Università degli Studi del Piemonte Orientale
"Amedeo Avogadro"

Dipartimento di Scienze del Farmaco

PhD program in Chemistry & Biology

XXXII ciclo a.a. 2016-2019

SSD MED/04

Decoding of epigenetic and metabolic events driving immune diversion of myeloid cells in cancer



Augusto Bleve

Supervised by Prof. Antonio Sica

PhD program coordinator: Prof. Luigi Panza

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"*People ask me often: « was the Nobel Prize the thing you were aiming for all your life? ». And I say that would be crazy. Nobody would aim for a Nobel Prize because, if you didn't get it, your whole life would be wasted.*

What we were aiming at was getting people well, and the satisfaction of that is much greater than any prize you can get."

Gertrude Belle Elion
Nobel laureate in Physiology or Medicine,1988

Cover picture: *Looking Forward*, by Dhruba Deb

"*In Looking Forward, Dhruba Deb depicts two cancer cells that sustain themselves by communicating with healthy cells and receiving nourishment via expanding networks of blood vessels—all while trying to escape detection by roving immune cells*."

(Landhuis E, *PNAS*, 2018)

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Chapter 1

1.1 Inflammation and cancer

The earliest mention of a link between cancer and inflammation was found in the Ebers Papyrus (c. 1500 BC). The ancient Egyptian physician Imhotep (c. 2600 BC) reported regression of tumors after infections induced by an incision in the tumor site[1,2,3]. Similar observations were noticed along the first millennium, albeit remained isolated and not investigated until the late 19th century. Two German physicians, Busch and Fehleisen, were the firsts to intentionally infect cancer patients with the erysipelas causative agent (S. pyogenes) and observe a tumor regression[4,5]. In 1891, the American physician William Coley accidentally experienced in a sarcoma patient the shrinkage of tumor mass partially excised by surgery. The extensive wound could not be successfully closed and became severely infected with erysipelas followed by a high fever and the complete disappearance of tumor[6]. From this evidence, Coley developed a series of experiments treating cancer patients with live or heat-inactivated Streptococcus pyogenes and Serratia marcescens bacteria – the so called Coley's toxin or vaccine – with pioneering results for that era[7].

In the meanwhile, alongside the first evidences of interaction and balance between cancer and inflammation in infective status, in 1863 the brilliant German pathologist Rudolf Virchow noted the presence of the so-called "lymphoreticular" cells infiltrate into malignant tissue[8,9]. Despite these discoveries, the elucidation of mechanism of immune control of neoplastic disease was neglected for decades. An important turning point arrived in the mid-1900s, when Macfarlane Burnet and Lewis Thomas elaborated the hypothesis of 'cancer immunosurveillance' according to which lymphocytes supervise homeostatic tissues identifying and eliminating nascent somatic cells transformed by spontaneous mutations[10].

In 1980s and 1990s a series of fundamental immunological discoveries (Table 1) provided the basis of the modern cancer immunology and immunotherapy.

Basically, inflammation consists of genetic programs acted by immune cells in response to harmful stimuli such as infections and tissue injury. The acute inflammatory response to infection or tissue injury requires a well-orchestrated delivery of blood components (leukocytes and their products) to the site of infection or injury[11].

Year	Event	Researcher	Ref.
1982	Cytotoxic T-Lymphocytes (CTLs) recognized new antigens of immunogenic cancerous cells	Boon T	12
1982	Identification of Interleukin-4	Paul W Howard M	13
1983	Interferon gamma (IFNγ) activates macrophages	Schreiber RD Katz DH	14
1983	Identification of T cell antigen Receptor (TCR)	Kappler J Allison JP	15
1985	Cloning of Transforming growth factor-beta (TGFβ)	Derynck R	16
1985	MHC role in the antigen processing and presentation	Unanue E	17
1985	Effective treatment with IL-2 and LAK of melanoma and renal cancer patients	Rosenberg SA	18
1986	Anti-CD3 monoclonal antibody (muromonab-CD3) approval for prevention of transplantation rejection	Kung P Schlossman S	19
1987	Identification of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)	Golstein P	20
1989	Role of CD28 as co-stimulator of T cell activation	June C Thompson CB	21
1990	Low and chronic levels of Tumor Necrosis Factor (TNF) as pro-tumoral agent	Balkwill F	22
1990	Highlights on pro-tumoral activity of Tumor-associated macrophages (TAM)	Mantovani A	23
1992	Identification of Programmed cell death protein 1 (PD-1) in T lymphocytes	Ishida Y Honjo T	24
1994	Description of CTLA-4 as immune checkpoint inhibitor of T cell activation	Uenaka A Nakayama E	25
1996	NF-kB pathway as regulator of macrophage inflammatory activity	Gerondakis S	26
1996	Enhancement of Antitumor Immunity by CTLA-4 Blockade	Allison JP	27
1998	PD-1 as negative regulator of immune response	Honjo T	28
2003	Tumor regressions in patients treated with the CTLA-4-inhibiting antibody	Dranoff G	29
2006	Cancer regression after autologous adoptive transfer of genetically engineered lymphocytes	Rosenberg SA Dudley M	30
2010	PD-1-specific monoclonal antibody treatment induces regression in patients with various cancers	Lowy I Topalian SL	31
2013	CAR-T cell therapy achieves a complete response in leukemia patients	Sadelain M Brentjens R	32
2013	Gut microbiota modulation of anti-tumoral immune response	Zitvogel L Trinchieri G	33,34

Table 1. ***Timeline of some of the key events in cancer-related immunology starting from 1980s***

This response has been characterized best for microbial infections (particularly bacteria), in which innate immune cells –including macrophages, mast cells, and dendritic and natural killer cells– interact and recognize the invading agent through specialized receptors, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), and thus leading to the release of chemical inflammatory mediators, including chemokines, cytokines, and reactive oxygen species (ROS)[35]. The main and most immediate effect of these mediators is to allow recruitment of leukocytes to the area of injury or infection and lead to the elimination of pathogens and/or tissue repair, mainly mediated by neutrophils, tissue-resident and recruited macrophages, with dendritic and natural killer cells initiating the adaptive immune response and T cell recruitment. If the acute inflammatory response fails to eliminate the pathogen, a chronic inflammatory state persists and produces the formation of granulomas and tertiary lymphoid tissues.[11]

The characteristics of this inflammatory state can differ depending on the effector class of the T cells that are present. In addition to persistent pathogens, chronic inflammation can result from other causes of tissue damage such as autoimmune responses (owing to the persistence of self-antigens) or undegradable foreign bodies[11].

The mechanisms of systemic chronic inflammatory process are not clearly understood, and the fact that these cells and mediators are present in the microenvironment of most tumors consistently corroborates the link between cancer and inflammation.

This relationship has a 'two-way' connection. Chronic inflammation can represent the triggering event or at least a co-factor causing neoplastic transformation of cells[36,37]. Mantovani A. defined this process as the 'extrinsic' pathway of the cancer-related inflammation[37]. Indeed, about 15-20% of cancer deaths are attributable to infections (both viruses and bacteria) and to the related chronic inflammation[9,38]. Examples of this process are human papillomaviruses, hepatitis C virus (HCV) and Helicobacter pylori,

which are important risk factors for cervical cancer, hepatocellular carcinoma (HCC) and gastric cancer, respectively. Moreover, increased risk of malignancy is associated with other sources of chronic inflammation such as biochemical agents (i.e. smoke, asbestos) or autoimmune disease (i.e. inflammatory bowel disease, IBD)[36,38,39]. Generally, it is thought that chronic inflammation results in DNA damage which increases the risk of malignant transformation[40,41].

