal cancer, in a cohort of non-small-cell lung cancer and malignant mesothelioma patients (Welsh et al., 2005; Alì et al., 2009). Similarly, large infiltration of MCs in the stroma of invasive breast cancers and in large B cell follicular lymphoma appears to correlate with a good prognosis (Hedström et al., 2007; Rajput et al., 2008). Reports on the protective role of MC in mouse model of cancer are rare and controversial. The case of discrepant results about MC role on colon cancer is the best example on the controversies about their protective role in murine carcinogenesis (as largely discussed in the next paragraph).

However, in the cancer setting, an immunotherapic strategy may target MCs with the outcome of modulate and reverse their pro-tumorigenic role. For example after activation of TLR2 on MCs, MC-derived IL-6 is involved in tumor growth inhibition. Direct antitumor effects in *vitro*, recruitment of NK and T cells, and decreased angiogenesis were observed (Oldford et al., 2010).

Another crucial point in understanding MC biology in cancer is their possibility to play opposite roles in different stages of the same tumor, also dependent on their localization. This possible alternative role of MC as dangerous promoter, or protective antitumor cells became clear in a recent work on mouse and human prostate cancer, a typical multifocal disease with heterogeneous characteristics. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, MMP-9-producing MCs accumulate around area of well-differentiated adenocarcinoma, but lack in poorly differentiated regions, a pattern found also in human biopsies. MC inhibition or ablation result in the reduction of well-differentiated TRAMP-derived tumor cell lines, but not of poorly ones. In testing effects of MC targeting in tumor-prone TRAMP mice, Pittoni and co-

workers observe a high incidence of aggressive neuroendocrine tumors, uncovering a protective role of MC against aggressive variants. These findings suggest that therapeutic inhibition of MCs could be efficient in patients with early-stage lesions, but would be useless for advanced disease (Pittoni et al., 2011; Pittoni and Colombo, 2012).

3.5.3 A focus on mast cell and colon cancer

The knowledge that patients with inflammatory bowel disease are at an increased risk of colon cancer has justified researches about the link between inflammation and colorectal cancer development.

Colorectal cancer (CRC) is the fourth commonest form of cancer occurring worldwide, with increasing numbers in westernized countries. More than 70% of colorectal cancers develop from sporadic adenomatous polyps, asymptomatic and multiple in most cases. Flat adenomas account for about 10% of all polyps and may have a higher rate of malignant change and predispose to a more aggressive cancer phenotype. Recognized familial syndromes, in *primis* familiar adenomatous polyposis and hereditary non-polyposis colon cancer, account for about 5% of colorectal cancers. Inflammatory bowel diseases, especially ulcerative colitis, are emerging as an important risk factor for colon carcinoma, representing one of the best-known models of inflammation-associated carcinogenesis. Important clinical and biological differences exist between the adenoma carcinoma sequence and colitis-associated cancer. In the classical adenoma carcinoma sequence, inactivation of the tumor suppressor gene APC is often the first event required for adenoma formation and is associated with accumulation of catenins, which lead to the transcription of c-myc, giving a proliferative advantage to the cell. Mutations in DCC (Deleted in Colon Cancer) and p53, probably the most important "guardian of the genome", occur later in the sequence, although the order in mutations may vary and they drive the transformation of adenoma to carcinoma. When IBD-associated colorectal cancer is compared to its sporadic counterpart, it is clear that it follows a different histological sequence, starting in the inflamed mucosa as a hyperplastic lesion, to develop through (flat) dysplasia into adenocarcinoma. The relative order in which mutations in genes such as APC, P53, SMAD4, or K-ras may differ; yet these mutations are observed with comparable frequencies in both disease entities.

Mast cells have important role as guardian at the intestine barrier: they mediate several antimicrobial functions and their total loss can be dangerous for the control of gut microflora. This protective behavior of MCs is lost with persistence of certain pathogens with excessive and inappropriate release of inflammatory mediators (Abonia et al., 2005; Groschwitz et al., 2009).

From the onset most colorectal cancers are infiltrated with MCs and their precursors in both human and mice. Several papers investigated the potential role of mast cells in both sporadic- and inflammation associated colorectal carcinogenesis. In the first functional experiment that addressed the potential role of mast cells in colon cancer development Kit^{W}/Kit^{W-v} mutant mice were exposed to dimethylhydrazine (DMH) and the genetic impairment of c-kit function was found to reduce the susceptibility of mice to DMH-induced colonic tumors, a result consistent with the possibility that mast cells promote the development of colonic epithelial tumors in mice (Wedemeyer and Galli, 2005). In addition, mast cell infiltration is associated with an increased polyp burden in $Apc^{min/+}$ mice lacking glutathione S-transferase with up-regulation of pro-inflammatory cytokines and several mast cell associated proteases (Ritchie et al., 2009).

Gounaris and co-workers demonstrated that MCs are an essential component for polyp development and tumor formation, since in $APC^{\Delta 468}$ mice model of sporadic colon carcinogenesis MC depletion attenuated polyposis which is partially dependent on MC-derived TNF-α (Gounaris et al., 2007). CRC are also enriched in IL-17-shifted regulatory T cells (Treg), with a cancer pro-inflammatory phenotype and the ability to promote local mastocytosis (Gounaris et al., 2009). In addition, tumor infiltrating Tregs switch from suppressing (Gri et al., 2008) to promote MC degranulation without losing T cell suppressive properties, escalating inflammation and growth of CRC (Blatner et al., 2010). Summarizing, Treg plasticity and cross-talk with mast cells seem to be crucial in establishing the optimal microenvironment for cancer development in colon (Colombo and Piconese, 2009).

One exception to the pro-tumoral role of MC is the more aggressive polyposis observed in mice generated from the cross of the polyposis-prone Min mice to the MC-deficient $Kit^{wsh/wsh}$ mice (Sinnamon et al., 2007). Discrepant results may be explain due to difference in tumor model and genetic background of MC deficient mice, that can harbor major abnormalities of their intestinal mucosal immune system other than just loss of mast cells, complicating the definition of MC causal role in these models.

Role of mast cells has also been investigated in different model of inflammation-associated colorectal neoplasia. First identified for their accumulation in carcinogenesis model induced by azoxymethane and dextran sodium sulphate (Tanaka et al., 2003), MC functional role has not been completely elucidated in colitis-associated cancer (CAC). Recently, in AOM/DSS induced model of CAC, Tanaka and Ishikawa demonstrated that mice lacking MCs are less susceptible to inflammation-associated colorectal carcinogenesis, partially due to reduced levels of cytokines and inflammatory enzymes in colonic mucosa (Tanaka and Ishikawa, 2012). Similarly, prevention of CAC with infliximab, an anti-TNF-α antibody, is associated with significant

reduction of MC number, suggesting a TNF-α-dependent MC pro-tumoral role in this setting (Kim et al., 2010). Conversely, MCs were shown to have a primarily protective role within the colonic microenvironment by enhancing the efficacy of the mucosal barrier but they did not affect the incidence or severity of colonic neoplasia in IBD-susceptible IL-10 deficient mice (Chichlowski et al., 2010).

Concluding, substantial evidences suggest that mast cell accumulation modulates the adenoma to carcinoma sequence of colorectal cancer development, but the role of MC infiltration is less clear in *"*inflammation-dysplasia-carcinoma*"* sequence that characterizes colitis-associated cancer and remarkably further studies need to be performed.

3.6 Mast cells and myeloid derived suppressor cells cross talk in cancer

Complex cellular networks are a relevant feature of tumor microenvironment and in this context activated-mast cells could remodel and affect other inflammatory cells. A closed loop between MCs and MDSCs, also involving regulatory T cells, was recently described. In a hepatocarcinoma model, microenvironment is characterized by SCF-dependent MC infiltration and activation, resulting in the release of pro-inflammatory factors, involved in exacerbating tumor immunosuppression (Huang et al., 2008). In particular, MC-secreted CCL-2 seems to be involved in the recruitment of IL-17-producing MDSCs that strengthen tumor inflammatory microenvironment. Up-regulation of CCL-17 and CCL-22 subsequently attracts the migration of Tregs. IL-17 signaling up-regulates CD39 and CD73 expression on highly suppressive Tregs and up-regulates their production of IL-9 that maintains the survival of MCs. This vicious axis develops an inflammatory and immunosuppressive microenvironment that promotes tumor growth (Yang et al., 2010; Liu et al., 2011). An alternatively way of MDSC recruitment in a murine model of colon polyposis involves MC 5-lipoxygenase activity, that affects intestinal epithelial cell proliferation and MDSC mobilization. In fact hematopoietic 5-LO deficiency, and particularly MC-derived 5-LO, reduces the number and arginase-1 activity of MDSCs and is associated with an attenuation of polyp development (Cheon et al., 2011). Since MCs were described for their implication in MDSC recruitment to the tumor site, Saleem and colleagues recently examined the contribution of these interactions to tumor biology. They described in a melanoma model that MDSCs, in particular the monocytic subset, promote tumor metastasis and compromise the efficacy of immunotherapy in a MC-dependent manner, given that *wt* mice are more susceptible to metastasis compared with $Kit^{wsh/wsh}$ mice. MDSC/MC interaction results in an increased production of cytokines, in a Th-2 skewed immune response. Although undesirable in the context of neoplasia, the observed response is ideal in helminthic infections. In fact MCs

augment MDSC activity in a model of parasite clearance, with MC required for granulocytic MDSC-mediated parasite clearance (Saleem et al., 2012).

Concluding, these recent reports highlight the existence of an important axis between mast cells and myeloid derived suppressor cells, with the development of both tumor inflammatory and immunosuppressive microenvironment. This relationship, which could be addressed as a novel approach to cancer therapies, needs to be further investigated in details, especially in term of mechanism regulating the fate of these immune populations in cancer.

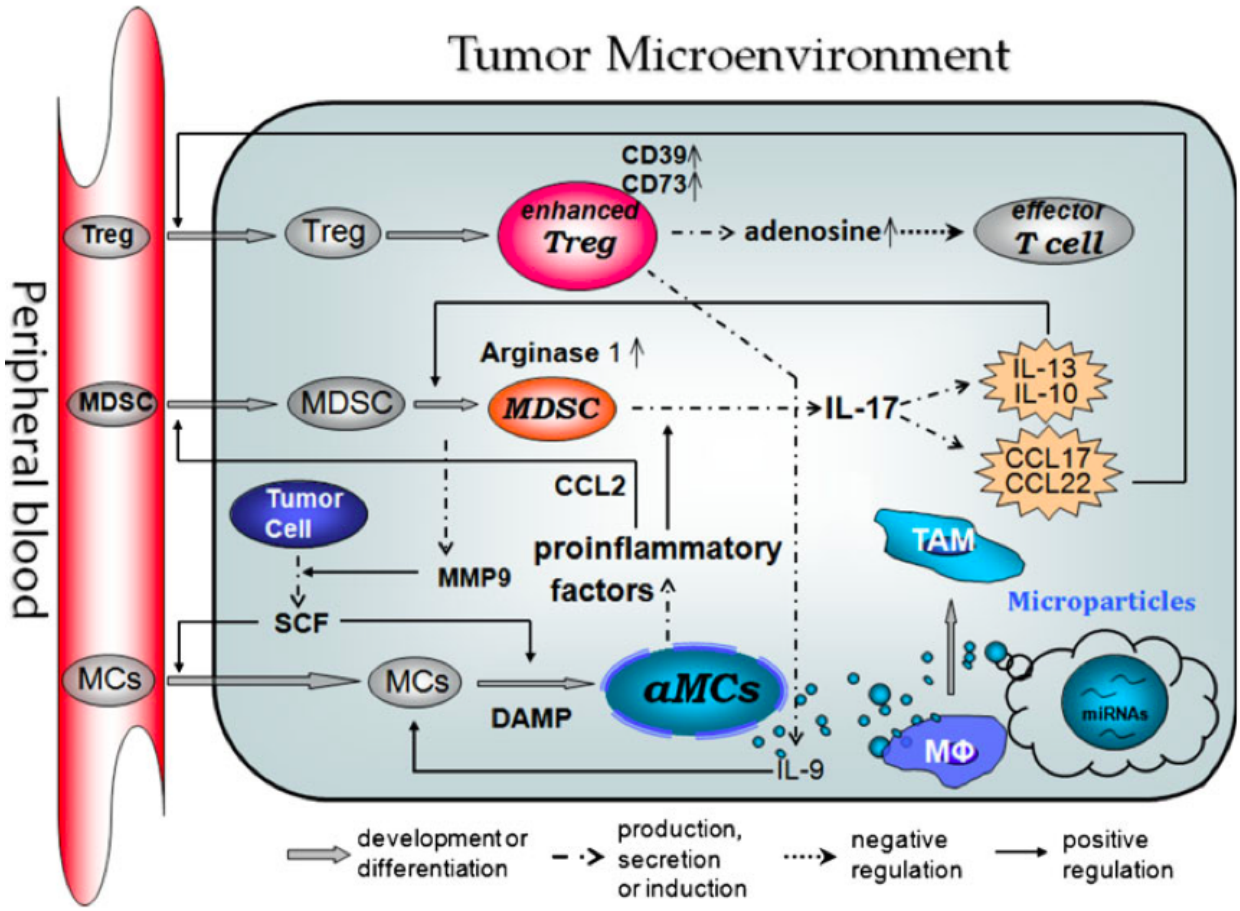

Figure 3.6: Mast cells cross talk with MDSCs and Tregs remodels tumor microenvironment. Within tumor microenvironment, activated MCs release a panel of factors leading to MDSC recruitment; IL-17-producing MDSCs strengthen tumor inflammatory microenvironment leading to Treg function enhancement. IL-9, produced by Tregs, increases the survival of MCs in the tumor microenvironment (Adapted from Liu *et al*., Cancer Metastasis Rev 2011).

Chapter 4: AIM OF THE WORK

Tumor-associated inflammatory response and tumor-induced immune suppression are likely to be an impediment to immunotherapy and immune-surveillance in patients and experimental animals with malignant tumors. Multiple mechanisms are thought to facilitate tumor-induced immune suppression and myeloid-derived suppressor cells (MDSC) are one of the major components of the immune-suppressive network responsible for T cell defects in cancer and for the pro-tumoral immune suppressive microenvironment (Gabrilovich and Nagaraj, 2009).

Mast cells (MC) act as potential regulatory linkers between innate and acquired immunity in the control of immune system homeostasis (Frossi et al., 2004), but they are also implicated in the promotion of tumor growth (Maltby et al., 2009). MCs play major roles in immune and tissue regulation in the gut and experimental evidences indicate their involvement in bowel inflammatory and carcinogenetic processes, but no conclusive data exist about their actual role in promoting or suppressing inflammation and cancer at this site (Heijmans et al., 2010).

Following these considerations, the aim of the present thesis was to characterize the role of MCs in a colon cancer model and to investigate the possibility of MC interaction with MDSCs in the control of tumor-induced immune response. In more details, in this work in *vitro* and in *vivo* characterization of MC and MDSC response to CT-26 colon cancer was performed. The evidence of the direct interaction between MC and MDSC in the spleen of CT-26 tumor-bearing mice led to investigate ex *vivo* if MCs could regulate MDSC suppressive functions and, vice versa, if MDSCs could influence the activation status of MCs, with particular attention to the molecular mechanisms involved.

Chapter 5: RESULTS

5.1 Pro-tumorigenic role of mast cells (MC) in colon cancer growth

Descriptive and experimental evidences in humans and in animal models have shown that mast cells (MC) accumulate in colorectal cancers and that their infiltration seems to modulate the adenoma to carcinoma sequence of colorectal cancer development. Several chemical and genetic models of adenoma development have been used to elucidate the role of MCs in colorectal carcinogenesis but actual data are still conflicting and some questions remain to be answered (Heijmans et al., 2010).

To address new issues in the role of the MC in colon cancer progression, the interaction between MCs and the colon cancer cell line CT-26 was initially studied in *vitro* and in a in *vivo* transplantable tumor model (through subcutaneous injection of tumor cells). CT-26 is a N-nitroso-N-methylurethane-(NNMU) induced, undifferentiated colon carcinoma cell line generated in Balb/c mice and largely used in the study of colon cancer biology.