Contrarily, the 'intrinsic' pathway of cancer-related inflammation is guided by genetic events, such as activation of oncogenes by mutation (e.g. *myc*, *ras*, *ret*), chromosomal rearrangement or amplification, and inactivation of tumor-suppressor genes (e.g. pVHL), that trigger both carcinogenesis and inflammatory programs. [37,42–44]

Mechanistically, both 'extrinsic' and 'intrinsic' pathways converge, resulting in the activation of transcription factors, among which nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α) play a central role in neoplastic cells. The activation of these molecular pathways in cancerous cells induces the production of inflammatory mediators, including cytokines, chemokines as well as the production of cyclooxygenase 2 (COX-2) (which, in turn, results in the production of prostaglandins). This inflammatory milieu promotes the recruitment and infiltration into the tumor microenvironment (TME) of leukocytes, most notably cells of myelomonocytic lineage. In some cancers, inflammatory conditions precede development of malignancy; in others, oncogenic changes generate a tumor-promoting "smouldering" inflammation, which aids proliferation and survival of malignant cells, angiogenesis, and metastasis, subverts adaptive immune responses and alters responses to chemotherapeutic agents[37,45].

Generally, this cancer-promoting inflammation precedes a process named immunoediting and proposed to consist of three phases (the three 'Es' of immunoediting')[46]. In the early phase, the immune surveillance is able to continuously recognize and destroy nascent tumor cells (elimination phase); due to the intrinsic genetic, epigenetic and metabolic instability of neoplastic cells, the arising of new cell clone variants with a non-immunogenic phenotype are selected for growth, concurrently, the host immune system exerts a selective pressure on the genetically unstable and rapidly mutating tumor cells, that is enough to contain, but not fully extinguish, the tumor bed (equilibrium phase). At this point, tumor cell variants selected in the equilibrium phase have acquired resistance to immune detection and/or elimination, and thus can grow in an immunosuppressed environment (escape phase)[10,46]. Generally, during this phase, tumors expand and become clinically detectable, increase the immunological request, and boost the inflammatory shaping involving both innate and adaptive immune cells (Fig. 1).

Several studies have emphasized that the "smouldering" inflammation associated with tumors is mainly oriented to tune the adaptive immune response. Thus, functional modulations of tumor-infiltrating lymphocytes have key roles in promoting tumor immune escape. Cancers also produce alterations of the myelopoietic output ('emergency' myelopoiesis) leading to accumulation of M2-polarized tumor-promoting myeloid cells.[47–50]

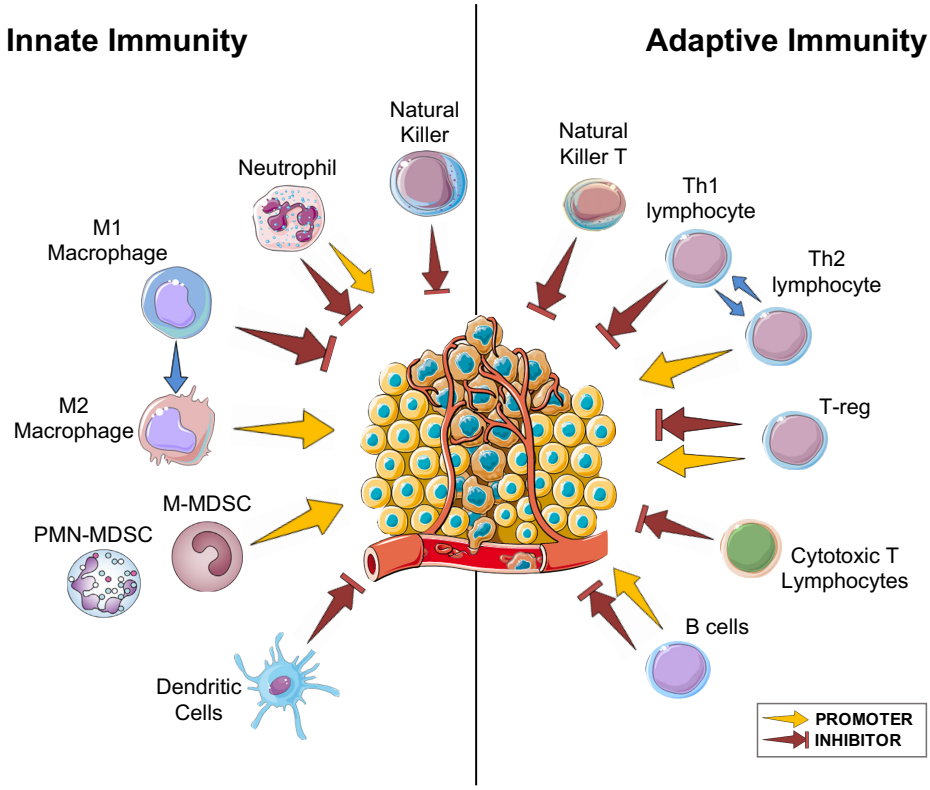

Figure 1. ***Innate and Adaptative immunity to cancer.*** Overview of key innate and adaptive immune cells that are present in the tumor microenvironment. Both myeloid and lymphoid cells can function as a double-edge sword by promoting or inhibiting tumor progression. *Abbreviations*: PMN-, polymorphonuclear; M-, monocytic; MDSC, myeloid-derived suppressor cells; Th1, CD4 T helper 1; Th2, CD4 T helper 2; T-reg, CD4 T regulatory cells.[51(adapted)]

Hence, the immune system plays a dual role in cancer: a) to suppress tumor progression by destroying cancer cells; b) to induce a disarmament of its anticancer capability, driven by the growing tumor. The driving mechanisms of this transition are not yet fully understood. In the last decade, cancer-related inflammation and immune-editing have attracted a lot of interest in cancer research, acquiring relevance in clinical practice. In 2011, Hanahan and Weinberg revised their "hallmarks of cancer" and have included the concept of 'immune escape' and 'tumor-promoting inflammation' as new cancer hallmarks and characteristics (Fig. 2)[52,53].

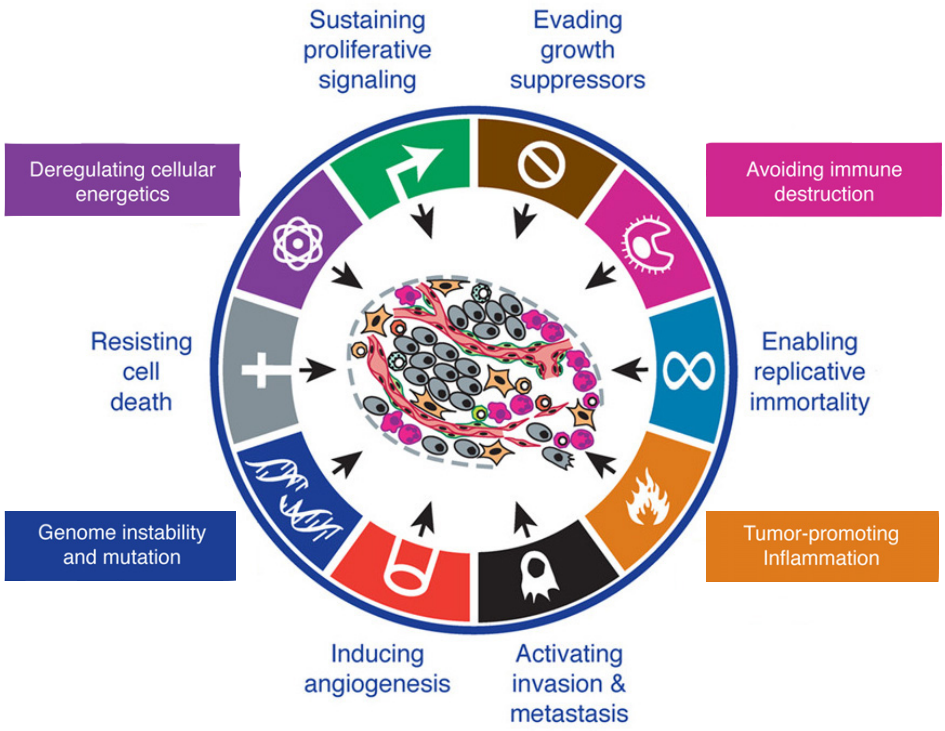

Figure 2. ***Hallmarks of Cancer: The Next Generation*****.** In 2000, in the first characterization of the cancer hallmarks, Hanahan and Weinberg highlighted six biological features, constituting an organizing principle for rationalizing the complexities of neoplastic disease. They included: sustained proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Conceptual progress in the last decade has added new emerging hallmarks and characteristics to this list: the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation; the ability of cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells; 'smouldering' inflammation supporting multiple cancer hallmark capabilities; genomic instability and thus mutability endowing cancer cells with genetic alterations that drive tumor progression. [52,53(adapted)]

1.2 Tumor-associated Myeloid Cells

In mammals, myeloid cells represent the most abundant hematopoietic cells in the human body and the first line of host defence against pathogens. These cells also play an essential role in tissue homeostasis and immune responses.[54] At the top of the hematopoietic hierarchy there are the hematopoietic stem cells (HSCs) which are quiescent cells with multipotent and extensive self-renewal potential, and reside in the bone-marrow (BM) niche. HSC differentiation into multipotent progenitor (MPP) cells is the initial step in a cascade of events resulting in terminal differentiation of mature hematopoietic cells, including cells of innate immunity, although tissue-resident macrophages may also derive from embryonic progenitors. MPPs subsequently give rise to progenitor cells with myeloid lineage commitment (common myeloid progenitors; CMPs) or lymphoid lineage commitment (common lymphoid progenitors; CLPs). Further steps of differentiation lead CMPs toward more restricted progenitors, e.g., granulocyte-macrophage progenitors (GMPs), erythrocyte-megakaryocyte progenitors, and monocyte-dendritic cell progenitors (Fig. 3). However, the commitments of myeloid progenitors do not form a hierarchical system, but can instead be considered as a network of cells that can differentiate into various more specialized myeloid cell subsets[55,56].

Generally, steady-state myelopoiesis is a continuous homeostatic process, occurring in the bone marrow, for replacing hematopoietic cells that are lost to normal programmed cell death. In contrast, in the presence of immunological stress, such as severe systemic infection or cancer disease, hematopoiesis sustains modifications of the magnitude and composition of the hematopoietic output to ensure proper supply of immune cells to increased demand. This process is termed ‘emergency myelopoiesis’ (or demand-adapted myelopoiesis)[57–60].

In the course of tumor development, cancerous cells are able to produce factors (tumor-derived factors, TDFs), which acts in a paracrine or systemic fashion to 'reprogram' non-cancerous host cells exacerbating rather than ameliorating disease progression. Many of these TDFs are myelopoietic stimulating factors, making the myeloid compartment a major target of this 'tumor reconditioning' not only to create a widespread tolerogenic environment by blocking T cell functions and proliferation, but also to drive tumor growth by promoting cancer stemness, angiogenesis, stroma deposition and metastasis formation[54,55].

Moreover, the continuous exposure to uncontrolled myelopoietic-like growth factors corrupts the normal process of myeloid cell development and differentiation. This phenomenon drives the increase of circulating myeloid cells, which are partially restrained in their differentiation, with a consequent accumulation of highly immunosuppressive, immature myeloid cells (iMCs)[55].

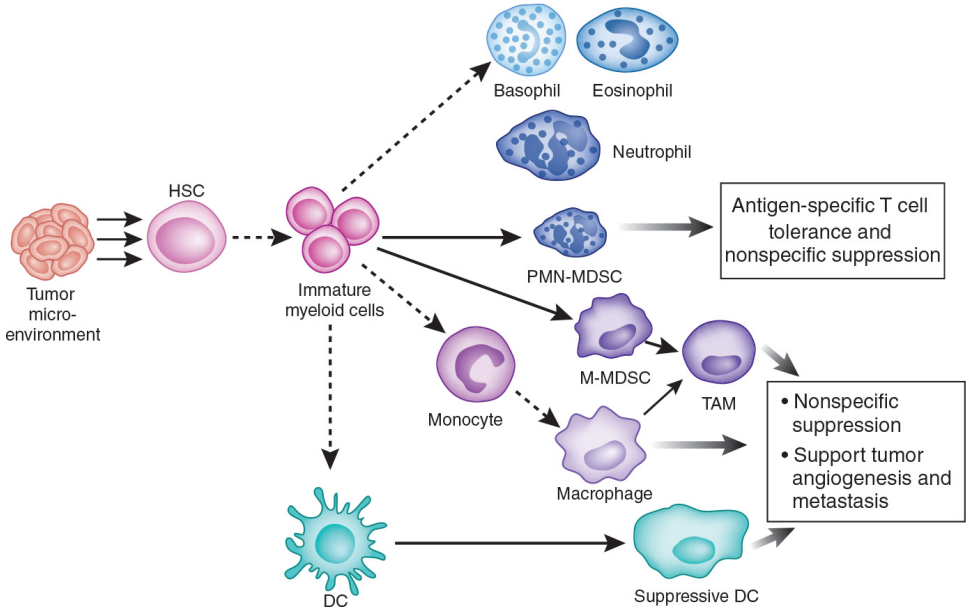

Fig. 3. ***Myeloid cells in cancer.*** Soluble mediators produced in the tumor microenvironment promote the aberrant differentiation of myeloid lineage cells. Dashed lines indicate the normal developmental pathway of immature myeloid precursor cells, which differentiate into DCs, monocytes-macrophages and granulocytes (basophils, eosinophils and neutrophils) in non-tumor-bearing hosts. Solid lines indicate the aberrant pathways of myeloid cell development.[61]

Indeed, different tumor-derived factors including VEGF, GM-CSF, IL-10, IL-6, TGFβ, prostaglandins and gangliosides have been implicated in this phenomenon and can affect myeloid cells at various stages of differentiation[62,63].

Myeloid alterations can occur at different level of development and functionality in all myeloid lineages. Generally, to distinguish the 'normal' myeloid cells, the dysfunctional population counterparts have been variously renamed myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), immature DCs or tolerogenic DCs. Among these, TAMs and MDSCs represent the major orchestrators of the immunosuppressive circuits arising in tumor bearers (Fig. 3)[48].

1.3 Myeloid-derived suppressor cells

The term "myeloid-derived suppressor cells" (MDSCs) was coined in 2007 to describe a non-lymphoid immune suppressor cell population of myeloid origin which expand in pathological conditions, such as infection, autoimmune diseases, trauma, and graft-versus-host disease. Cancer is the predominant condition where MDSCs have been described.[64–67].

MDSCs constitute a population of myeloid cells with heterogeneous morphology, surface phenotype, functions, and immune-suppressive properties. They generate in the bone marrow (BM) from common myeloid progenitor cells and accumulate during cancer progression. MDSCs migrate to peripheral lymphoid organs and tumor tissues, where they inhibit T-cell responses, contributing to the formation of a pro-tumor microenvironment[68]. In addition, MDSCs are able to promote cancer development inducing tumor angiogenesis and matrix remodelling. Indeed, MDSCs have been implicated in the formation of pre-metastatic niches, tumor cell migration and invasion, tumor angiogenesis and growth and survival of metastatic tumor[69,70].

1.3.1 Phenotypical features of MDSCs

MDSCs consist of a heterogeneous population conventionally divided into two major subsets based on their phenotypic and morphological features: polymorphonuclear (PMN)-MDSC, with some phenotypic feature similar to neutrophils, and monocytic (M)-MDSC, more similar to monocytes[71]. In mice, MDSCs historically were defined as cells expressing both CD11b and Gr-1 markers, the latter used to distinguish the two major subsets of MDSCs according to its level of expression ($Gr1^{high}$ cells are mostly PMN-MDSCs and $Gr1^{low}$ cells are mostly M-MDSC)[72]. Since the Gr-1 marker is a combination of the Ly6C and Ly6G markers, these subsets can be now more accurately identified based on Ly6C and Ly6G expression: PMN-MDSC ($CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{lo}$) and M-MDSC ($CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{hi}$)[66,69].