Since MC-migration to the tumor site and the following activation may be the prerequisite for their promoting effect on tumors, this work started from the in *vitro* investigation of MC response to the CT-26 colon cancer cell line. As a starting point, to evaluate whether colon carcinoma cells could recruit MCs, in *vitro* chemotaxis assays were performed using bone marrow-derived mast cells (BMMC). CT-26 cell conditioned culture media (CM) induced migration of BMMCs in a transwell migration assay in a dose-dependent manner (Fig. 5.1A). Not-conditioned media and SCF-supplemented media were respectively used as negative and positive control of MC-migration.

Mast cells are known to secrete a large array of mediators in response to a plethora of stimuli. To evaluate the behaviour of BMMCs stimulated with colon cancer conditioned media, we verified that CM could induce a transcriptional response in MCs, both directly and with additive effects respect to MC specific IgE-dependent stimulus. In fact, resting BMMCs treated with CT-26 CM up-regulated gene expression of pro-inflammatory cytokines (IL-6 and TNF-α) and chemokines (CCL-2 and CCL-5) (Fig. 5.1B). Conditioned media had a significant additive effect on MC IgE-dependent (IgE/Ag) stimulation on IL-6, TNF-α and CCL-5 gene expression (Fig. 5.1B) and on IL-6 and CCL-3 secretion (Fig. 5.1C). The same trend was obtained for TNF-α production but results were not statistically significant; conversely it was not possible to validate data about CCL-2 because conditioned media contained high levels of the chemokine (data not shown). Addictive effect of CT-26 CM on classical IgE/Ag activation was responsible also for dose-

dependent increased release of histamine (Fig. 5.1D). This last result indicated that mediators produced by cancer cells could affect also early events of MC degranulation.

In *vitro* data indicate that soluble products released by CT-26 tumor cells are able to: i) induce MC migration; ii) modulate MC gene expression; iii) induce MC release of early and late mediators.

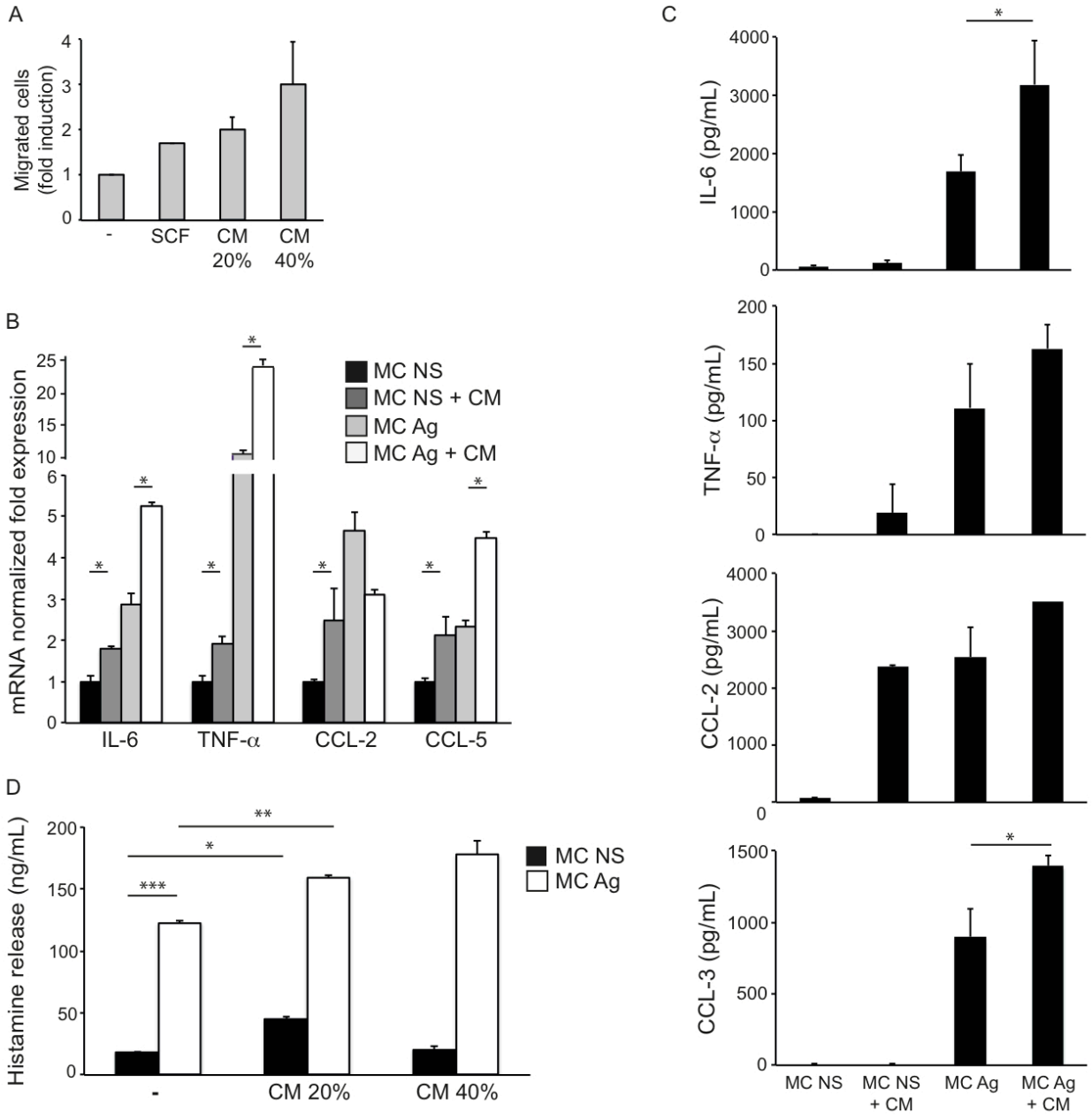

Figure 5.1: Characterization of mast cell response to colon carcinoma cell line-conditioned media

(**A**) 7,5 x 10^4 BMMCs were plated on the top of a transwell chamber and incubated for 4 hours with CT-26 colon cancer cell line conditioned media (CM), corresponding to 20% and 40% of total chamber volume, or with not-conditioned medium (-). 20 ng/mL SCF was used as positive control for mast cell migration. Migrated cells were counted and results (mean+s.d. of two separated experiments performed in duplicate) are shown as fold induction compared to cells migrated in response to non-conditioned media (-).

(**B**) 10 x 10^6 IgE-sensitized BMMCs were incubated for 4 hours with CT-26 conditioned media (CM; 30% of total culture volume) and, where indicated, challenged with 100 ng/mL DNP (MC Ag). Total RNA was extracted, reverse transcribed and the generated cDNA was amplified with Real Time PCR for the indicated genes. G3PDH transcript levels were used as normalizers. Results are expressed as normalized fold expression (mean+s.d. of two representative experiments) compared to resting MC transcript levels (MC NS). * $p<0,05$
(**C-D**) BMMCs (2 x 10^6 cells/mL) were incubated with different concentration of CM (30% in C; 20% or 40% in D) and challenged with 100 ng/mL DNP antigen. Supernatants were collected after 1 hour and 24 hours to determine respectively cytokines (**C**) and histamine (**D**) levels by ELISA. Dataset are representative of at least two experiments. Mean+s.d. are shown. * $p<0,05$; ** $p<0,01$; *** $p<0,001$.

To demonstrate that MCs could be recruited into tumor site and could influence cancer growth in *vivo*, 2 x 10^5 CT-26 cells were subcutaneously injected in Balb/c mice. MCs (MC number = 37,0±5,9/mm^2) were present in CT-26 solid tumors and accumulated preferentially in the periphery of the lesion (Fig. 5.2A), with rare MCs scattered inside tumor in proximity of emerging vessels. In *vivo* MC-recruitment was further confirmed by endovenous injection of Fast DiO (a lipophilic fluorescent viable-cell labeling tracker) labeled-BMMCs that were identified in tumor sections of CT-26 bearing mice 24 hours later (Fig. 5.2B). To study the involvement of MC in cancer growth, CT-26 tumor-bearing mice were treated with sodium cromoglycate (cromolyn), a compound that inhibits MC degranulation and release of inflammatory mediators (Thompson et al., 1983). In *vitro* experiments showed that cromolyn did not affect cancer cell proliferation (data not shown). Mice treated daily with cromolyn (for two weeks from day +5 to day +15 after tumor cells injection) showed a significant tumor growth reduction compared to untreated mice (Fig. 5.2C).
Taken together these results suggested a pro-tumoral role of MC in the CT-26 model of colon carcinoma.

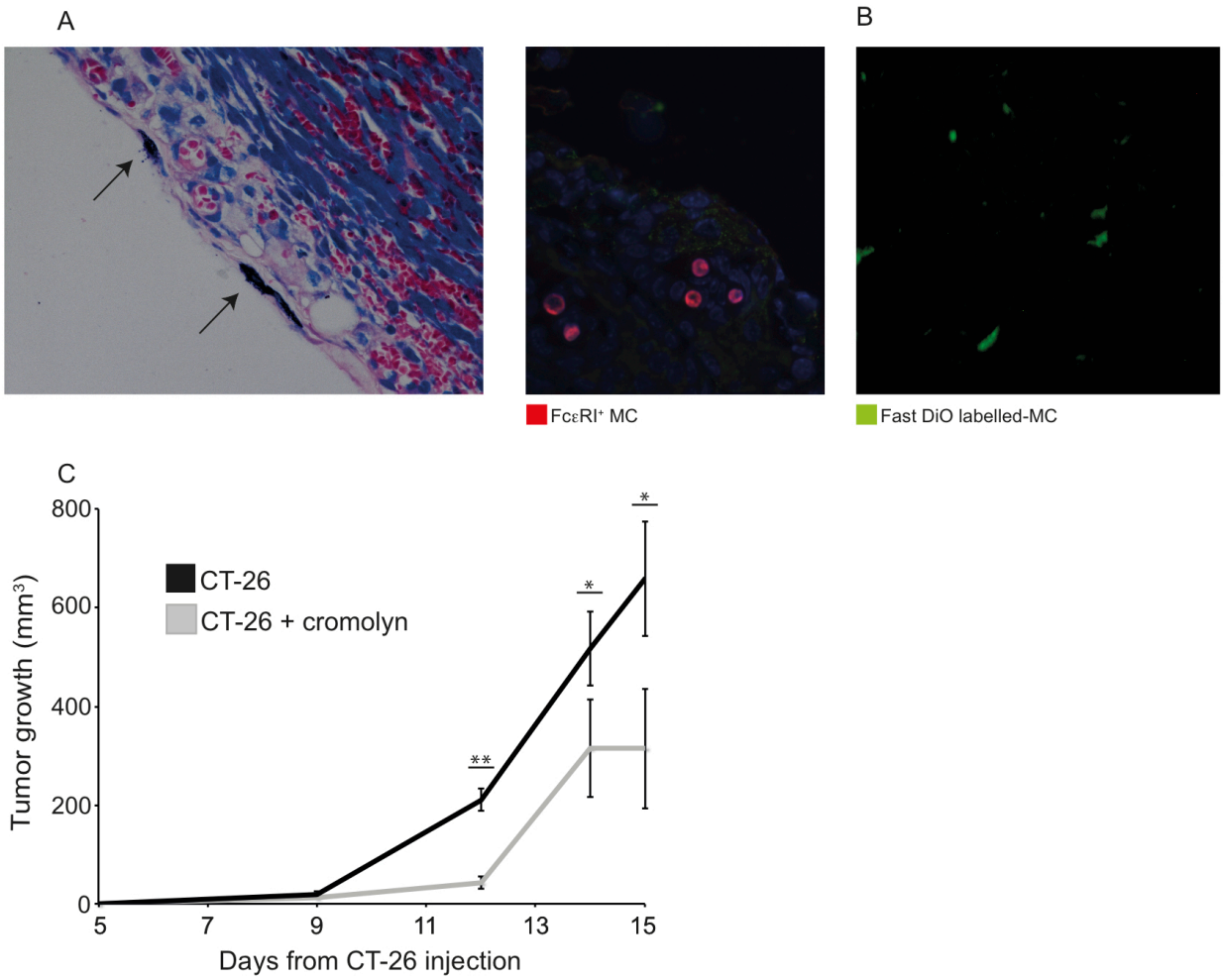

Figure 5.2: MCs promote colon cancer growth

(A-C) Balb/c mice were injected subcutaneously with 2 x 10^5 CT-26 cells; tumor growth was monitored and mice were sacrificed within 3-4 weeks from tumor injection. (**A**) MCs infiltrate CT-26 tumor-bearing mice. Giemsa staining (left image, arrows indicate MCs) and immunofluorescence staining (right image) for the detection of Fc**ε**RI$^+$ cells (the marker is specifically expressed by MCs, in red; nuclei were counterstained with DAPI, in blue) of one representative tumor section from CT-26 tumor-bearing mice are shown (400X).

(B) 2 x 10^5 Fast DiO-labeled BMMCs were injected into the tail vein of CT-26-tumor bearing mice when the tumor mass became palpable. 24 hours later mice were sacrificed, tumors were removed and freezed for analysis with fluorescent microscope. One representative tumor section infiltrated by BMMCs (in green) is shown.

(**C**) Tumor size (mean+s.e.m) in Balb/c mice injected subcutaneously with 2 x 10^5 CT-26 cells and 1 x 10^5 BMMCs, eventually treated with cromolyn (n=5) from day 5 to day 15 after cell injection. * p<0,05; ** p<0,01.

5.2 Mast Cells potentiate the suppressive function of Myeloid Derived Suppressor Cells (MDSC)

A major characteristic of mast cell biology in cancer is their ability to influence other immune cells that are part of the tumor microenvironment, such as T cells or eosinophils. In fact MCs can influence tumorigenesis in several ways: they can directly affect tumor growth, angiogenesis and

tissue remodeling but they can also exert an indirect function by immune-modulating cancer microenvironment (Maltby et al., 2009).

Recent findings suggest that mast cells mobilize myeloid derived suppressor cells (MDSC) by producing 5-lipoxygenase and other mediators (i.e. CCL-2) and further enhance their suppressive activities to drive immune escape in a tumorigenic context (Yang et al., 2010; Cheon et al., 2011; Saleem et al., 2012).

In order to elucidate and analyze the possible functional relevance of MDSC-MC interaction, the cross talk between these two immune populations was examined in the CT-26 colon cancer model. To address the question of this study, mice were injected s.c. with 2 x 10^5 tumor cells and all the mice used were sacrificed at development of similar size (1.5 cm in diameter) tumors within 3 weeks of inoculation. The choice of this condition was due to the fact that it is routinely used in experiments aimed to study MDSC-mediated immune suppression. It has been shown that there is a rate of variability in MDSC frequency between different tumor models. Some murine tumors are associated with a substantial (>15%) expansion of MDSCs, some have limited (<5%) expansion and some have intermediate (10–15%) levels of MDSCs (Youn et al., 2008).

To examine ex *vivo* properties of MDSCs and their interplay with MCs, we took advantage of the CT-26 colon carcinoma model because it showed a relatively strong accumulation of MDSCs (percentage of spleen-derived MDSCs: 13,5±1,0% in CT-26 tumor bearing-mice vs 3,8±0,8% in Balb/c control mice) (Fig. 5.3A).

To investigate the possible occurrence of cellular interactions between MCs and MDSCs in an inflammatory cancer microenvironment, we performed double immunofluorescence staining for the MC marker FcεRI and for the MDSC markers Gr-1 or CD11b on tissue sections from spleens of CT-26 tumor-bearing mice. Several MCs clearly showed signs of cell-to-cell interaction with Gr-1^+ and $CD11b^+$ cells (Fig. 5.3B).

Following this evidence, spleen-derived MDSCs were isolated as purified polymorphonuclear Gr-1^{high} $Cd11b^+$ myeloid cells (PMN-MDSC) and monocytic Gr-1^{dim} $CD11b^+$ cells (M-MDSC) (isolation strategy is described in Methods 7.3). These two distinct subsets differ not only for the levels of Gr-1 expression (Fig. 5.3C) but also for their phenotype, morphology and preferential suppression mechanisms (Movahedi et al., 2008).

As mentioned above, two recently published reports (Yang et al., 2010; Cheon et al., 2011) showed that MCs contribute to MDSC recruitment to the tumor site via 5-lipoxygenase and through the production of other mediators, with suppressive population showing an increased arginase-1 activity. To verify the chemoattractant potential of MCs in the CT-26 colon cancer model, in *vitro* migration assay was performed. Supernatant from IgE/Ag-activated BMMCs

induced migration of both PMN- and M-MDSCs and had an addictive effect with CT-26 conditioned culture media (CM)-induced migration (Fig. 5.3D). These results confirmed the known (Cheon et al., 2011) role of MCs in the recruitment of MDSCs.