MDSC subset	Mouse Phenotype	Human Phenotype
PMN-MDSC	$CD11b^+$ $Ly6G^+$ $Ly6C^{lo}$	$CD11b^+$ $CD14^-$ $CD15^+$ ($CD66^+$)
M-MDSC	$CD11b^+$ $Ly6G^-$ $Ly6C^{hi}$	$CD11b^+$ $CD14^+$ HLA-$DR^{-/lo}$ $CD15^-$.
eMDSC	Not defined	Lin^- ($CD3/14/15/19/56^-$) HLA-DR^- $CD33^+$

Table 2. ***Minimal surface markers identifying MDSC subset in mouse and human.***

In human, the equivalent to PMN-MDSC are defined as $CD11b^+$ $CD14^-$ $CD15^+$ or $CD11b^+$ $CD14^-$ $CD66b^+$, and M-MDSC as $CD11b^+$ $CD14^+$ HLA-$DR^{-/lo}$ $CD15^-$. Prior to CD11b, CD33 was used as myeloid marker. CD33 myeloid marker can be used instead of CD11b since very few $CD15^+$ cells are $CD11b^-$. While M-MDSC express the myeloid marker CD33, PMN-MDSC display $CD33^{dim}$ expression[73]. In humans, but not yet in mice, it has been identified a mixed group and more immature subset of MDSCs, named 'early-stage MDSC' (eMDSC) identified as Lin^- ($CD3^-$, $CD14^-$, $CD15^-$, $CD19^-$, $CD56^-$) HLA-DR^- $CD33^+$ (Table 2)[66].

1.3.2 MDSCs immunosuppressive functions in cancer

Immune suppression by MDSCs is exerted through several direct or indirect, antigen-specific or -non-specific mechanisms that could be recapitulated in four main groups: 1) depletion of essential extracellular metabolites; 2) production of immunosuppressive molecules; 3) interference with T cell migration and viability; 4) induction of immunoregulatory cells (Fig.4)[70,74,75].

Depletion of essential extracellular metabolites (Fig.4.1)

- Cysteine. T cells strictly need L-cysteine to sustain their proliferation and activation. Unlike other cells, T cells cannot produce cysteine either by the intracellular conversion of methionine (lack of cystathionase enzyme) or by import of extracellular oxidized cysteine (lack of x_c^- transporter). The only way to collect cysteine is during the immunologic synapse occurring between antigen-presenting cell (APC) and T cell. During this process, APCs cells, through the ASC transporter, couple the import of extracellular oxidized

cysteine with the export of reduced cysteine, which can be taken up by T cells. By contrast, MDSCs lack the ASC transporter and do not export cysteine, thus limiting the supplying of this amino-acid required for T cell fitness.[76]

- Tryptophan. MDSCs express high levels of the isoenzyme indoleamine 2,3-dioxygenase 1 (IDO1) that catabolize the L-Tryptophan into kynurenines, the degradation products. These metabolites have immune-modulatory and tolerogenic properties: inhibit CD8 T-cell proliferation by causing cell cycle arrest and inducing anergy; direct CD4 T-cell differentiation toward the Treg phenotype by Foxp3 upregulation; reprogram DCs toward a tolerogenic phenotype by binding the aryl hydrocarbon receptor; and concomitantly depriving T cells of an essential nutrient.[77]

- Arginine. Metabolic conversion of L-arginine (L-Arg) through either iNOS or Arg1 is the first and the main mechanism associated with the immunosuppressive activities of MDSCs[78]. iNOS generates nitric oxide (NO) which is able to inhibit T-cells signalling pathways responsible of their activation and to induce T-cell apoptosis[78,79]. Arg1 activity, converting L-arginine to urea, L-ornithine and polyamines, causes the depletion of this amino-acid, which induces the translational blockade of the ζ chain of CD3, preventing T cells from responding to various stimuli[80]. Moreover, ornithine and polyamines can support directly tumor cell proliferation[81].

- NAD. CD38 is an ectoenzyme belonging to the ADP-ribosyl cyclase family and, consuming extracellular nicotinamide adenine dinucleotide (NAD), leads to mitochondrial dysfunction of surrounding cells, as observed in metabolic diseases and cancer[82]. CD38 was found up-regulated in MDSCs from various preclinical tumor models and cancer patients[83]. Along with the detrimental effects associated with depletion of microenvironmental NAD, CD38 generates second messengers associated with calcium signalling, resulting in an increased amount of NO that favours tumor growth[83]. NAD is the substrate for numerous NAD-consuming enzymes that participate in cell signalling,

including sirtuin 1 (SIRT1) whose lack in MDSCs fuels the glycolytic mTOR-HIF1α pathway associated with a functional switch toward a pro-inflammatory (NO, TNFα, IL-12) and anti-tumor phenotype[84]. The expression of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD salvage pathway converting nicotinamide into nicotinamide mononucleotide (NMN), precursor of NAD, is elevated in MDSCs. Inactivation of NAMPT causing reduced NAD availability showed reactivation of specific antitumor activity of T cells.[85]

- Fatty acids. In both tumor bearing mice and humans, tumor-derived cytokines induce expression of cell surface lipid transport receptors by MDSCs. This results in increased fatty acid uptake and oxidative metabolism in association with activation of MDSCs' immunosuppressive mechanisms. Therefore, hampering the intracellular accumulation of lipids as well as pharmacological inhibition of fatty acid oxidation (FAO) blocks the immunosuppressive functions of MDSCs, improving the efficacy of either immunotherapy or low-dose chemotherapy.[86,87]

Production of Immunosuppressive Molecules (Fig.4.2)

- Adenosine. MDSCs express high levels of Ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1, CD39) and the ecto-5'- nucleotidase, which convert the extracellular ATP released by dying cells in adenosine. Extracellular adenosine is a powerful immunosuppressive factor that impairs differentiation of naïve $CD8^+$ T cells in effector cells, inhibits cytolytic activity of NK and T cells, and it promotes the immunosuppressive functions of tumor-associated macrophages (TAM) and expansion of PMN-MDSCs.[88]

- Cytokines. MDSCs also produce copious amounts of immunosuppressive cytokines, such as TGFβ and IL-10, which induce the generation of Treg cells, differentiation of pro-tumoral TAMs and direct suppressive effects on T effector cells. Mechanistically, it was confirmed that tumor-derived TGFβ triggers CD39 expression on circulating and tumor-infiltrating MDSCs via activation of mTOR/HIF1α-signaling.[89]

- ROS. High arginase activity and NO production by MDSCs leads to augmented reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as the free radical peroxynitrite ($ONOO^-$). These radical species, interacting with macromolecules (DNA, lipids, proteins), disrupt their structure and biological processes, including immune responses. NADPH oxidase 2 (NOX2), the main actor in ROS production in leukocytes is induced by cytokines (i.e., TNFα and IFNγ) and growth factors (VEGF). NOX2-dependent ROS production supports MDSCs expansion and recruitment in tumors through the up-regulation of VEGF receptors[90]; its lack impaired both generation of ROS by MDSCs and their ability to suppress $CD8^+$ T cells[91]. Myeloperoxidase is another ROS-producing enzyme contributing to the suppression of antigen-specific T cell responses in tumor bearers[75]. Tumor-infiltrating MDSCs produce large amounts of H_2O_2, which affect T-cell fitness by downregulating CD3 ζ-chain expression and reducing cytokine secretion, as observed in pancreatic cancer and melanoma[92].