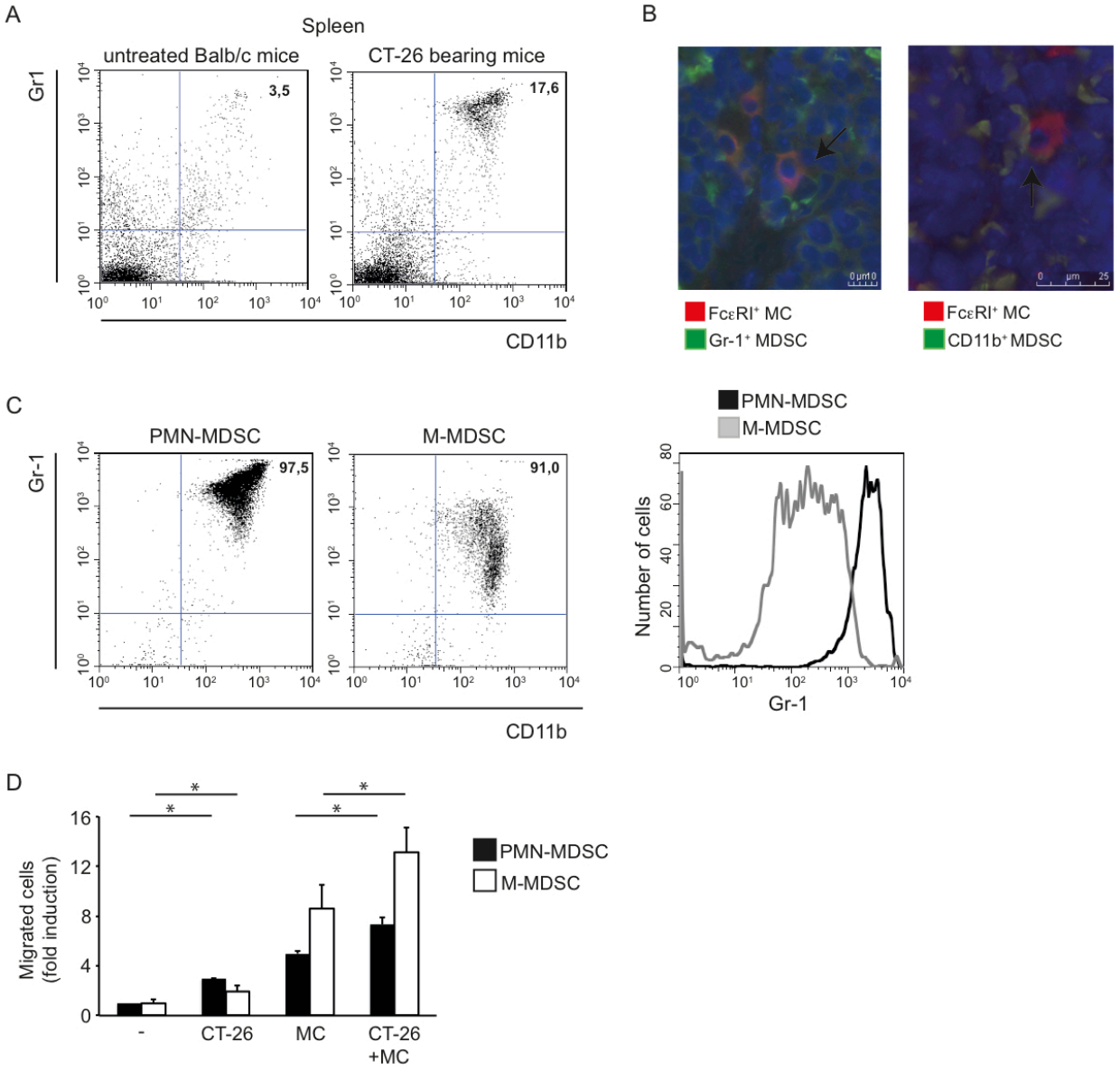

Figure 5.3: MDSC characterization in CT-26 tumor-bearing mice and their interaction with mast cells.

(**A**) 2×10^5 CT-26 colon carcinoma cells were injected s.c. into Balb/c mice. After 3 weeks, spleens were collected and mechanically digested. Gr-1^+CD$11b^+$ MDSCs were evaluated as percentage of total splenocytes. Spleens from untreated mice were used as control.

(**B**) Formalin-fixed and paraffin-embedded sections from spleen of CT-26 tumor-bearing mice were immunohistochemically stained for Gr-1 or CD11b (green) and FcεRI (red) expression.

(**C**) Spleens from CT-26 tumor-bearing mice were digested and used for purification of MDSCs. Dot plots represent the purified Gr-1^{high} Ly6G$^+$ CD$11b^+$ PMN-MDSC (left panel) and Gr-1^{dim} Ly6G$^-$ CD$11b^+$ M-MDSC (middle panel) subsets from spleen of the same mice; histogram (right panel) shows the differential expression of Gr-1 marker in purified PMN- (black line) vs M- (grey line) MDSCs.

(D) Migration of 1 x 10^5 PMN-MDSCs (black histograms) and M-MDSCs (white histograms) purified from spleen of CT-26 tumor bearing mice were performed in presence of 30% CT-26 conditioned media (CT-26) and/or equal volume of supernatants from IgE/Ag-activated BMMCs (MC). Migrated cells were counted and results (mean+s.d. of two separated experiments performed in duplicate) are shown as fold induction compared to cells migrated in response to non-conditioned media (-). * $p<0,05$.

Since previous works did not mechanistically describe the interaction between these two immune cell populations, the possibility of an interplay between MCs and MDSCs was analyzed ex *vivo* to assess whether the contribution of MCs on the MDSC-dependent immune suppression is due only to their recruitment or may be relevant for their biological functions. It is well known that MCs and MDSCs have an opposite effect on T cells, since the first enhance (Nakae et al., 2005; 2006), while the second inhibit their proliferation (Gabrilovich and Nagaraj, 2009). To examine the cooperative effect of MCs and MDSCs on T cell proliferation, total splenocytes from *naïve* Balb/c mice were labeled with CFSE and polyclonally activated with anti-CD3 (5 μg/mL). Proliferation assay was performed by addiction of spleen-derived MDSCs from CT-26 tumor-bearing mice in the absence or presence of resting (MC NS) or activated BMMCs (MC IgE/Ag). After 72 hours, proliferation of T cells stained for CD4 and CD8 was detected by FACS analysis using CFSE dilution. In these experimental conditions M-MDSCs, but not PMN-MDSCs, acted as weak suppressors of non-antigen-specific $CD4^+$ T cell proliferation, whereas MCs alone did not significantly affect T cell proliferation (Fig. 5.4A and data not shown). As shown in figure 5.4A, resting BMMCs significantly enhanced the ability of monocytic MDSCs (M-MDSC) to suppress the proliferation of $CD4^+$ T cells. When IgE/Ag activated, the BMMC-dependent increase of M-MDSC inhibitory potential was further amplified for $CD4^+$ T cells and became significant also for the $CD8^+$ cell compartment (Fig. 5.4B).

By contrast polymorphonuclear MDSCs (PMN-MDSC) were not suppressive in these experimental conditions and their inhibitory ability was not affected by co-culture with MCs (Fig. 5.4).

Following these first intriguing data, the loop between MCs, monocytic-MDSCs and $CD4^+$ T cells was specifically dissected in search of molecular mechanisms regulating these previously undescribed “partnership” in the control of antitumoral immune response.

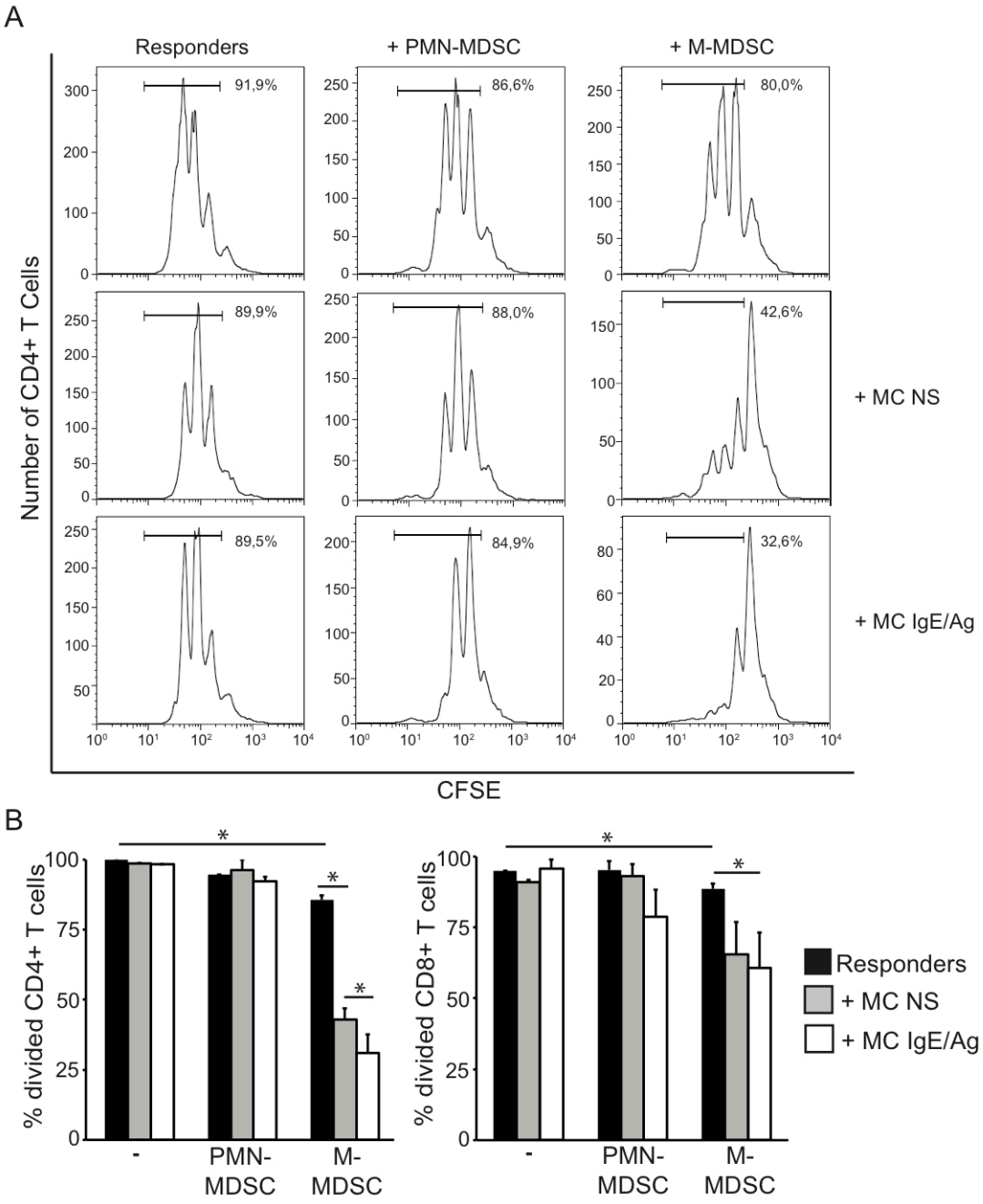

Figure 5.4: MCs enhance M-MDSC suppressive activity

(A-B) 1,5 x 10^5 total splenocytes (Responders) from untreated mice were CFSE-labeled and anti-CD3 activated in the presence of 1 x 10^5 purified PMN-MDSCs or M-MDSCs isolated from CT-26 tumor bearing mice, and/or of the same number of resting (+ MC NS) or IgE/Ag-stimulated BMMCs (+ MC IgE/Ag). After 72 hours, CFSE dilution in gated $CD4^+$ and $CD8^+$ T cells was evaluated by flow cytometry as a function of proliferation and results were expressed as percentage of divided T cells. (**A**) Representative histogram plots shows CFSE dilution in $CD4^+$ T cells in the different conditions. Percentages indicate the fraction of divided $CD4^+$ T cells. (**B**) Histograms represent the percentage of divided $CD4^+$ T (left panel) or $CD8^+$ T cells (right panel). Data (mean+s.e.m) are from at least three independent experiments. * $p < 0,05$.

5.3 MC-dependent MDSC suppressive activity is nitric oxide-mediated and IFN-γ-dependent

It has been shown that MDSCs can suppress T cell function through different mechanisms among which the production of soluble factors such as TGF-β, arginase 1, reactive oxygen species (ROS) and nitric oxide (Ostrand-Rosenberg and Sinha, 2009). Since the activity of monocytic-MDSCs is typically nitric oxide (NO)-dependent (Gabrilovich and Nagaraj, 2009), to verify NO relevance in the co-culture system, supernatants were collected after 72 hours of anti-CD3 stimulation and tested for nitrites production with Griess reaction. Both resting (MC NS) and activated (MC IgE/Ag) BMMCs were able to increase basal NO release by M-MDSCs (Fig. 5.5A), in direct ratio with inhibition of T cell proliferation (Fig. 5.4). To assess the specificity of NOS signaling on the M-MDSC activity, L-NG-monomethyl-arginine (L-NMMA, a nitric oxide synthase inhibitor) was used. As shown in figure 5.5B, the L-NMMA completely abolished the nitrites production and re-established T cell proliferation despite the presence of MC-MDSC.

Since freshly isolated MDSCs from tumor-bearing mice required IFN-γ to become fully suppressive and activate iNOS machinery (Gallina et al., 2006; Haverkamp et al., 2011), the possible contribution of this cytokine in the co-culture system was investigated. As expected, anti-CD3-polyclonally-activated splenocytes (responders) produced IFN-γ but, interestingly, a significant increase in IFN-γ levels was found when the responder population was co-cultured with resting or IgE/Ag-activated-BMMC either alone (as already described in Nakae et al., 2005) or together with M-MDSCs (Fig. 5.5C). Therefore, in the described in *vitro* microenvironment IFN-γ production was sustained despite the inhibition of T cell proliferation. On the opposite, for example, the release of IL-4, produced by proliferating Th-2-skewed T cells, followed the profile of T cell proliferation, with the lowest level found in the co-culture of M-MDSCs with activated MCs (Fig. 5.5C).

At this point, to understand the importance of IFN-γ in the co-culture system, a neutralizing anti-IFN-γ antibody was used: its addition significantly inhibited the acquisition of MC-dependent M-MDSC-suppression properties (Fig. 5.5B), underlining an important role for IFN-γ in M-MDSC activation.

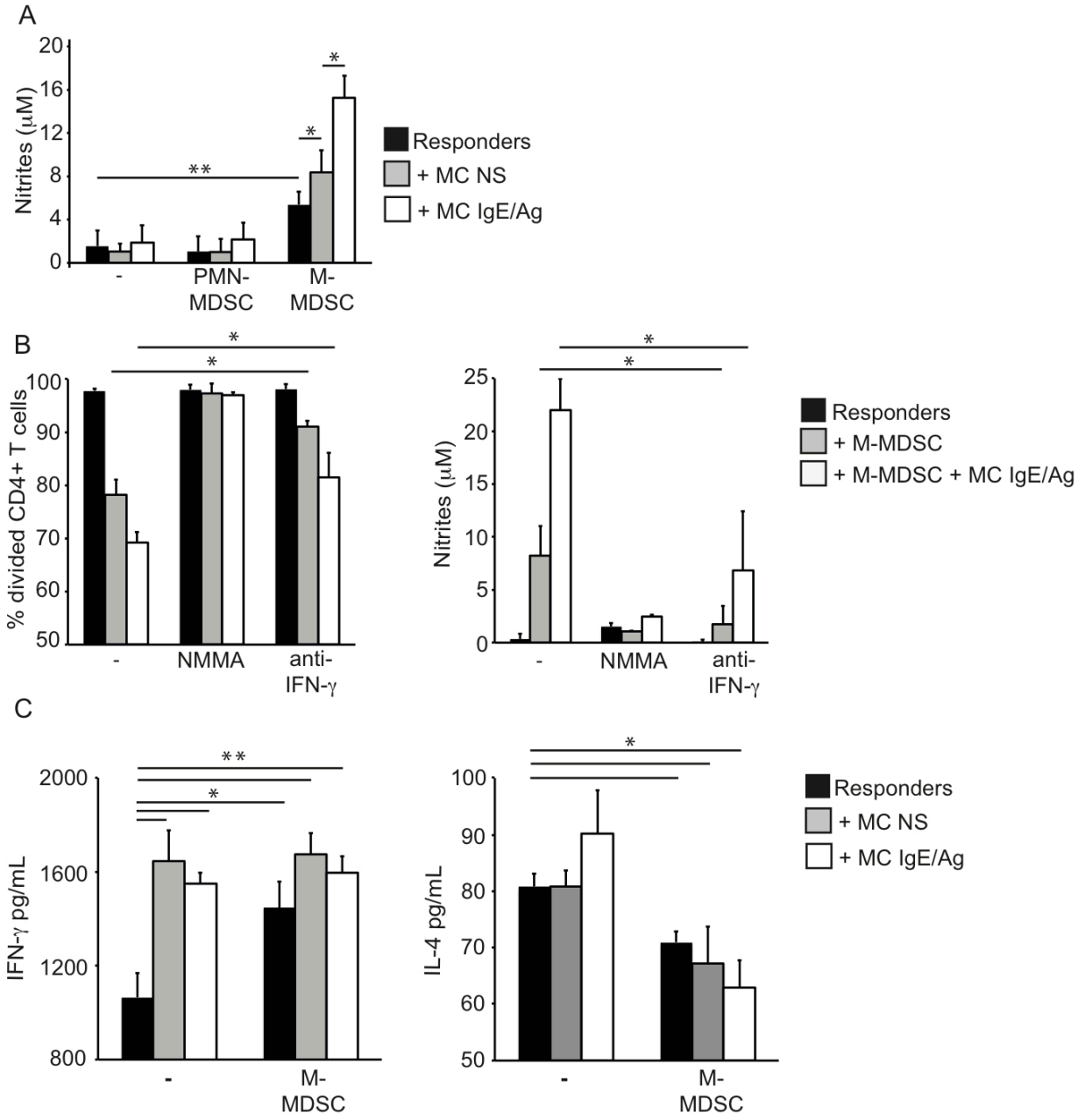

Figure 5.5: Role of nitric oxide and IFN-γ in MC-dependent-MDSC suppressive activities

(**A**) Supernatants from co-culture experiments of the proliferation assay (as in figure 5.4) were collected and tested for nitrites levels with Griess reagent. * $p<0,05$; ** $p<0,01$, n=3 experiments.

(**B**) Total splenocytes (Responders) were anti-CD3-stimulated in presence of M-MDSCs with (+ M-MDSC + MC IgE/Ag) or without (+ M-MDSC) IgE/Ag-stimulated BMMCs. Where indicated, an iNOS inhibitor (L-NMMA) and the neutralizing antibody anti-IFN-γ (10 μg/mL) were added. Results from one representative of three independent experiments are expressed as percentage of divided $CD4^+$ T cells (left panel) and nitrites production (right panel).

(**C**) Supernatants, as in A, were analyzed for the production of IFN-γ and IL-4. * $p<0,05$; ** $p<0,01$ versus anti-CD3-stimulated responder cells. Mean+s.e.m. of three independent experiments are shown.

Since activated-MDSCs are known producers of IFN-γ (Gallina et al., 2006), *gamma knock out* (*gko*) mice, that are deficient for IFN-γ expression, were used to discriminate the different contributes of IFN-γ production in the co-culture assay. Single cell populations (responder cells, M-MDSCs or BMMCs) from *gko* mice were used for the proliferation assay in the presence of other cellular components derived from *wt* Balb/c mice. IFN-γ deficiency in responder cells reduced almost completely MC-dependent M-MDSC activation, emphasizing the crucial role of IFN-γ in NOS machinery activation (Fig. 5.6A). Interestingly, in the presence of *gko*-derived M-MDSCs nitrites production was not affected compared to *wt* MDSCs, and consequently they still suppressed proliferation of $CD4^+$ T cells (Fig. 5.6B). In addition, *gko*-derived-BMMCs were still able to potentiate MDSC-nitrites production similarly to the wild type counterpart (Fig. 5.6C), suggesting that their potential source of IFN-γ was not necessary in this setting.
To confirm the importance of T cells as source of IFN-γ, we purified and used $CD4^+$ $CD25^-$ T cells from spleen of untreated *wt* mice as CFSE-labeled responder population in presence of *wt* mytomicin-treated APCs (as described in Methods 7.9). In these experimental conditions, $CD4^+$ T cell proliferation was inhibited at high levels by M-MDSCs alone, independently from MC presence. Despite MCs did not play a crucial role in T cell suppression, unstimulated or IgE/activated BMMCs continued to increase NO levels when co-cultured with M-MDSCs (Fig. 5.6D). To exclude IFN-γ-production by other non-T cells, proliferation of *wt* $CD4^+$ T cells was performed in the presence of *gko*-derived APCs. Figure 5.6D shows that M-MDSCs, either alone or together with MCs, were still competent in the suppression of $CD4^+$ T cell proliferation, indirectly confirming that anti-CD3-activated-$CD4^+$ T cells represent the crucial initial source of IFN-γ required to fully activate MC-MDSC suppressive axis.

Both in *vitro* and in *vivo*, MCs can be activated in an Ag-dependent or independent manner. Since in the co-culture system Ag-not specific stimuli can activate other cell populations present among the splenocytes used as responder cells, the experiments described so far were performed with IgE/Ag-stimulated MCs. Therefore, to investigate if the M-MDSC suppressive activity is affected by the activation status of the different cellular immune populations existing in the ex *vivo* generated inflammatory microenvironment, different stimulatory patterns were investigated. Proliferation assays were performed in the presence of the TLR4 activator lipopolysaccharide (LPS) and of phorbol myristate acetate (PMA), known activator of both MCs and MDSCs (Klemm et al., 2006; Bunt et al., 2009; Corzo et al., 2009; Greifenberg et al., 2009; Hochdörfer et al., 2011), as well as a mitogen for T cell proliferation in case of PMA (Kay, 1991).

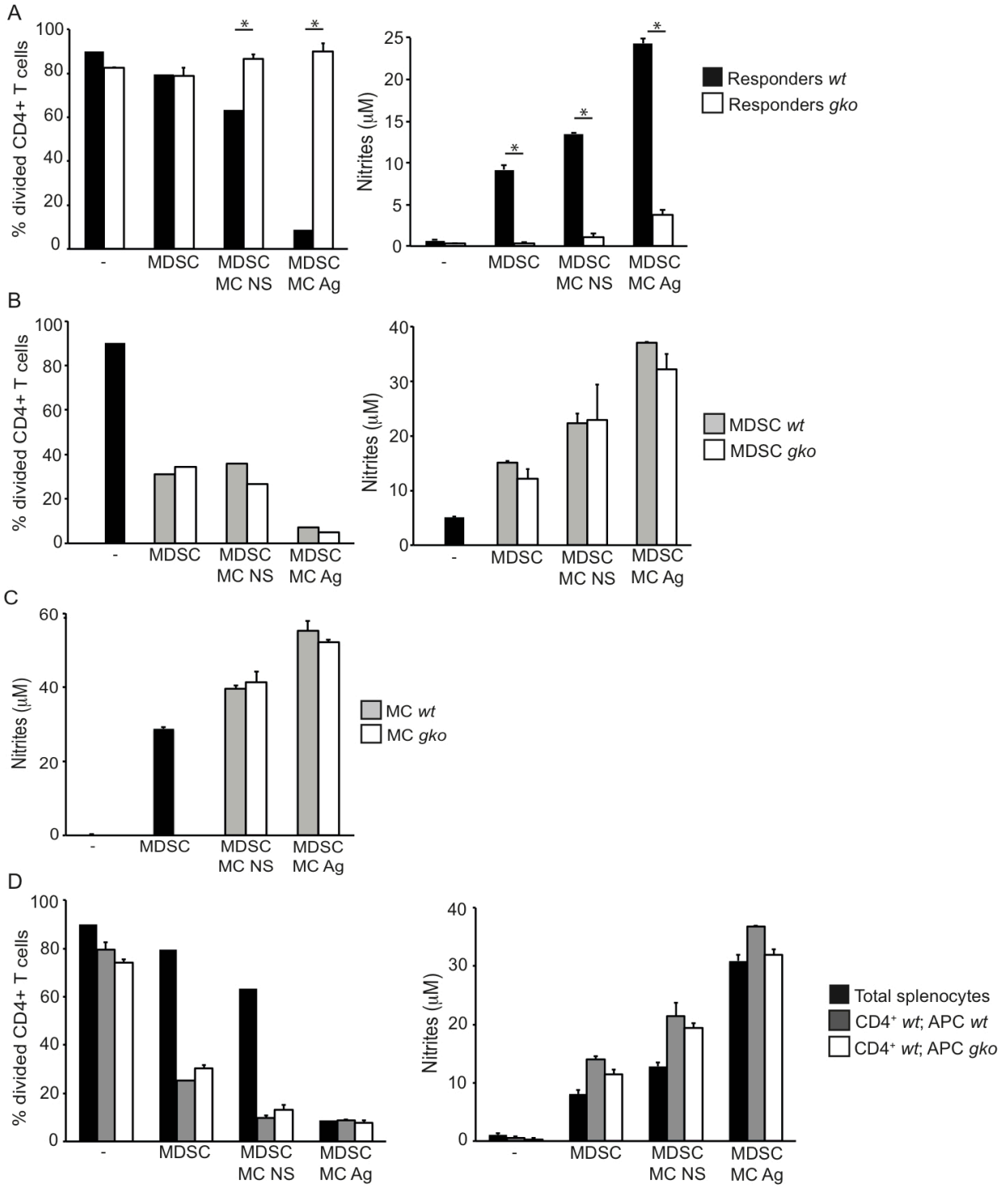

Figure 5.6: IFN-γ from anti-CD3-activated T cells is required for activation of MC-MDSC axis.

(A-C) Proliferation assay was performed as explained in figure 5.4 using respectively total splenocytes (Responder *gko* in **A**), M-MDSCs (in **B**) or BMMCs (in **C**) derived from *gko* Balb/c mice while the other cellular components were derived from *wt* Balb/c mice. Results (mean+s.e.m.) from three independent experiments are expressed as percentage of divided $CD4^+$ cells and/or nitrites production and are compared to proliferation of the *wt* system. * $p < 0,05$. (**D**) 1,5 x 10^5 $CD4^+$ $CD25^-$ T cells, purified from *wt* Balb/c mice, were anti-CD3-stimulated in presence of 7,5 x 10^4 mytomicin-treated APC derived from *wt* (grey bars) or *gko* (white bars) mice, with or without M-MDSCs and resting or IgE/Ag-stimulated MCs. Data from one representative of two experiments were shown and compared with proliferative response of total splenocytes from *wt* mice (black bars).

As shown in figure 5.7, both LPS and PMA stimulation raised the basal level of NO secretion by M-MDSCs, phenomena that was completely (for PMA) or partially (for LPS) reverted by using a neutralizing anti-IFN-γ antibody. As for IgE-dependent activation, LPS- or PMA-activated BMMCs boosted M-MDSC production of NO. Worthy of remark, pattern reversion by addition of neutralizing anti-IFN-γ demonstrated that mechanism of MC-dependent M-MDSC suppression was commonly mediated by IFN-γ for IgE-dependent and -independent MC activation (Fig. 5.7).

Overall these data suggest that in a Th1-skewed-microenvironment, MCs may actually switch on M-MDSC suppressive function, inhibiting Teff proliferation in a NO dependent manner. Since M-MDSCs were not competent in the presence of *gko*-derived proliferating T cells, the initial burst in IFN-γ production by anti-CD3 stimulated T cells seemed to be necessary to activate this previously undescribed MC-MDSC suppressive axis.

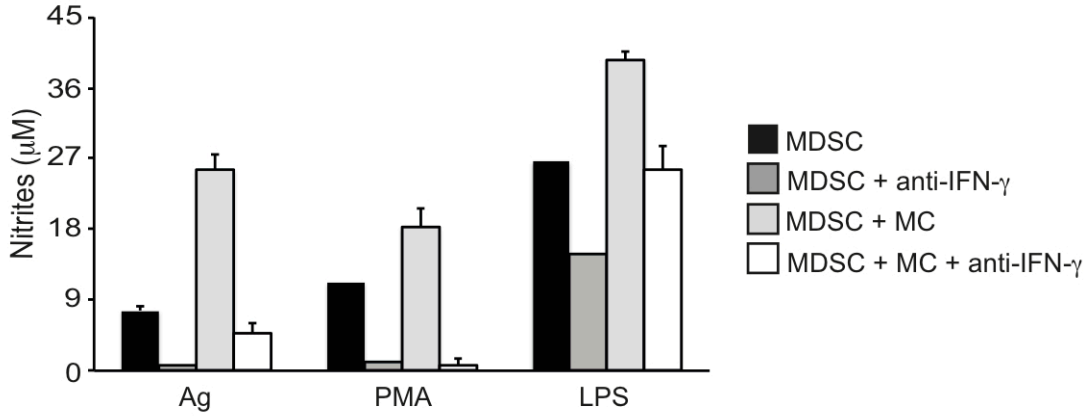

Figure 5.7: MCs maintain their ability to increase M-MDSC suppressive potential in presence of IgE-independent stimuli.

1,5 x 10^5 anti-CD3 activated-total splenocytes from untreated *wt* mice were stimulated in the presence of 1 x 10^5 M-MDSCs alone (MDSC, black histograms) or together with an equal number of IgE-sensibilized BMMCs (MDSC+MC, clear grey bars). Where indicated, the neutralizing antibody anti-IFN-γ (10 μg/mL) was added to the co-culture (MDSC+anti-IFN-γ, dark grey bars; MDSC+MC+anti-IFN-γ, white bars). 100 ng/mL IgE-specific Ag DNP (Ag), 100 ng/mL PMA or 5 μg/mL LPS were used as stimuli. After 72 hours, supernatants were collected and nitrites levels assayed with Griess reagent. Results (mean+s.d.) from one representative experiment are shown.

To confirm that IFN-γ was a limiting factor in MC:MDSC cross talk, we examined the outcome of the direct interaction between M-MDSCs and BMMCs, in the absence of anti-CD3-activated splenocytes. Equal numbers of BMMCs and M-MDSCs were cultured either alone or together for 72 hours, at the end of which supernatants were collected and nitrites levels were measured. As shown in figure 5.8A, resting or IgE/Ag-activated BMMCs were not sufficient alone to switch on M-MDSC activation but activated BMMCs contributed to generate NO-competent M-

MDSCs in presence of soluble IFN-γ. Therefore, similarly to what happens in the presence of IFN-γ producing proliferating T cells, MCs are potentially able to activate and amplify the suppressive function of M-MDSCs.