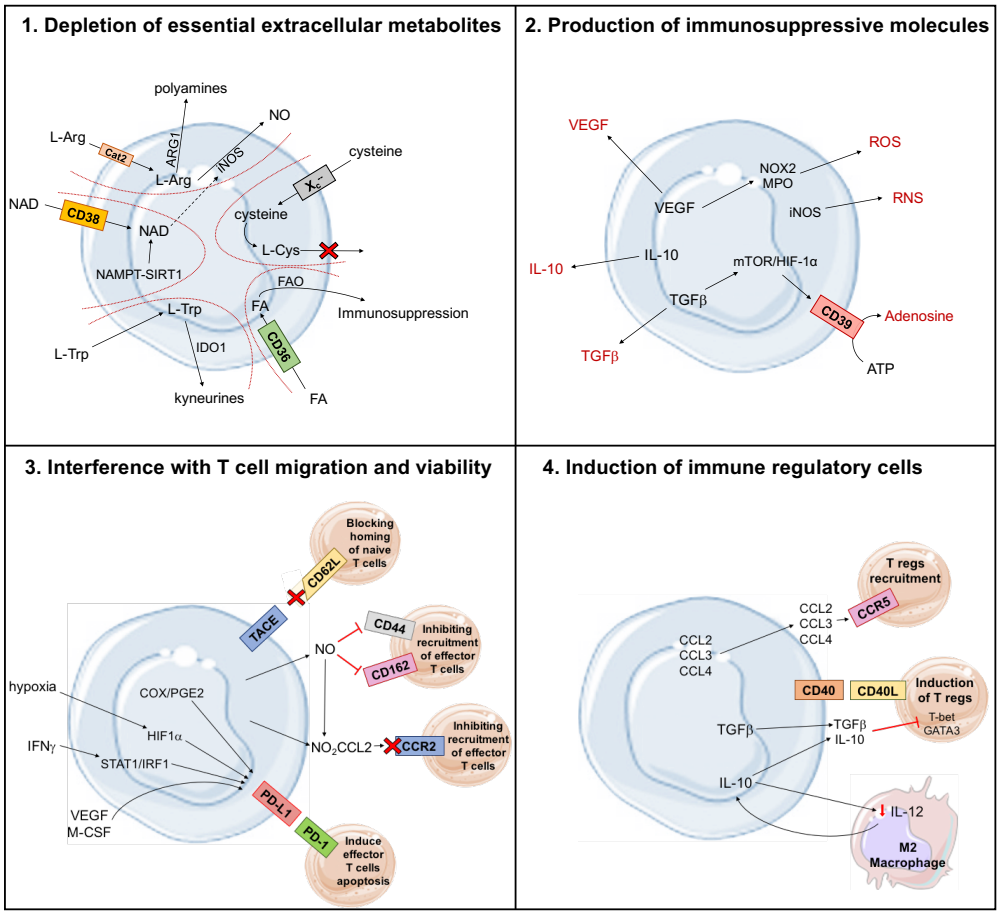

Fig. 4. ***MDSCs immunosuppressive mechanisms.*** (1) MDSCs deplete essential extracellular metabolites for T cells. Through the up-regulation of metabolic enzymes (e.g. ARG1, iNOS, IDO1, NAMPT, CD38) MDSCs consume L-Arg, L-Trp and NAD, and concomitantly produce immunomodulatory agents (NO, polyamines and kynurenines). Further, MDSCs internalize cysteine without releasing the oxidized form, and up-take and oxidize fatty acids (FA/FAO) inducing immunosuppressive activities. (2) MDSCs release a range of immunosuppressive molecules. They produce ROS and RNS through NOX-2, iNOS and MPO, adenosine via CD39, anti-inflammatory cytokines (IL-10, TGFβ) and growth factors (VEGF). TGFβ induces CD39 via HIF1α, promoting expression of immunosuppressive molecules (IL-10, VEGF). (3) MDSCs interfere with T-cell migration and viability. MDSCs express the metalloproteinase TACE/ADAM17, able to cut the integrin CD62L on the T-cell membrane. RNS promote homing of Tregs by nitration of selective chemokines (like CCL2) and limit recruitment of effector T cells by down-regulation of CD162 and CD44. MDSCs up-regulate PD-L1 in response to multiple signals, including hypoxia-HIF1α, IFNγ via STAT1/IRF1, COX2/PGE2, M-CSF and VEGF. PD-L1 can induce T-cell apoptosis by engaging PD-1. (4) MDSCs drive specific subsets of immune-regulatory cells. MDSCs produce CCL3, CCL4, CCL5 which drive differentiation and recruitment of $CCR5^{+}$ Treg cells. Tregs differentiation is also induced by direct interaction between CD40 and CD40L and by inhibition of effector T cells-driving transcription factors (Tbet, GATA3) through TGFβ and IL-10. The latter inhibit the IL-12 production by macrophages which in turn acquire an M2 immunosuppressive phenotype and induce further IL-10 production by MDSCs. For abbreviations and details, see the text.[75(adapted)]

Interference with T cell migration and viability (Fig.4.3)

T-cell activation and effector functions require trafficking to lymph nodes and tumor sites dependent by the expression of L-Selectin (CD62L) homing marker of naïve T cells. MDSCs highly express the metalloproteinase TNFα-converting enzyme (TACE/ADAM17) which directly mediates the CD62L cleavage and, thus, interfering with T cell recruitment[93]. In addition, MDSCs can hamper the recruitment of circulating effector T cells into tissues by inhibiting the expression of CD162, a ligand of P-selectin and CD44, the receptor for the extracellular matrix component, hyaluronic acid (HA)[94]. MDSCs and cytotoxic T lymphocytes (CTLs) trafficking to the tumor is regulated by the CCL2/CCR2 axis. Intra-tumoral production of RNS induces nitration/nitrosylation of CCL2 that no longer attract tumor-specific CTLs, but can still recruit MDSCs[95].

Beyond the recruitment into the tumor site, MDSCs can hamper the activation of effector T cells through the expression of immune checkpoint inhibitors (ICIs). Programmed death-ligand 1 (PD-L1) is an inhibitory ligand, abundantly expressed in MDSCs that binds to PD-1 and, thus, suppressing T cell activation[96]. Several mechanisms can trigger its expression through different pathways and stimuli, such as: hypoxia-HIF1α [97]; IFNγ via STAT1-IRF1 axis[96]; M-CSF and VEGFA[98]; cyclooxygenase-2 (COX2)/prostaglandin E2 (PGE2) axis[99].

Induction of immune regulatory cells (Fig.4.4)

The fourth group includes the diversion of immune functions toward immunosuppression. As described above, reprogramming of MDSCs produce large amounts of TGFβ and IL-10 in the tumor microenvironment[89]. TGFβ exerts direct anti-proliferative effects on T cells, arresting their cell cycle in the G1 phase or inhibiting the IL-2 secretion[100]. Notably, TGFβ was shown to inhibit the differentiation of $CD4^+$ T cells into Th1/Th2 cells by suppressing the expression of T-bet and GATA-3, master regulators of Th1 and Th2 conversion, respectively[101]. MDSCs also promote the clonal expansion and

differentiation of $CD4^+$ T cells to Treg cells directly, via cell–cell interactions (including CD40–CD40L), or indirectly through production of soluble factors such as IFNγ and IL-10[102], or CCL3, CCL4 and CCL5 which drive the recruitment of immune-modulatory $CCR5^+$ Treg cells[103]. Further, through an IL-10- or cell contact-dependent mechanism, MDSCs skew macrophages toward an M2 phenotype, resulting in IL-12 downregulation[104]. Macrophages, in turn, promote the production of IL-10 by MDSCs, creating a self-maintaining negative loop[104].

1.3.3 MDSCs expansion and activation in cancer

Accumulating evidence from tumor-bearing mice and human cancers indicates that MDSCs occurrence is governed by a network of transcriptional regulators that could be combined into two partially overlapping groups of factors: a) promoting myelopoiesis and preventing differentiation of mature myeloid cells; b) promoting pathologic activation of MDSCs[105,106].