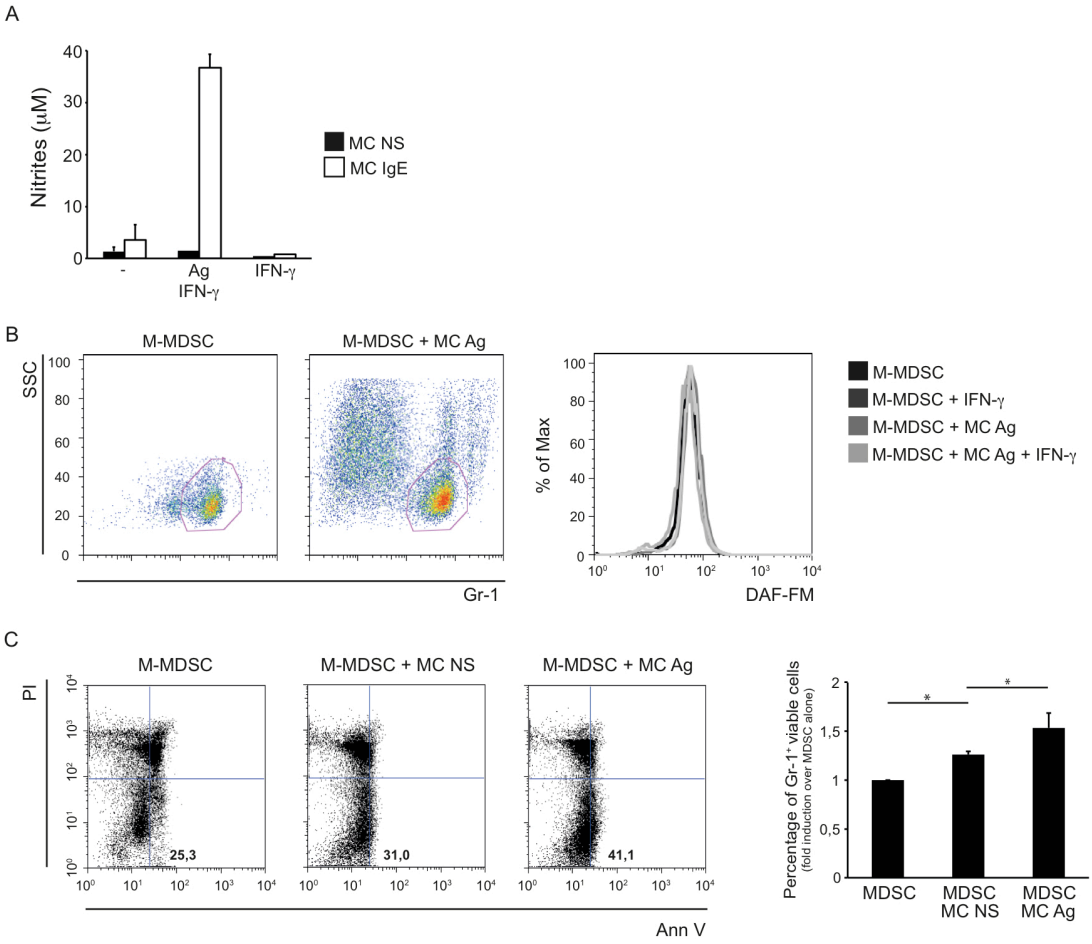

Figure 5.8: MCs promote nitrites production and survival of M-MDSC

(**A**) 2 x 10^5 M-MDSCs were cultured with an equal number of resting (MC NS, black histograms) or IgE-sensitized (MC IgE, white histograms) BMMCs without additional stimuli (-) or in the presence of 20 ng/mL IFN-γ (IFN- γ), and eventually 100 ng/mL IgE specific Ag DNP (Ag IFN-γ). After 72 hours nitrites levels in supernatants were measured with Griess method.

(**B**) 2 x 10^5 M-MDSCs were cultured alone or with an equal number of IgE/Ag stimulated BMMC without additional stimuli or in presence of 20 ng/mL IFN-γ as indicated. After 5 hours of co-culture, cells were harvested, washed and stained with the NO indicator DAF-FM diacetate. The levels of DAF-FM in Gr-1^+ M-MDSCs (gating strategy is shown in the dot plots, left panel) in the different experimental conditions are shown in the histogram (right panel)

(**C**) 2 x 10^5 M-MDSCs were incubated alone (-) or co-cultured with an equal number of resting (MC NS) or IgE/Ag stimulated (MC Ag) BMMCs. Then, after 24 hours, they were stained with Annexin V and Propidium Iodide (PI) for detection of viable cells. Representative dot plots of AnnV/PI staining for Gr-1^+ gated MDSC cells (numbers indicate the percentage of viable cells) and results (mean+s.d. of two independent experiments) expressed as percentage of viable cells ($AnnV^{neg}$ PI^{neg} gated cells) in terms of fold induction over M-MDSCs alone are shown. *p<0,05.

A possible explanation for the observed increase in nitrites secretion in the co-culture systems could be that cell-to-cell contact or MC-derived soluble factors promote signals in monocytic-MDSCs that lead to increased nitric oxide levels. To test this possibility, M-MDSCs were co-cultured with resting or IgE/Ag stimulated MCs in the presence of soluble IFN-γ and then labeled with the nitric oxide indicator DAF-FM diacetate. Levels of intracellular NO were detected after 5 (Fig. 5.8B), 24 and 72 hours (data not shown) of co-culture, but no significant difference was seen in terms of quantity of intracellular NO in MDSCs. Of note, this last result did not exclude the possibility that MCs could favor the secretion of NO from MDSCs.

Since MCs did not seem to be able to up-regulate NO expression at the single cell level, it was investigated the possibility that MCs could improve the survival of the monocytic-MDSC population. As showed in figure 5.8C, when M-MDSCs were cultured with resting MCs, there were significantly more viable Gr-1^+ cells compared to M-MDSCs incubated alone. Survival of MDSCs was further improved by soluble factors produced by IgE/Ag stimulated-MCs (Fig. 5.8C).

5.4 MC-dependent MDSC suppressive activity is TNF-α dependent

MCs are able to influence the biology of several immune cell types both through cell-to-cell contact and through the release of soluble mediators (Gri et al., 2012). Therefore to investigate if the MC-dependent increase of M-MDSC suppressive function was dependent on cell-to-cell contact, co-culture experiments were performed in the presence or absence of a transwell that physically separates the cell populations. As shown in figure 5.9A, when BMMCs were separated from M-MDSCs and proliferating T cells by a transwell membrane filter, no significant differences in T cell suppression were observed and the profile of nitrites production was maintained.

Despite the transwell result, the requirement of CD40:CD40L axis in the activation of M-MDSC in this setting was evaluated. The choice of taking into account the role of this interaction was due to the important role of CD40 expression and ligation in the activation and in the immunosuppressive function of MDSCs (Pan et al., 2010) and to the known constitutive expression of its ligand, CD40L, on mast cell surface (Merluzzi et al., 2010),. To this end, the proliferation assay was repeated using BMMCs obtained from *cd40l*$^{-/-}$ mice. Similarly to the transwell experiment, no significant differences in terms of MDSC-dependent T cell suppression or nitrites productions were found compared to *wt* BMMCs (Fig. 5.9B). All these findings revealed that cell-to-cell contact between M-MDSCs and MCs was not necessary to up-regulate nitrites production in the suppressive population, suggesting the involvement of soluble

mediators in MC-dependent M-MDSC activation and inhibition of T cell proliferation.

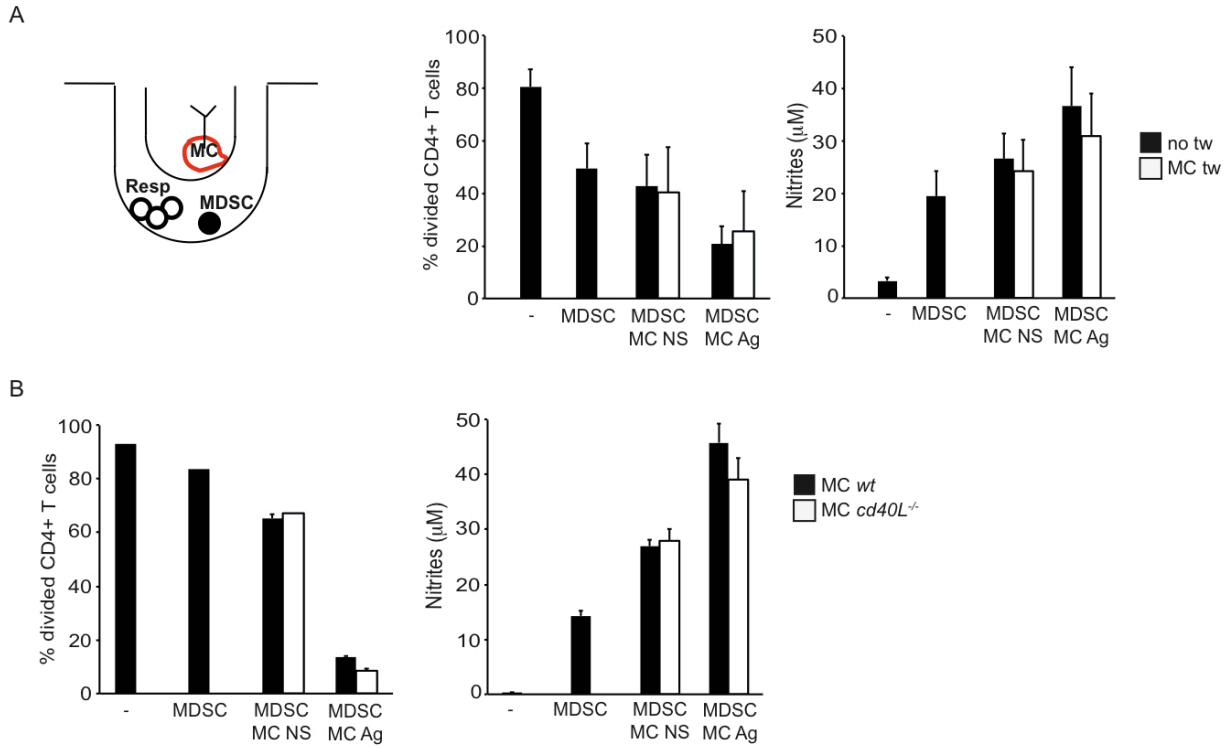

Figure 5.9: MC-MDSC contacts are not functionally necessary to exacerbate M-MDSC suppressive potential

(**A**) 3 x 10^5 CFSE labeled splenocytes from untreated *wt* Balb/c mice (Resp) were anti-CD3-stimulated in the presence of 2 x 10^5 M-MDSC and/or of an equal number of resting or IgE/Ag-stimulated-BMMCs. A transwell system was used to physically separate different cell populations. BMMCs were added in direct contact with T-MDSC co-culture (no tw, black bars) or separated by membrane (MC tw, white bars). After 72 hours, the percentage of divided $CD4^+$ T cells and the levels of nitrites were evaluated. Data are from three independent experiments.

(**B**) Proliferation assay was performed as in figure 5.4 comparing the effect of *wt* (black bars) and *cd40l*$^{-/-}$ (white bars) BMMCs on MDSC-dependent T-cell suppression. One representative of three experiments is shown and results (mean+s.d.) are expressed as percentage of divided $CD4^+$ T cells (left panel) and levels of nitrites (right panel).

Since mast cell-derived TNF-α is required for T cell activation (Nakae et al., 2005; Nakae et al., 2006) and as this cytokine is involved in the activation of nitric oxide-producing early myeloid cells (Angulo et al., 2000), the role of TNF-α in the co-culture system was tested. For this purpose the proliferation assay was performed in the presence of a neutralizing anti-TNF-α antibody. A decreased response of MC-dependent M-MDSC suppressive activity was observed following TNF-α blocking (Fig. 5.10A). *Tnf-α*$^{-/-}$-derived BMMCs showed no differences in their ability to amplify M-MDSC inhibitory activity compared to *wt* BMMCs; in addition MC-MDSC suppressive axis was similarly inhibited by using the neutralizing anti-TNF-α antibody (nitrites levels are shown in figure 5.10B), suggesting that in the proliferation assay the crucial TNF-α source responsible for MDSC activity was not directly represented by MCs.

Notwithstanding, these evidences prompted to further investigate the relevance of TNF-α in this experimental setting. When IgE/Ag activated-BMMCs were co-cultured with M-MDSCs, the secretion of TNF-α was significantly higher compared to cytokine levels produced by IgE/Ag-stimulated MCs alone (Fig. 5.10C). It must be emphasized that M-MDSCs themselves may be an important source of TNF-α: even if their basal secretion levels were almost undetectable, these cells released a large amount of TNF-α when LPS-activated (data not shown). To understand the real source of TNF-α and to try to elucidate the mechanism of the synergism in TNF-α production, the co-culture experiment was performed with *tnf-α*$^{-/-}$ BMMCs. In this condition, IgE/Ag-activated-*tnf-α*$^{-/-}$ BMMCs were not able to produce the cytokine and, interestingly, the level of TNF-α in M-MDSC/MC co-culture was similarly undetectable, suggesting that MCs constitute the crucial source of TNF-α (Fig. 5.10C).

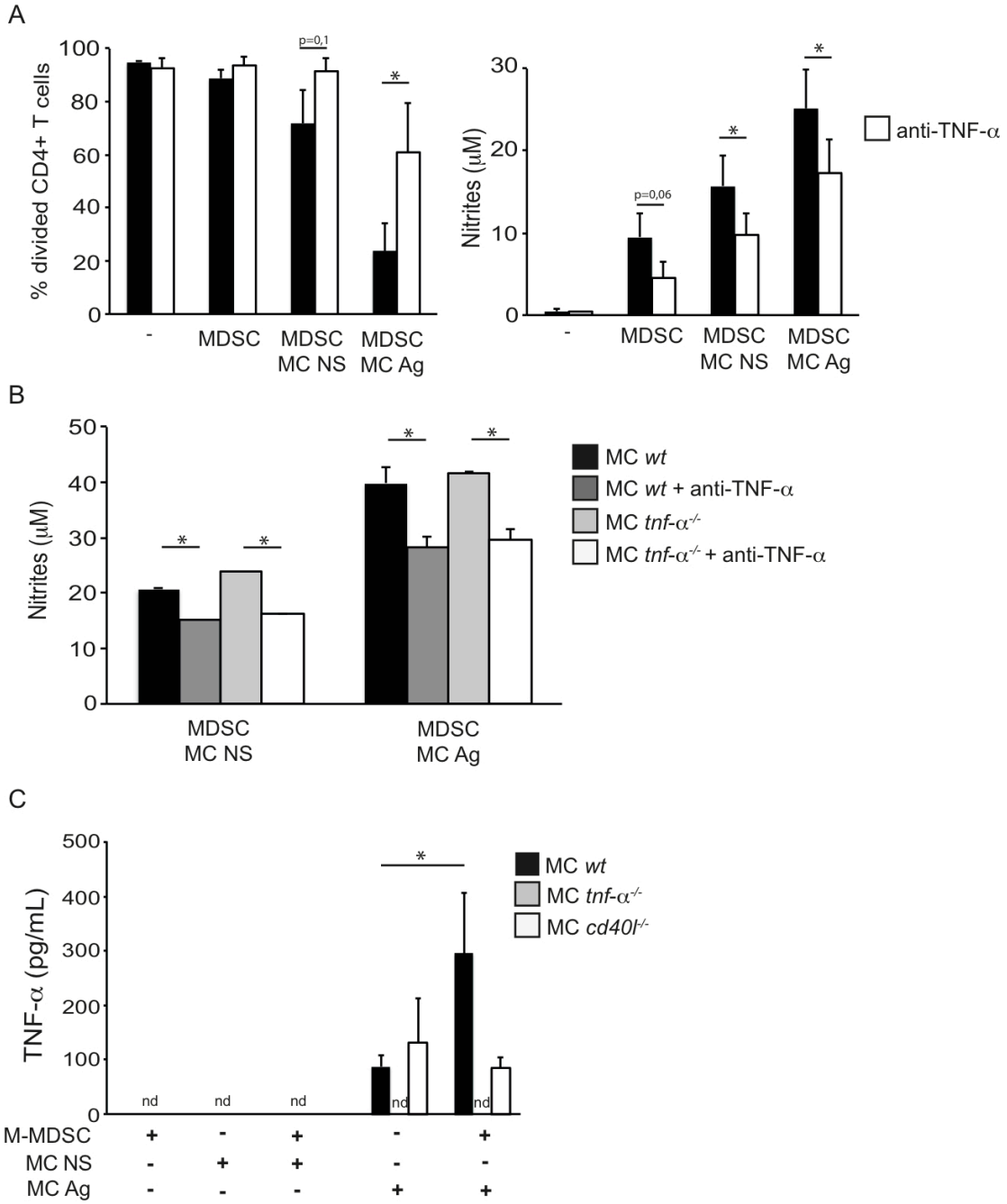

Figure 5.10: Role of TNF-α in MC-dependent MDSC suppressive properties

(**A)** Anti-CD3-stimulated responder splenocytes were co-cultured with M-MDSCs and/or resting or IgE/Ag activated-BMMCs. Proliferation assay was performed in the presence of 10 μg/mL of neutralizing anti-TNF-α antibody. Means+s.e.m. of three independent experiments are shown as percentage of divided $CD4^+$ T cells (left panel) and as levels of nitrites production (right panel). * $p < 0,05$. (**B**) The same experiment was performed also in the presence of *tnf-α*$^{-/-}$ BMMCs and results were compared with the ones obtained with *wt* BMMCs. One representative of three independent experiments is shown in terms of nitrites levels. *$p < 0,05$.

(**C**) 2 x 10^5 M-MDSCs were cultured with an equal number of resting (MC NS) or IgE/Ag-activated (MC Ag) BMMCs derived from *wt* (black bars), *tnf-α*$^{-/-}$ (grey bars), or *cd40l-/-* (white bars) mice. Supernatants were collected after 24 hours of co-culture and TNF-α levels were measured by ELISA. Data are the mean+s.e.m. of three independent experiments. n.d.: not detectable.

5.5 CD40:CD40L interacting MDSCs and MCs acquire an enhanced activation state

The finding of synergism between MCs and MDSCs in the context of TNF-α production led to investigate if the increase in cytokine production was a general outcome of their interaction.

Whereas resting BMMCs, either alone or together with M-MDSCs, released low or undetectable levels of the investigated mediators, co-culture between activated BMMCs and MDSCs resulted in the significant increase in IL-6 and CCL-2 release, similarly to what happened for TNF-α production (Fig. 5.11A). Interestingly, when the same experiment was performed with *cd40l*$^{-/-}$ BMMCs, the amount of mediators produced by activated MCs was not affected by the presence of M-MDSC (Fig. 5.11A). Neither resting or IgE/Ag-activated BMMCs, or M-MDSC alone, produced detectable levels of CCL-3, IL-2, IL-4, IL-10 or IL-17, and a synergistic effect in the production of these cytokines was not observed in the M-MDSC:MC co-culture (data not shown).

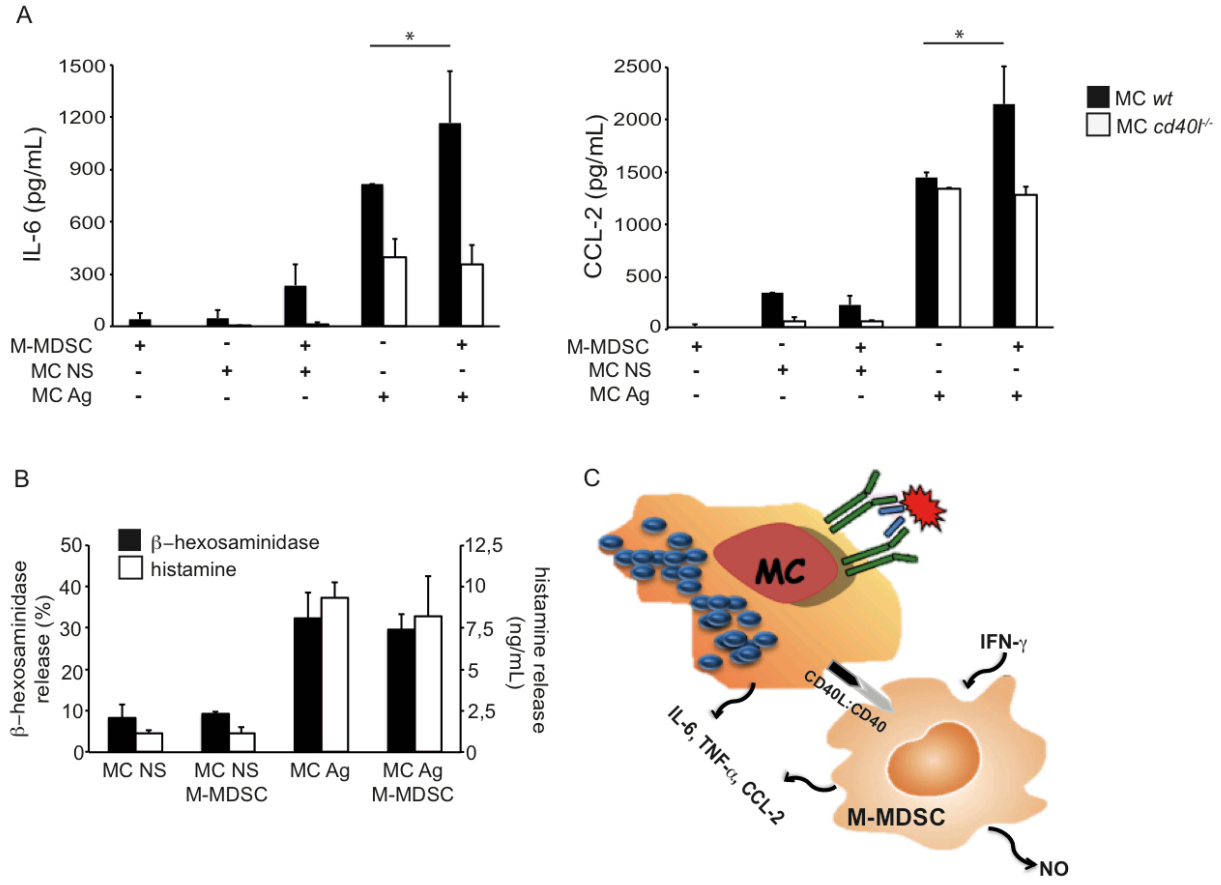

Figure 5.11: CD40:CD40L interacting M-MDSC:MC increase cytokine production

(**A**) 2 x 10^5 M-MDSCs were cultured with an equal number of resting (MC NS) or IgE/Ag-activated (MC Ag) BMMCs derived from *wt* (black bars) or *cd40l*$^{-/-}$ (white bars) mice. Supernatants were collected 24 hours after the beginning of co-culture. IL-6 and CCL-2 levels were measured by ELISA. Data are the mean+s.d. of at least two independent experiments. n.d.: not detectable.

(**B**) BMMCs sensitized with IgE (MC NS) and challenged with Ag (MC Ag) in the presence or absence of equal amounts of M-MDSCs were examined for release of b-hexosaminidase (expressed as percentage of the cells total

mediator content-black bars), and histamine (white bars), respectively after 30 minutes and 1 hour of co-culture. Shown are the means±s.d. of three independent experiments.

(**C**) Schematic representation of the cross talk between MCs and monocytic MDSCs. CD40:CD40L interacting M-MDSC:MC showed a synergistic increase in the production of pro-inflammatory mediators, such as TNF-α, IL-6 and CCL-2. In the presence of IFN-γ, activated-MCs were able to stimulate nitric oxide (NO) production in M-MDSCs, rendering them competent to inhibit T cell proliferation. The last effect was not directly dependent on the CD40:CD40L axis (data not shown).

Therefore, these results suggest that the M-MDSC:MC axis, through CD40:CD40L interaction, was shown to induce a selective and preferential production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6 and CCL-2, that could sustain an immune response supporting tumor promotion.

As a last point, the possibility that M-MDSCs could influence also immediate and early responses of MCs was evaluated. In this experimental setting, FcεRI-initiated degranulation, in terms of both release of β-hexasominidase and histamine production, was not affected by IgE/Ag-activation in the presence of M-MDSCs (Fig. 5.11B)**.**

Summarizing, MC cytokine production, but not degranulation events, are influenced by cross talk with M-MDSCs.

5.6 MC role in an inflammation-associated colon cancer model

Inflammation is a well-known enabling characteristic of tumor and increases cancer initiation and progression. About 25% of tumor types are associated with chronic inflammation sustained by infection or inflammatory conditions of different origin, such as inflammatory bowel disease (IBD) and the related risk of colitis-associated colorectal cancer (CAC) development.

Contribution of MCs to the development of CAC is not well understood and no conclusive data are reported.

To determine whether MCs were involved in CAC, colorectal tumors were induced in the colon mucosa of mice by using the colonic carcinogen azoxymethane (AOM) and the tumor-promoter dextran sodium sulfate (DSS). In this model, an important infiltration of MCs within and around adenocarcinomas has been previously described (Tanaka et al., 2003).

The induction of CAC was based on a single i.p. injection of azoxymethane (AOM, 10 mg/kg) and by three cycles of 1-week oral exposure to dextran sodium sulfate (DSS, 2%) followed by 2 weeks of normal drinking water. To dissect MC role in this model of carcinogenesis, AOM/DSS treatment was performed in C57BL/6 control mice (*wt*, aged 10-12 weeks), and in syngeneic and age-matched C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice (*wsh*), which are genetically deficient for MC

population (Grimbaldeston et al., 2005). To determine if any differences in tumorigenesis actually depend on MC behaviour, Wsh mice were reconstituted with bone marrow-derived mast cells (*MC --> wsh*, as described in Methods 7.13). Starting from general observations, it was reported that several mice of each genotype had bloody stools during DSS treatment and in the following days, with peaks of weight loss during DSS cycles. Nevertheless no clinical symptoms were observed thereafter. During acute phases of treatment, two *wt* animals and one *wsh* mouse died and were not considered in further analysis. Starting from week 12, mice were sacrificed and colons were collected and analyzed for tumor lesions, which located preferentially in the distal region of colon. These macroscopic tumors were histopathologycally determined to be intramucosal adenocarcinomas, while microscopic lesions were low-grade adenomas. MC presence and localization was examined in colons of treated mice. Giemsa staining (Fig. 5.12A) revealed that in *wt* mice rare MCs were present within tumors (and in the adjacent normal mucosa) but they were found isolated or in foci infiltrating peri-tumoral muscularis propria and adipose tissue. As expected, no MCs were present in treated MC-deficient *wsh* mice (data not shown), while in reconstituted *wsh* mice higher numbers of MCs were found in intra- and peri-tumoral regions compared to *wt* mice: this result confirmed both the efficiency of reconstitution protocol and the tumor-dependent recruitment of MCs (Fig. 5.12C) To evaluate the functional importance of MC presence in tumor, total numbers of polypoid lesions, also according to size and total volume, were enumerated and mice genotypes were compared to dissect a specific role of MCs in carcinogenesis. Despite the depicted trend in which MC-deficient *wsh* mice presented higher adenocarcinoma multiplicity and higher mean volumes of tumors compared to *wt* MC-competent mice and MC-reconstituted *wsh* mice, differences were not statistically significant (Fig. 5.12B). Despite the need to increase the numerosity of mice used for experiments to confirm these results, these findings suggest that the increased susceptibility of MC-deficient mice to AOM/DSS-induced colorectal carcinogenesis could be dependent on the primarily protective role of MC as “guardian” of the efficacy of the mucosal barrier, as suggested for other inflammation-associated models (Chichlowski et al., 2010). Therefore, MC-deficient *wsh* mice seemed to be more susceptible to DSS-induced damage and inflammation that switched on carcinogenesis. Nevertheless it was not possible to exclude that MCs, once recruited, could play a pro-tumoral role through pro-inflammatory cytokines and enzymes production and secretion.

To overcome experimental limits of *wsh* MC-deficient mice, that present other *kit*-dependent MC-independent abnormalities, the future perspective is to repeat the induction of the AOM/DSS model of colon cancerogenesis in a conditional MC-deficient mouse model, as a new approach in the study of MC biology.

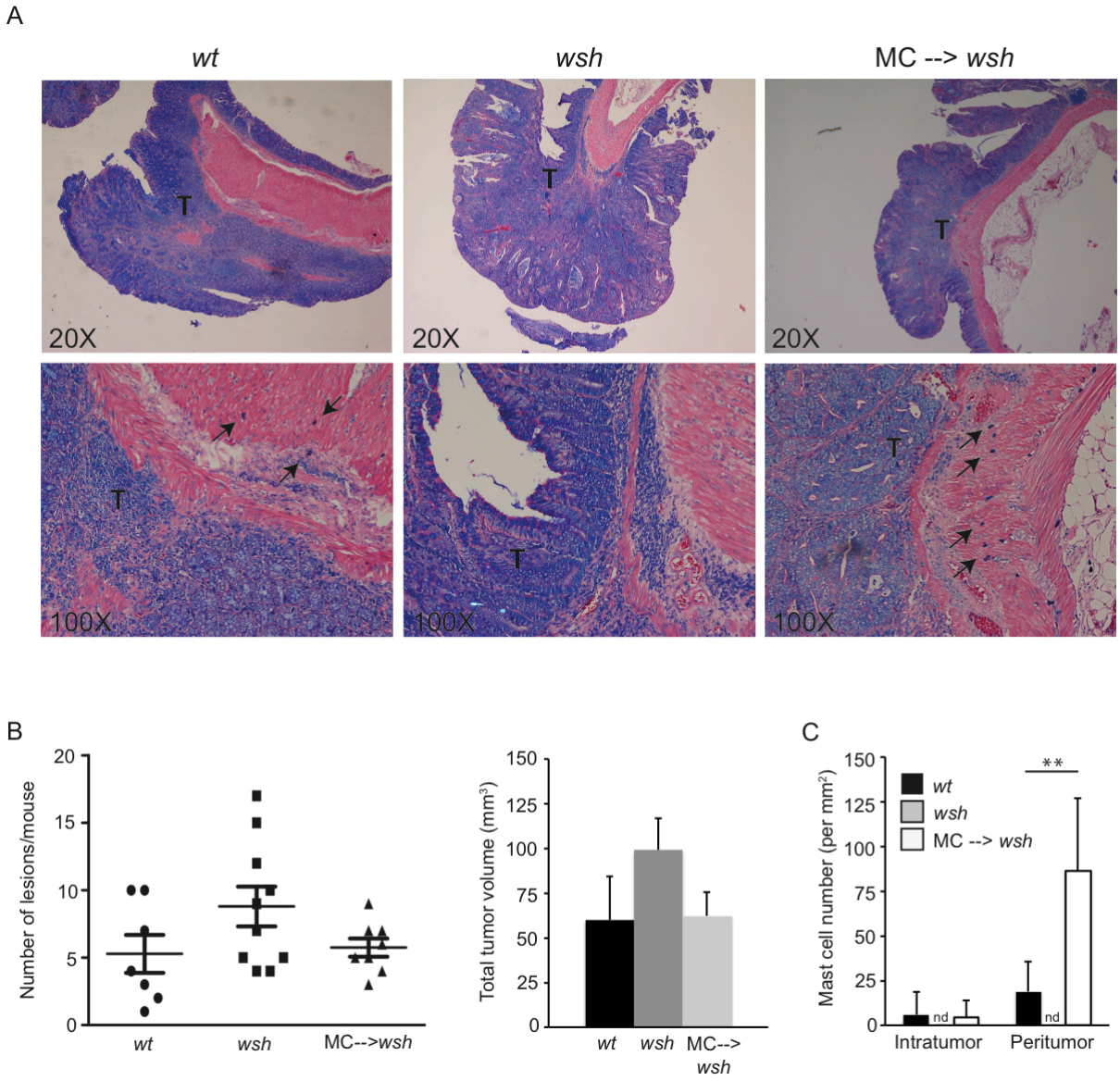

Figure 5.12: AOM/DSS-induced colon cancer development in MC-deficient mice.

(**A-C**) Wild type C57BL/6 mice (wt, n=7), syngeneic genetically mast cell-deficient *wsh* mice (wsh, n=10), and *wsh* mice that have been repaired of their MC deficiency by intraperitoneally reconstitution with BMMCs at 4-6 weeks of age (MC --> wsh) were AOM/DSS treated to induce colon cancer formation. Mice were sacrificed 12 weeks after the beginning of AOM/DSS treatment.

(**A**) Representative images of tumor sections (Giemsa staining) from *wt*, *wsh* or MC-reconstituted *wsh* AOM/DSS treated mice are shown. T indicate tumor areas; arrows indicate MCs. (**B**) Numbers of total colon lesions (left panel, each symbol in graphs represents the total number of lesions per mouse) and total tumor volume (mean+s.e.m.) are shown. Data were pooled from two independent experimental groups and were analyzed using both the ANOVA parametric test and the non-parametric Krustal-Wallis test.

(**C**) Mast cells were counted through Giemsa staining in formalin-fixed paraffin-embedded section of colon from all studied animals. Data (mean+s.d) are expressed as number of MCs per mm^2. *p<0,05.

Chapter 6: CONCLUSIONS AND DISCUSSION

Mast cell (MC) is widely recognized as an early and persistent infiltrating cell in many types of tumors. MC can influence tumor growth either through direct effect on tumor cells or through indirect mechanisms, such as MC-driven angiogenesis, tissue remodeling or modulation of the recruitment and functionality of other immune cells in the neighboring tumor-associated microenvironment (Maltby et al., 2009).

Within tumor-infiltrating immune cells, myeloid-derived suppressor cells (MDSCs) are primarily recognized and studied for their immunosuppressive properties that operate to inhibit the antitumor immune response (Ostrand-Rosenberg and Sinha, 2009). However, their interactions with other innate immune cells in the tumor-associated microenvironment are not well characterized.