Regulation of MDSCs expansion

STAT3 was the first transcription factor characterized in MDSCs expansion and accumulation in cancer[107]. In addition to the well-described myeloid-specific growth factors GM-CSF and G-CSF, several other factors have been implicated in the activation of STAT3. Among these, high levels of IL-6 produced by MDSCs showed a critical role in the activation of STAT3 not only in MDSCs[108], but also in cancer cells, increasing tumor invasiveness and metastatic potential[109]. STAT3 activation in MDSCs was reported also in response to hepatocyte growth factor, tumor-derived exosomes, and IL-11.[106] Interestingly, Yan et al. recently reported that exposure to a diet rich in polyunsaturated fatty acids (PUFAs) significantly increased the accumulation of MDSCs in tumor-bearing mice in a STAT3-dependent manner.[110] IRF8 is a transcription factor implicated in the terminal differentiation of monocytic/macrophage lineage, which, interacting with the transcription

factor C/EBPα, prevent its binding to DNA, and thus resulting in the inhibition of the granulocytic differentiation program in favour of monocytic differentiation.[111] The absence of IRF8 in tumor-bearing mice results in increased accumulation of MDSCs, while its overexpression is correlated with a decreased accumulation of MDSCs.[112] The molecular mechanisms of IRF8-dependent expansion of MDSCs are not fully understood, but evidences indicate that the *Irf8* gene is negatively regulated by tumor-derived G-CSF and GM-CSF, through STAT3/STAT5.[112]

Interleukin-17A (IL-17A) promotes both granulocyte-colony stimulating factor (G-CSF)- and stem-cell-factor-mediated neutrophilia[113] and supports G-CSF-driven "emergency" myelopoiesis[114]. Terminal macrophage differentiation is instead induced by macrophage-CSF (M-CSF) through activation of the transcription factors PU.1 and IRF8[115]. The C/EBPβ transcription factor isoform is implicated in the regulation of emergency myelopoiesis, both in response to infection[58] and cancer[116]. Recent evidences have shown that, whereas C/EBPα appears to be a major regulator of "steady-state" granulopoiesis[117], C/EBPβ[118] promotes the expansion and maturation of neutrophils in emergency conditions.

The retinoic acid-related orphan receptor (RORC1/RORγ) orchestrates emergency myelopoiesis in cancer-bearers by suppressing negative (Socs3 and Bcl3) and promoting positive (C/EBPβ) regulators of granulopoiesis, as well as the key transcriptional mediators of myeloid progenitor commitment and differentiation to the monocytic/macrophage lineage (IRF8 and PU.1).[119] The retinoblastoma protein RB1 was recently implicated in MDSCs expansion in mice and humans through a process mediated by tumor-derived G-CSF.[120] NLRP3 inflammasome is an intracellular sensor which regulates the production of IL-1β and IL18. NLRP3 is up-regulated in MDSCs and its lack correlates with decreased MDSCs in the tumor site.[121]

Regulation of MDSCs activation

The NF-κB pathway (see below) is activated in MDSCs through various mechanisms which showed to increase their suppressive activity. Although controversial evidences, TLRs-mediated activation of NF-κB through MyD88/HSP70 pathway appear to contribute to the suppressive activities of MDSCs.[122,123] The IL-1β overexpression by tumor cells increases production of peroxynitrite by MDSCs.[124] TNFα, another well-known activator of the NF-κB pathway, up-regulates iNOS expression in an NF-κB- and p38 MAPK-dependent manner.[125] Several studies have linked the IFNγ-STAT1 pathway in the increase of iNOS and ARG1 expression in M-MDSCs, inducing their immunosuppressive activity.[94] The STAT6 pathway can be activated in MDSCs through engagement of CD124 (IL-4Rα) by IL-4 or IL-13; this leads to the up-regulation of ARG1[126], as well as increased production of TGFβ[127]. The COX2/PGE2 pathway is implicated in a positive-feedback loop regulating MDSCs suppressive functions. PGE2 increases the expression of COX2 in monocytes, converting them to M-MDSC, and further increases the production of PGE2.[128,129] This induces to the up-regulation of ARG1 and iNOS suppressing T cell activity.[130] PGE2 is also involved in the recruitment of MDSCs to the tumor site by inducing the expression of CXCL12.[131]

1.3.4 Role of myeloid-derived suppressor cells in tumor metastasis

Tumor metastasis remain the major cause of cancer-related mortality.[132] Accumulating evidence from preclinical and clinical studies highlight that MDSCs play an important role in all steps of metastasis formation (Fig. 5). Although the immunosuppressive activity of MDSCs is critically important for the formation of a metastatic niche, these cells employ a number of other mechanisms promoting metastasis.[133]

MDSCs migration to pre-metastatic niche

The pre-metastatic niche is the receptive tissue microenvironment undergoing a series of molecular and cellular changes to form the metastatic-designated sites, or the fertile 'soil' for metastatic tumor cell 'seed' colonization, thus supporting tumor settlement and metastasis at distant organs.[132]

Several chemokines and chemokine receptors are involved in the recruitment of MDSCs to the pre-metastatic niche. Among these CXCL1, CXCL2, and CXCL5 have been shown to recruit MDSCs to the pre-metastatic niche through the interaction with the same receptor, CXCR2.[134–136]

Pro-inflammatory proteins S100A8 and S100A9 are potent chemoattractants for MDSCs and have been implicated in the promotion of tumor growth and metastasis by MDSCs.[137–139] Moreover, S100A8/A9 induces serum amyloid A (SAA) 3 which in turn directly attracts MDSCs to pre-metastatic lungs, stimulate NF-κB signalling in a TLR4-dependent manner, and facilitate metastasis[140]. Thus, it appears that MDSC recruitment to tumor sites generate a chain reaction: MDSCs initially recruited to the tumor site by tumor-derived chemokines can facilitate the recruitment of other MDSCs via release of S100A8/A9 proteins[134].

MDSCs effects on angiogenesis

Rapid growth of solid tumors results in hypoxia, which induces upregulation of proangiogenic factors such as VEGF, PDGF, basic fibroblast growth factor (b-FGF), and angiopoietins[141], and enhances MDSCs migration to the tumor site via HIF1α-mediated production of chemokines[134]. Inhibition of MDSC recruitment in the tumor sites prevents tumor angiogenesis.[142] The myeloid CSFs-dependent up-regulation of STAT3 in MDSCs induces the secretion of pro-angiogenic factors, such as VEGF, bFGF, and Bv8.[143] Bombina variegata peptide 8 (Bv8) production by PMN-MDSCs has been shown to promote lung metastasis and its blockade is correlated with the inhibition of angiogenesis and tumor growth.[144]

MDSCs can promote tumor neovascularization by secreting matrix metallopeptidase 9 (MMP-9), whose activity promotes the bioavailability of VEGF in the tumor microenvironment.[145] MDSCs can potentially incorporate into the vascular endothelium by differentiating into endothelial-like cells expressing VE-cadherin and VEGF-R2.[145]

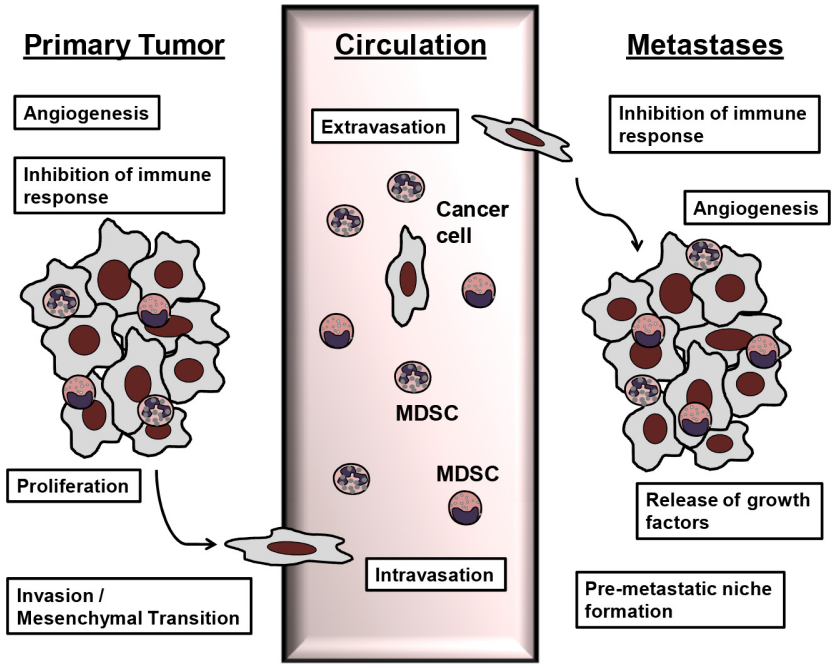

Fig. 5. ***Role of MDSCs in the metastatic process.*** MDSCs promote angiogenesis, tumor cell invasion, and metastasis through a variety of different soluble factors. MDSCs play an important role in the formation of the pre-metastatic niche and in promoting tumor metastasis.[146]