The idea that MCs could mobilize and enhance the immunosuppressive function of MDSCs recently emerged (Yang et al., 2010; Cheon et al., 2011). Mast cells constitute a selective source of 5-lypooxigenase that significantly potentiates the growth of the intestinal polyps, with a mechanism involving the recruitment of MDSCs to the polyp site and the increase of the activity of the immune suppressive enzyme arginase-1 (Cheon et al., 2011). Further supporting this hypothesis, Conrad's group described the critical capacity of MCs to augment MDSC activity in *vivo*. This work demonstrated that the suppression of antitumor immune response mediated by monocytic-MDSCs is MC-dependent, since wild type mice were more susceptible to tumor metastasis compared to mast cell-deficient mice. In addition M-MDSC:MC interaction determined a synergistic Th2-skewed immune response, undesirable in the context of cancer immune response (Saleem et al., 2012). All together these data revealed the existence of an interesting closed loop between MCs and MDSCs that provides a new insight into the relationship between inflammation and immunosuppression in tumor microenvironment.

On the basis of these evidences, the present work aimed to investigate and learn more about the nature of the MDCS:MC axis in cancer microenvironment. For this reason, the role of MCs in a colon cancer model was investigated, as well as their interaction with MDSCs during tumor-associated immune response. The carcinogen-induced undifferentiated colon carcinoma cell line CT-26 was adopted for in *vitro* and in *vivo* studies. This cell line, largely adopted in the study of colon cancer immune response, was originally selected also because it showed a relatively strong accumulation of MDSCs (Fig. 5.3A; (Cheng et al., 2008)). CT-26 cancer cell line and bone marrow derived mast cells (BMMC) were used for in *vitro* experiments and the obtained results

demonstrated that tumor cells were able to induce MC migration, to modulate MC gene expression and the release of early and late mediators (Fig. 5.1). In *vivo,* MCs were recruited and infiltrated CT-26 growing tumors, and their pro-tumoral role was demonstrated since mice pharmacologically treated with a MC stabilizer showed reduced tumor growth (Fig. 5.2).

In addition, Gr-1^+ and $CD11b^+$ cells were found to physically make contacts with $Fc\varepsilon RI^+$ MCs in the spleen of CT-26 tumor bearing mice (Fig. 5.3B). Following this observation, the relevance of the interplay between these two immune populations and the molecular mechanisms involved were investigated ex *vivo* by using BMMCs and MDSCs derived from the spleen of CT-26 tumor bearing mice, purified as polymorphonuclear $Ly6g^+$ Gr-1^{high} $Cd11b^+$ (PMN-MDSC) and monocytic $Ly6g^-$ Gr-1^{dim} $CD11b^+$ (M-MDSC) cells (Fig. 5.3C). In the anti-CD3-dependent proliferation assay (Methods 7.9), peripheral isolated MDSCs had a low ability to suppress polyclonal antigen-nonspecific activation of $CD4^+$ T cells. Interestingly, when the monocytic-MDSC population was co-cultured with resting MCs, their suppressive function was significantly increased. IgE/Ag-dependent and -independent activation further increased the potential of MCs to boost M-MDSC suppression of T cell proliferation (Fig. 5.4 and 5.7), with a mechanism involving the production of nitric oxide (Fig. 5.5). Furthermore, it was shown that IFN-γ production by activated $CD4^+$ T cells was required to sustain initial activation of M-MDSCs and render functional the M-MDSC:MC suppressive axis (Fig. 5.5 and 5.6). MC-dependent M-MDSC suppressive function was partly soluble mediated, involving TNF-α production (Fig. 5.10). Further experiments showed that cell-to-cell contact was not necessary to activate M-MDSC:MC suppressive axis (Fig. 5.9), but that CD40:CD40L axis was required to confer an enhanced activation status regarding the selective release of pro-inflammatory mediators such as TNF-α, IL-6 and CCL-2 (Fig. 5.11) which could drive an immune response supporting tumor growth and likely create a positive feedback loop that enhances monocyte progenitor recruitment. In fact, IL-6 is known to induce MDCS accumulation and enhance tumor progression (Bunt et al., 2007). Likewise, CCL-2 has multiple roles in cancer progression and its crucial involvement in the accumulation of MDSCs at the cancer site is one of the described protumoral effects of the chemokine (Huang et al., 2007). It is possible to speculate that the importance of CD40 could be "lost" in the proliferation assay because of the presence of other CD40L positive cells, such as T cells and macrophages, in the splenocytes preparation, with a possible redundant role in CD40 activation of MDSCs respect to MCs.

The molecular mechanisms proposed in the model of MC-dependent MDSC-activation (depicted in figure 6.1) were not dissimilar to what described previously. In fact, Angulo *et al*. demonstrated that NO-suppressive inducible early myeloid cells depend on trigger by activated T

cells through CD40 and IFN-γ derived signals, in a mechanism involving the production of TNF-α, to become actually active (Angulo et al., 2000). Similarly, Gallina and co-workers described that in a "triggering phase" inflammatory monocyte precursors are activated by IFN-γ-producing T lymphocytes to release IL-13 and IFN-γ. Subsequently these cytokines are able to induce the activation of the enzymes inos and arg1, which confer immunosuppressive properties to myeloid cells. In this context, the initial activation of antitumor-specific T cells and other immune effector cells able to recognize transformed cells and to produce IFN-γ (reviewed in Dunn et al., 2002) may become a double-edged sword in tumor bearing host because IFN-γ cascade could contribute to MDSC functional activation, further sustained by signals derived from infiltrating MCs.

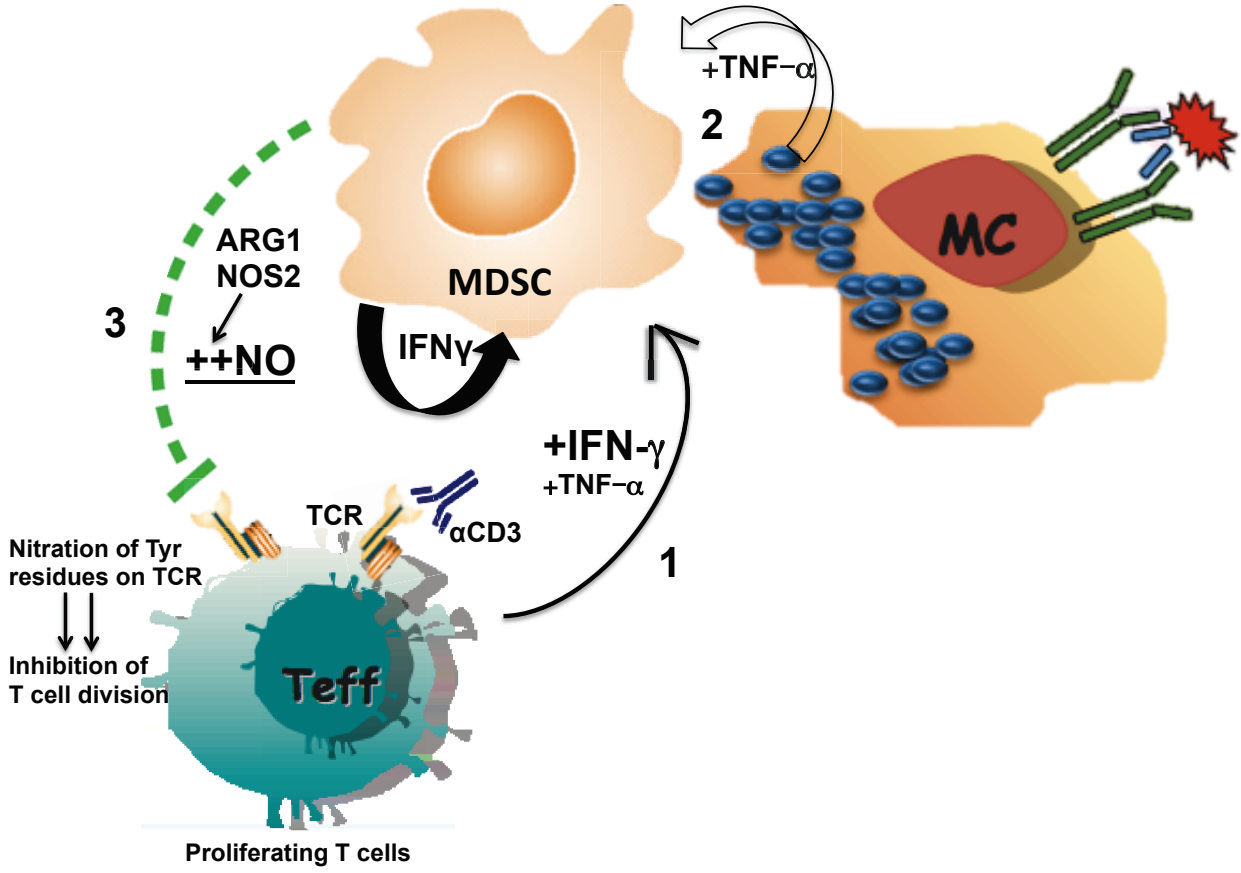

Figure 6.1: Schematic representation of MC-dependent M-MDSC-activation

The presented results suggest an alternative MDSC-activation model dependent on MC. It is possible to speculate that, in peripheral lymphoid organs or directly in the periphery of tumor, (1) recruited monocytic-MDSCs were firstly activated by T cell-derived IFN-γ and switched on iNOS signaling. (2) once contacted by MCs, M-MDSC activation was further amplified and they became fully competent suppressor cells. (3) the high levels of nitric oxide that were released by M-MDSCs inhibited $CD4^+$ T-cell function in a non-antigen specific manner. TNF-α produced in the system was involved in the complete activation of M-MDSCs.

To further contextualize these results, it is important to consider the complexity of MDSCs biology in cancer. Recently Gabrilovich's group proposed a model of MDSC differentiation and function in cancer in which they sustain that in lymphoid organs, as in spleen, MDSCs are not completely activated and failed to suppress antigen-nonspecific activation of T cells. In contrast, at the tumor site, through a mechanism dependent on hypoxia via HIF-1α, MDSCs up-regulate the expression of inos and argI and acquire the ability to suppress antigen-nonspecific T cell functions (Corzo et al., 2010).

Summarizing, results presented in this thesis work demonstrated that MCs were recruited in the tumor and had potential pro-tumoral functions in the growth of CT-26 colon cancer. The tumor induced accumulation of $CD11b^+$ $Gr\text{-}1^+$ immature MDSCs, which caused profound alterations of the antitumor immune response. In the spleen, conditioned by the inflammatory response to tumor growth, MCs seemed to be able to directly contact accumulating MDSCs and direct their activation triggered by tumor specific IFN-γ-producing $CD4^+$ T cells. Ex *vivo* MCs emerged for their ability to switch on the suppressive properties of spleen-derived monocytic-MDSCs, rendering them able to inhibit polyclonal antigen-non-specific activation through increased production of nitric oxide. In addition, the CD40:CD40L cross talk between the two populations was responsible for the instauration of a pro-inflammatory microenvironment, with the exacerbation in the production of mediators that can further support MDSC mobilization and tumor growth.

Despite an extremely limited accumulation of immature myeloid cells, draining lymph nodes are "historically" considered the primary site for tumor antigen presentation and tolerance induction (Nagaraj et al., 2007), but recent data underlined that MDSC-dependent immune tolerance to tumor antigen occurs efficiently in specialized environments of the spleen (Ugel et al., 2012). In light of this knowledge, the evidence, that in the spleen MCs can further amplify the potential of monocytic-MDSCs to switch off the antitumor response of $CD4^+$ T cells, could be an effective novel mechanism to amplify tumor-induce tolerance in the spleen. This possibility do not exclude that once activated M-MDSCs, driven by MCs, could migrate toward lymph nodes and in the tumor mass and exert their suppressive functions directly in these sites.

Taken together, it is possible to speculate that MCs are not only important in the mobilization of MDSC from peripheral lymphoid organs to tumor site, but that they can also actively participate in MDSC acquisition of suppressive functions. In light of these results, interfering with MDSC:MC axis could be a promising approach to abrogate MDSC-related immune suppression and to improve antitumor immune response.

Chapter 7: METHODS

7.1 Mice, cancer cell lines and conditioned media, transplantable tumor induction

BALB/c and C57BL/6 mice were purchased from Harlan Laboratories. All mice were maintained in DSMB (University of Udine) animal facility and used from 8 weeks of age.

Gamma knock out (*gko)*, *tnf-α*$^{-/-}$, and *cd40l*$^{-/-}$ mice on Balb/c background and mast cell-deficient C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice (*wsh*) were kindly provided by Dr. M. Colombo (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan).

CT-26 is a carcinogen-induced, undifferentiated colon carcinoma cell line on BALB/c background. Tumor cells were cultured in DMEM (Euroclone) supplemented with fetal calf serum (10%), L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO_2 atmosphere. To generate tumor cell-conditioned media (CM), sub-confluent cancer cells were further kept in DMEM medium for 48 h. After that time, supernatants were collected and stored at -20 °C until use.

For in *vivo* experiments, 2×10^5 CT-26 cells were inoculated subcutaneously (s.c.) in mice flank. Tumor growth was monitored twice-three times per week and recorded as [longest diameter × (shortest diameter)^2/ 2] (mm^3). Where indicated, tumor-bearing mice were treated with cromolyn (Sigma) by daily i.p. injection of 10 mg/kg (in PBS 1x) per mice from day +5 to day +15 after cancer cell injection.

PBS 1x: (at pH 7.4) 137 mM NaCl; 27 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4
(all products purchased from Sigma Aldrich)

7.2 Histopathology, immunohistochemistry and immunofluorescence

Sections from formalin-fixed, paraffin-embedded tissues were stained with haematoxylin and eosin for histological characterization and/or with Giemsa for specific evaluation of MC distribution on mouse tissues. MCs were counted out of 5 high-power microscopic fields (400X), and counts were expressed as average of at least three fields. For in situ double-marker immunofluorescence, sections underwent two sequential rounds of single-marker immunostaining as reported in Tripodo et al., 2010. Anti-Gr-1 (e-Bioscience), anti-CD11b (e-Bioscience) and anti-FcεR (IRK clone, described in Rivera et al., 1988) antibodies were adopted and Alexa Fluor-conjugated secondary Abs (Invitrogen) were used. DAPI was used to counterstain nuclei. Slides were analyzed by an expert pathologist (Professor Laura Mariuzzi, DSMB-University of Udine) in a blinded fashion respectively under a Leica DM2000 optical

microscope and a Leica DMI6000 fluorescent microscope, and microphotographs were collected using a Leica DFC320 digital camera.

7.3 Myeloid derived suppressor cell purification

Myeloid derived suppressor cells (MDSC) were purified from spleens of tumor-bearing mice with mouse Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec) developed for the isolation of Gr-1^{high} Ly-6G^{+} CD11b^{+} (indicated as PMN-MDSC) and/or Gr-1^{dim} Ly-6G^{-} CD11b^{+} (indicated as M-MDSC) myeloid cells, according to the manufacturer's instructions.

Briefly, within 3 weeks from cancer cell subcutaneous inoculation, CT-26 tumor-bearing mice were sacrificed; spleens were collected and mechanically digested and perfused to obtain a homogenous single-cell suspension. After centrifugation at 350xg for 5 minutes, 1 ml/spleen cold ACK lysis buffer (BioWhittaker) was added to the cell pellet and the suspension was incubated at 4°C for 5 minutes to allow osmotic lysis of red blood cells. After centrifugation, the obtained cellular suspension was resuspended in 350 µL of MIN buffer (a solution containing PBS, 0,5% BSA and 2 mM EDTA) per 10^8 total cells and FcR Blocking Reagent (50 µL for 10 minutes) and anti-Ly6G-Biotin antibody (100 µL for 10 minutes) were sequentially added. Subsequently, washed cells were resuspend in 800 µL of buffer and incubated with 200 µL of anti-Biotin MicroBeads for 15 minutes. After washing in buffer, anti-Ly6G-Biotin and anti-Biotin MicroBeads magnetic labelled Gr-1^{high} Ly-6G^{+} PMN-MDSCs were positive selected through magnetic separation. Magnetic purification was performed in a Miltenyi Biotec LS column, placed in the magnetic field of a MACS separator. Cell suspension was applied onto the column and the flow-through containing unlabelled cells, that represent the pre-enriched Gr-1^{dim} Ly-6G^{-} fraction, was collected. After removing from separator, the magnetically labelled PMN-MDSCs were flushed out by pushing the plunger into the column and were resuspend in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 200 U penicillin, and 200 mg/mL streptomycin.

At this point, unlabelled pre-enriched Gr-1^{dim} Ly-6G^{-} fraction was resuspended in 400 µL of buffer and 100 µL of anti-Gr-1-Biotin were added for 10 minutes. Finally washed cells were resuspended in 900 µL of buffer and 100 µL of Streptavidin MicroBeads were added for 15 minutes. At this point, Gr-1^{dim} Ly-6G^{-} M-MDSCs were positive selected through magnetic separation (that works as for PMN-MDSCs) and resuspend in RPMI medium supplemented with 10% fetal calf serum.

All incubations and centrifugations were respectively performed in the refrigerator (2-8 °C) and at 300xg for 5 minutes at 4°C.

7.4 BMMC differentiation and IgE-dependent activation

Bone marrow-derived mast cells (BMMC) were obtained by *in vitro* differentiation from *wt*, *gko*, *cd40l*$^{-/-}$, and *tnf-α*$^{-/-}$ Balb/c mice as described (Gri et al., 2008). Bone marrow precursors were obtained from femurs of mice, and were cultured in RPMI medium supplemented with 20% FCS, L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), non essential amino acids, Hepes buffer (20 mM), Pyruvate sodium (1 mM) (all purchased from EuroClone), β-mercaptoethanol (50 mM, Sigma-Aldrich). To obtain mast cell differentiation the described medium were further supplemented with IL-3 (20 ng/mL, Peprotech) and Stem Cell Factor (20 ng/mL, Peprotech, only for the first two weeks of culture). BMMC maturation was monitored for c-kit and FcεRI expression by flow cytometry after 4-5 weeks with purity usually more than 95%.

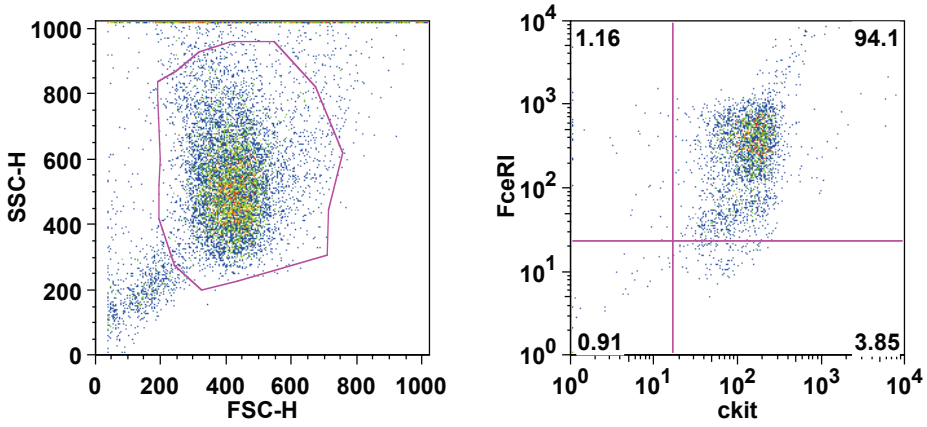

Figure 7.1: Purity of the bone marrow-derived mast cell (BMMC) population. Representative example of FACS analysis to evaluate BMMC maturation. Mature BMMCs, gated on the basis of their size and granularity (FSC vs SSC plot, left side), are positively stained for both FcεRI and c-Kit (right panel).

For IgE dependent activation, BMMCs were sensitized with 1 µg/mL of dinitrophenol(DNP)-specific IgE in medium (RPMI with 10% FCS) without IL-3 for 3 hours and then eventually treated with IgE specific antigen DNP (Sigma-Aldrich).

In some experiments, BMMCs were labelled with the fluorescent dye Fast DiO (Invitrogen, 50 µg/ml for 15 minutes at 37 °C) and subsequently 2 x 10^5 Fast DiO-labelled cells were injected into the tail vein of CT-26-tumor bearing mice with palpable growing tumors.

7.5 β-hexosaminidase- and histamine-release assay

IgE-pre-sensitized BMMCs were challenged in Tyrode's buffer (135 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 1,8 mM $CaCl_2$; 5,6 mM Glucose; 20 mM Hepes pH 7.4) with DNP antigen (50 ng/ml) for 30 minutes. When indicated, pre-sensitized BMMCs were plated with equal number

of MDSCs for 10 min before Ag challenge. At the end of incubation, samples were placed on ice and immediately centrifuged to pellet cells. The enzymatic activities of β-hexosaminidase in supernatants and in the cell pellets, after solubilization with 0.5% Triton X-100 in Tyrode's buffer, were measured with p-nitrophenyl N-acetyl-b-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C. The release of the product 4-p-nitrophenol was detected by absorbance at 405 nm after addition of carbonate/bicarbonate tampon. The extent of degranulation was calculated as the percentage of 4-p-nitrophenol absorbance in the supernatants over the sum of absorbance in the supernatants and in cell pellets solubilized in detergent.

Histamine secretion was measured in supernatants of IgE/Ag-activated BMMCs (at least 2 x 10^6 cells/mL) 1 hour after Ag challenge. Concentration was evaluated with the immunoenzymatic *Histamine Cell Culture ELISA* (DRG) assay, according to the manufacturer's instructions.

7.6 Migration assay

Chemotaxis was performed in a 96-transwell insert (Corning) with 5 μm pore-size filters. 7,5-10 x 10^4 cells (in 75 μL) were plated in the upper compartment and different stimuli were added in the lower compartment (for a total volume of 310 μL).

After 4 hours, cells migrated in the lower chamber were counted and results expressed as fold induction compared to number of cells migrated in response of not-conditioned medium.

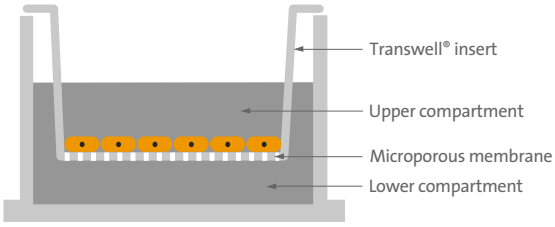

Figure 7.2: Schematic representation of a Transwell insert in a well plate.

7.7 Flow cytometry

PE-Cy7-conjugated anti-CD4 (L3T4 clone), PE-Cy7-conjugated anti-CD8 (53-6.7 clone), FITC-conjugated anti-CD11b (M1/70 clone), PE-conjugated and PE-Cy7-conjugated anti-Gr-1 (RB6-8C5 clone), FITC-conjugated anti-CD117 (c-kit, 2B8 clone), PE-conjugated anti-FcεRIα (MAR-1 clone) were purchased from eBioscience.

To assess cell-surface expression of different co-stimulatory molecules and/or activation markers, 3-4 x 10^5 cells were collected into polystyrene tubes (Sarstedt), washed with PBS and then incubated in the dark for 30 minutes at 4°C with a fluorescent mAb. In case of multicolor staining, multiple fluorescent antibodies were added at the same time. After the incubation, the

cells were washed with PBS, resuspended in 0.3 ml of PBS and kept at 4°C until flow cytometric analysis

Flow cytometry data were acquired on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Version 8.8.6; TreeStar).

7.8 Detection of necrotic and apoptotic cells

Necrotic and apoptotic cells were detected by labeling with annexin V and Propide Iodide using Annexin V-FITC Apoptosis detection Kit (eBioscience), as indicated. Briefly, 2-4 x 10^5 cells were resuspend in the provided Binding Buffer 1x and 5 μL of Annexin V-FITC were added to 195 μL of cell suspension for 10 minutes at room temperature. After a wash, cells were resuspend in 200 μL Binding Buffer with 10 μL of Propidium Iodide before performing FACS analysis.

If required, cell surface staining was performed (as indicated in 7.7) before Annexin staining.

7.9 T-cell proliferation assay

To induce T cell proliferation, 1,5 x 10^5 responder cells consisting of whole splenocytes from untreated *wt* or *gko* Balb/c mice, labeled by incubation with 5 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) for 15 minutes at 37°C, were stimulated with 5 μg/mL of anti-CD3 (*145-2C11 clone,* eBioscience). In the proliferation assay, responder cells were co-cultured with 1 x 10^5 purified splenic MDSCs derived from CT-26 tumor bearing mice with or without 1 x 10^5 BMMCs. Where indicated, IgE-sensitized BMMCs were treated with 100 ng/mL of DNP (Sigma-Aldrich) (IgE/Ag). In independent experiments effects of L-NMMA (0,5 mM, Sigma-Aldrich), 10 μg/mL neutralizing antibody anti-IFN-γ (XMG.1 clone, Harlan) and anti-TNF-α (Miltenyi Biotech), PMA (100 ng/mL, Sigma-Aldrich) and LPS (5 μg/mL, Sigma-Aldrich) were tested on the co-culture system. In some co-culture experiments BMMCs were separated from responder cells and MDSCs by a transwell polyester membrane using 96-well plates (Costar, Euroclone) with 0.4 μm pore size, following the manufacturer's recommendations. For transwell experiments the number of origin cells used has been doubled. After 72 hours of stimulation, CFSE dilution in gated $CD4^+$ and/or $CD8^+$ responder T cells was evaluated by flow cytometry as a function of proliferation, and results were expressed as percentage of divided cells. Proliferation experiments were performed in RPMI 1640 (Sigma-Aldrich) complete medium containing 10% FCS (Sigma-Aldrich), 2 mM L-glutamine, 200 U/mL penicillin, 200 mg/mL streptomycin, 20 mM Hepes, 1mM sodium pyruvate, non-essential amino acids (all purchased from EuroClone) and β-mercaptoethanol (50 mM, Sigma-Aldrich).

Alternatively instead of whole splenocytes, where indicated, 1 x 10^5 highly purified $CD4^+CD25^-$ T cells were used as responder population for CFSE-labeling and anti-CD3-activation in presence of 7,5 x 10^4 mytomicin C treated antigen-presenting cells (APC). $CD4^+CD25^-$ T were obtained through immunoaffinity purification with the $CD25^+$ T-cell isolation kit (Miltenyi Biotec). T cell purification started from whole splenocytes (obtained as described in Methods 7.3) that were loaded and eluted on a nylon column to eliminate B cells through electrostatic interaction with nylon polymers. Then, the obtained cell suspension was incubated at 4°C for 20 minutes in MIN buffer with anti-CD8 antibody conjugated with magnetic beads (80 µl antibody/100 million cells) and anti-CD45R/B220 antibody conjugated with magnetic beads (60 µl antibody/100 million cells) to positive selection of $CD8^+$ T cells and of remaining $B220^+$ B cells. After incubation step, the suspension was washed and loaded on a LS magnetic column (Miltenyi Biotec). The unlabelled eluted $CD4^+$ fraction was subsequently incubated at 4°C for 20 minutes with anti-CD25-phycoeritrin (PE) conjugated antibody, washed and incubated with anti-PE antibody conjugated with magnetic beads for additional 20 minutes. Finally, cell suspension was applied onto a LS column and the flow-through containing $CD4^+CD25^-$ T cell population was collected and cells were resuspend in RPMI media with 10% FCS to be used in proliferation assay. Before loading on the nylon column a fraction of whole splenocytes were separated and treated with 50 µg/mL Mytomicin C (Sigma Aldrich) in PBS at 37°C for 20 minutes to block cell proliferation. After extensive washing, cells were resuspend in RPMI media with 10% FCS to be used as APC in proliferation assay.

7.10 Nitric Oxide (NO) detection

To detect secreted nitrites, equal volumes of culture supernatants (50 µl) were mixed with Griess reagent (Sigma-Aldrich). After 15 minutes of incubation at room temperature, the absorbance at 560 nm was measured using a microplate plate reader (Bio-Rad Laboratories). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 100 µM sodium nitrite.

Alternatively, to detect intracellular concentration of nitric oxide (NO), cells were labeled with DAF-FM diacetate (Molecular Probes), a cell-permeant reagent that passively diffuses across cellular membranes and that after deacetylation reacts with NO to form a fluorescent benzotriazole that can be detected with a flow cytometer. Briefly, cells in suspension were resuspend in PBS and incubated with 10 µM DAF-FM diacetate for 30 minutes at 37 °C. After a wash to remove the excess probe, cells were replaced with fresh buffer and incubated an additional 15 minutes at RT (this incubation time allowed the surface staining of cells) to allow

complete de-esterification of the intracellular diacetates before flow cytometry analysis.

7.11 RNA preparation and Quantitative PCR assays

Total RNA was extracted from 10 x 10^6 cells by using the EuroGold Trifast™ kit (EuroClone) according to manufacturer's instructions. 1 μg total RNA was reverse transcribed using iScript cDNA Synthesis kit (BioRad). cDNA generated was amplified by quantitative real-time PCR (with a BioRad iQ5 device) using iQ™ SYBR Green Super Mix (BioRad). Data from the reaction, each performed in triplicate, were collected and analyzed by the complementary computer software. Results were expressed as fold induction compared to control condition. G3PDH transcripts levels were used as normalizer for sample. Primers used were the following: **IL-6** Forward: ACCACTTCA CAAGTCGGAGGCTTA, Reverse: TCTGCAAGTGCATCATC GTTGTTC; **CCL-2** Forward: AGCAGGTGTCCCAAAGAAGCTGTA, Reverse: AAAGGTGC TGAAGACCTTAGGGCA; **CCL-5** Forward: TCGTGCCCACGTCAAGGAGTATTT, Reverse: TCTTCTCTGGGTTGGCACACACTT; **TNF-α** Forward: TCTCAGCCTCTTCTCATTCCTGC T, Reverse: AGAACTGATGAGAGGGAGGCCATT; **G3PDH** Forward: CATGACCACAGTC CATGCCA, Reverse: TCAGATCCACGACGGACACA.

7.12 Cytokine and chemokine ELISA assays

Supernatants from different experimental settings were collected at the indicate times for quantification of IFN-γ, TNF-α, CCL-2, and CCL-3 (all kits were purchased from Peprotech); IL-4 and IL-6 (e-Bioscience), through ELISA test according to manufacturer's protocol. In addition IL-2, IL-4, IL-6, IFN-γ, TNF-α and IL-17A levels were monitored with the BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson), following manufacturer's instructions.

7.13 AOM/DSS treatment to induce colitis-related colorectal cancer

Mice (aged 10-12 weeks) were given a single intraperitoneal injection of AOM (10 mg/kg body weight diluted in saline). Seven days after, mice were exposed to drinking water containing 2% DSS for a period of seven days, followed by 2 weeks of normal drinking water. DSS exposure was repeated for a total of three cycles. Mice were observed daily for any relevant clinical signs and were weighed 2-3 times a week. Colonic carcinogen AOM was purchased from Sigma-Aldrich; DSS with a molecular weight of 36000-50000 was from MP Biomedicals and was dissolved in water to a concentration of 2% (w/v).

Starting from 90 days after AOM injection, mice were sacrificed and colons were removed, opened longitudinally and macroscopically inspected. Tumor lesions were quantified and dimension of each tumor was measured. Tumor volume was calculated using the formula (a x $b^2/2$), where a and b represent respectively the long and the short diameters. The opened colon was subsequently fixed in formalin, and embedded in paraffin for histopathological and immunohistochemical analysis performed by an expert pathologist (Professor L. Mariuzzi, DSMB University of Udine).

Where indicated, the mast cell population was reconstituted in MC-deficient C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice (*wsh*) by injecting *in vitro* differentiated BMMCs derived from C57BL/6 mice. The cells (5 x 10^6 cells in 0.2 ml PBS) were intraperitoneally transferred into 4-6-weeks-old female *wsh* mice and AOM/DSS treatment was initiated 6–8 weeks after adoptive transfer of the BMMCs.

7.14 Statistical analysis

Results are expressed as the mean plus standard deviation (or standard error of the mean where specifically indicated). Data were analyzed using two-tailed Student *t* test.

$p < 0,05$ was considered statistically significant.

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Chapter 9: PAPERS

1. Frossi B, D'Incà F, Crivellato E, Sibilano R, Gri G, Mongillo M, **Danelli** L, et al. Single-cell dynamics of mast cell CD4+ CD25+ regulatory T cell interactions. Eur. J. Immunol. 2011;41:1872–82.

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4. Mion F, D'Incà F., **Danelli** L, Toffoletto B, Gerdes N, Guarnotta C, et al. Mast cells contribute to the expansion and differentiation of IL-10^{+} regulatory B cells (submitted)

5. **Danelli** L, et al. Interacting mast cells and myeloid derived suppressor cells in the regulation of tumor-specific immune response (in preparation)

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