Influence of MDSCs on metastasis formation

The current concept suggests that MDSCs reach the pre-metastatic site before the tumor cells. Once into the site, MDSCs promote tumor seeding and growth through various mechanisms. MDSCs' role in the promotion of metastasis was extensively investigated in mouse models of breast cancer and melanoma. MDSCs in 4T1 model of breast cancer upregulated the expression of several MMPs, which was critical in mediating invasiveness of tumor cells.[147]

MDSCs also downregulates protease inhibitors such as the neutrophilic granule protein, an inhibitor of tumor invasiveness and metastasis.[148]

TGFβ showed a direct, although controversial, role in the MDSCs-mediated metastatic process. Inhibition of TGFβ receptor II signalling increased MDSCs recruitment and tumor invasion mediated by SDF-1, CXCL5 and CCL9.[147,149]

In contrast, a specific deletion of TGFβ receptor II in myeloid cells significantly inhibits tumor metastasis.[150] MDSCs recruited to pre-metastatic lungs stimulate the migration of tumor cells by secreting TNFα, CXCL2, TGFβ, IL-6, and monocyte chemotactic protein-1 (MCP-1).[109,138,151]

To disseminate, invade tissues and metastasize, some tumor cells undergo epithelial-mesenchymal transition (EMT), in which polarized epithelial cells lose epithelial markers and differentiate into cells with mesenchymal features.[152] Recently, several studies have shown the role of MDSCs in EMT. Indeed, MDSCs recruited to the tumor site can produce hepatocyte growth factor (HGF) and TGFβ inducing EMT of primary tumor cells.[136]

Because most of the metastasis are represented by epithelial cells, similar in morphology to the primary tumor, but not mesenchymal cells, it is suggested that EMT is a temporary event and that after arriving at a metastatic site, tumor cells undergo reverse transition from mesenchymal to epithelial phenotype (mesenchymal-epithelial transition, MET) in order to colonize the niche. In one model, MDSCs accumulated in the pre-metastatic lung secrete versican, an extracellular matrix proteoglycan, which contribute to MET and the formation of macro-metastasis in the lungs.[153] MDSCs also promote cancer metastasis by inducing stemness of cancer cells or by expanding the cancer stem cell population.[154,155]

1.3.5 MDSCs as target for cancer therapy

The expansion of MDSCs has been demonstrated to be associated with tumor progression, metastasis, and recurrence of many types of human tumors.[69,156] In addition, the efficacy of immunotherapy was negatively correlated with an elevated MDSCs frequency and activity not only in solid tumors[157,158] but also in blood of non-Hodgkin lymphoma and multiple myeloma patients.[159] Importantly, the frequency of circulating MDSCs was found to correlate with disease stage. It was reported that patients with stage III and IV of a variety of cancer types have higher frequencies of MDSCs in the peripheral blood, as compared to stage I and II patients.[160,161] In addition, a negative association between MDSCs numbers and clinical response to radio-, chemo-, and immunotherapy was reported.[162] Therefore, MDSCs could become not only promising biomarkers for the survival of patients and treatment efficacy, but could also serve as a valuable target in combined immunotherapy of cancer patients.

Three main strategies to modulate MDSCs are under development in several preclinical and clinical studies: 1) inhibition of immunosuppressive functions; 2) inhibition of MDSCs trafficking; 3) regulation of myelopoiesis and/or depletion of MDSCs (Fig.6).[163]

Inhibition of MDSC-mediated immunosuppression

Recent preclinical and clinical studies showed that treatment with tadalafil, an inhibitor of phosphodiesterase-5, significantly inhibited MDSC functions by the downregulation of iNOS and ARG1 activities, leading to an increased T cell reactivity and improved outcome of advanced cancer bearers.[164–166] A class I histone deacetylase inhibitor, entinostat, in combination with anti-PD-1 antibodies, reduces the expression of ARG1, iNOS, and COX2 in MDSC subsets, inducing a strong reduction of tumor-infiltrating macrophages and a significantly delayed tumor growth in mice.[167,168]

Inhibitors of the activation of STAT3, others than old detrimental small molecule inhibitors, are showing promising therapeutic possibilities. Indeed, interfering with STAT3 mRNA through STAT3 siRNA or decoy oligonucleotides is applied in the combination with immune checkpoint inhibitors in the frame of the phase I/II clinical trial.[169] In another approach, selective delivery to MDSCs of STAT3 inhibitors, through their coupling to CpG oligonucleotides, which are well-known agonists of TLR9 (highly expressed by myeloid cells), showed decreased immunosuppressive activity.[170] A further possibility to target MDSCs is the modulation their metabolic pathways. Pharmacological inhibition of the fatty acid oxidation (FAO) decreased the immunosuppressive capacity of MDSCs and, in combination with low-dose chemotherapy and adoptive cellular therapy, resulted in antitumor effect in different cancer models.[171]

Inhibition of MDSCs trafficking

Myeloid-derived suppressor cells exhibit their main immunosuppressive activity within the tumor microenvironment. Therefore, intensive investigations were performed to block the chemokine-dependent migration of MDSCs to the tumor site. The blockade of CXCR2-CCL2 interaction with a CXCR2 antagonist in combination with chemotherapeutic drugs showed significant therapeutic effect.[172] Another chemokine receptor, CCR5, is expressed on a broad spectrum of immune cells.[173] Many evidences suggest that targeting CCR5 on MDSCs could be applied not only to prevent their recruitment but also to reduce their immunosuppressive functions in cancer patients[174,175]. Patients with a mutated CCR5 variant were reported to be resistant to the prostate cancer development; blocking the CCR5–CCR5 ligand interaction improved the survival of tumor-bearing mice; $CCR5^{+}$ MDSCs from melanoma patients displayed an increased immunosuppressive pattern compared to the $CCR5^{-}$ MDSC counterpart.[174,176]

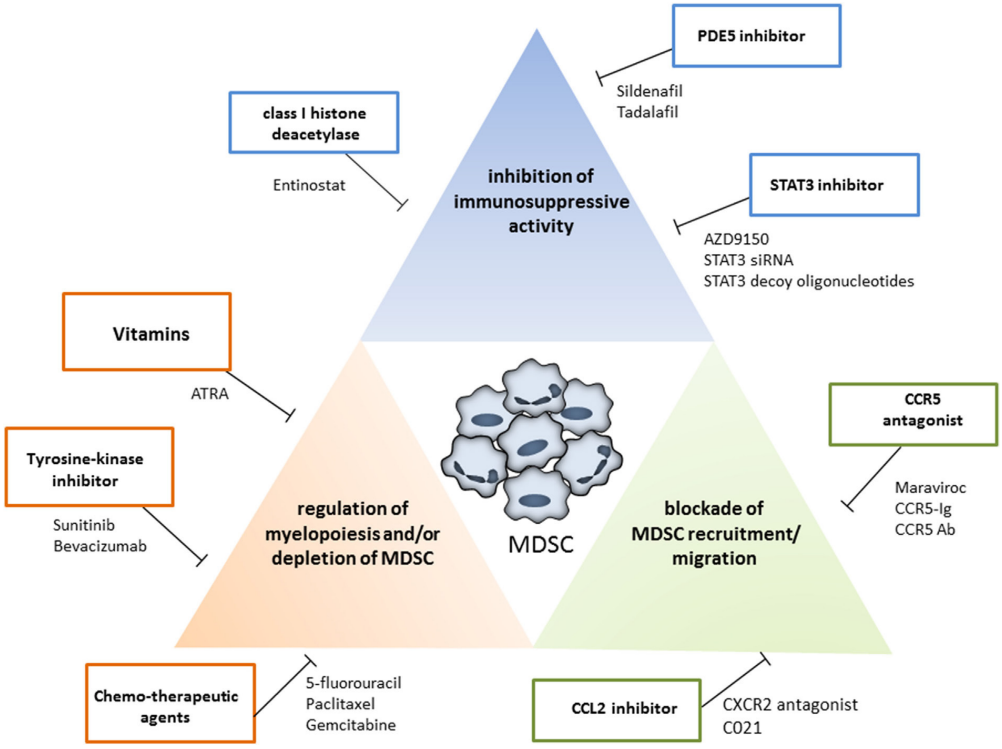

Fig. 6. ***Strategies for MDSC targeting.*** The MDSC modulation could be achieved by the inhibition of their immunosuppressive activity (blue box), by blockade of MDSC recruitment to the tumor site (green box), and by the regulation of myelopoiesis and/or depletion of MDSC (red box). Examples of each therapeutic approach are shown.[163]

Regulation of myelopoiesis and/or depletion of MDSCs

The number of MDSCs in tumor-bearing hosts could be reduced by the normalization of myelopoiesis, the inhibition of the conversion of immature myeloid cells (IMC) into MDSCs, and the differentiation of MDSCs into mature myeloid cells like DC or macrophages. Supporting evidences were shown by all-trans retinoic acid (ATRA), a vitamin A derivative binding to the retinoic acid receptor. ATRA alone, or in combination with other agents (i.e. IL-2, p53-DCs vaccine), decreased the frequency of MDSCs, induced their differentiation into dendritic cells or macrophage, downregulated ROS production by MDSCs.[177,178]

Tumor-derived extracellular vesicles (EV) were reported to induce the conversion of non-immunosuppressive IMCs into MDSCs and further to activate their immunosuppressive functions.[179,180] Dimethyl amiloride or omeprazole, inhibitors of the EV release, reduced MDSCs expansion and immunosuppressive activity.[181]

Tyrosine kinase inhibitors (such as sunitinib), which can block VEGF and c-kit signalling, decreases the number of circulating MDSCs, reducing STAT3 activation and ARG1 expression, while increasing the activity and proliferation of CD8 T cells. However, no significant prolongation of the overall survival was observed.[182,183]

Some chemotherapeutics showed positive effects. Gemcitabine and 5-fluorouracil induces apoptosis of MDSCs in mouse cancer models and reduces the frequency MDSCs in patients, but with poor effect on T cells activity.[163,184] Administration of docetaxel induced a decrease of tumor burden accompanied by the conversion of MDSCs into M1-like cells. Further, doxorubicin led to the reduction of MDSCs frequency and immunosuppressive activity, with an enhancement of granzyme B and IFN-γ production by effector T and NK cells. Paclitaxel, administrated at ultra-low, non-cytotoxic doses, reduced MDSCs number, and increased their differentiation into DCs. Treatment of mice with paclitaxel elevated the activity of CD8 T cells, correlating with prolonged survival.[163]

1.4 Tumor-associated Macrophages

Macrophages are an essential component of innate immunity and play a central role in inflammation and host defence, with decisive functions in both innate and adaptive responses. Resident macrophages provide immediate defence against foreign pathogens and coordinate leukocyte trafficking.[185] They mediate the degradation of apoptotic cells, microbes and possibly neoplastic cells. Moreover, they collaborate with T and B cells in triggering, instructing and terminating the adaptive immune response.[185] Further, they play a role in tissue remodelling and wound healing.[186]

Macrophages have remarkable functional plasticity that allows them to differently set their functions in response to environmental signals, such as microbial products, damaged cells, activated lymphocytes.[185,187]

Tumors are abundantly infiltrated by macrophages. Although macrophages were originally thought to be part of an anti-tumor response, clinical and experimental data indicate that, in the large majority of cancers, tumor-associated macrophages (TAMs) support cancer initiation, progression and metastasis.[187,188]

1.4.1 Macrophage polarization

Macrophages can respond to endogenous stimuli that are rapidly generated following injury or infection. These early stimuli are typically produced by innate immune cells and can exert a marked effect on the physiology of macrophages. The current consensus identifies the opposite extremes of macrophages as: 1) M1-polarized or classical activated macrophages and 2) M2-polarized or alternatively activated macrophages (Fig. 7).[186,189,190]

The terms M1 has been used to designate the classically activated effector macrophages that are produced in response to inflammatory signals. In particular, the combination of two signals, interferon-γ (IFNγ) and tumor-

necrosis factor (TNFα), results in a macrophage population that has enhanced microbicidal or tumoricidal capacity and secretes high levels of pro-inflammatory cytokines and mediators. Typically, a TLR ligand (e.g. LPS), acting in a MyD88-dependent manner, induces the transcription of TNFα gene, which can then cooperate with IFNγ to activate the M1 program, associated with production of pro-inflammatory cytokines (e.g IL-12, IL-23, TNFα), nitric oxide (via iNOS) and ROS. Generally, the activation of M1 macrophages requires activation of the transcription factors STAT1 (following IFNγ receptor ligation) and nuclear factor-κB (NF-κB), obtained in response to TLR or TNF receptor ligation.[186]

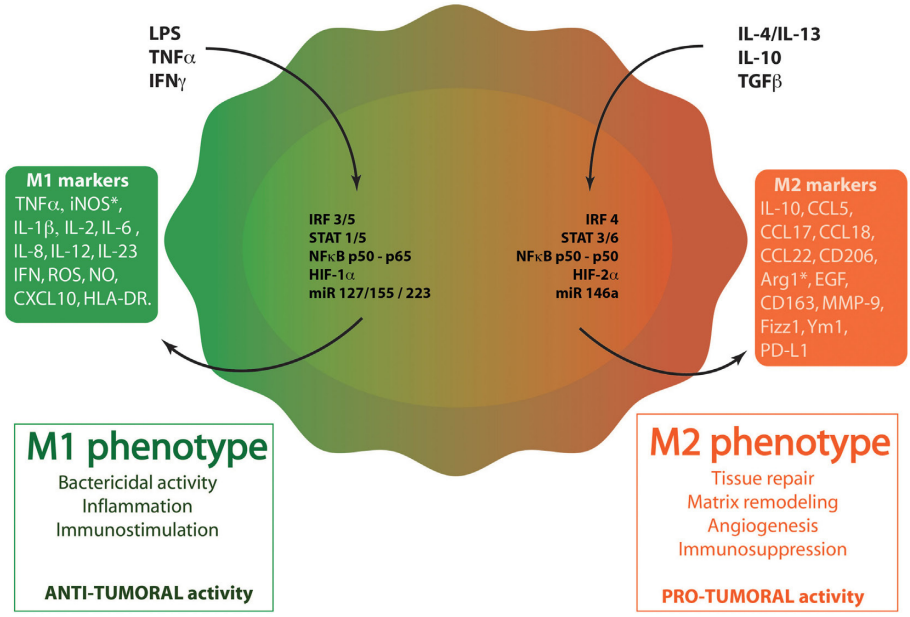

Figure 7. ***Macrophage polarization.*** M1 stimuli (LPS, TNFα, IFNγ) trigger the activation of several transcription factors, including, STAT1 and NF-κB (p65–p50 heterodimer). M1-polarized macrophages produce specific cytokines (e.g. TNFα, IL-1β, IL-12, IL-23, IFNγ), chemokines (CXCL10), ROS and nitric oxide (NO). M1 phenotype plays key roles in inflammation, immunostimulation and in antibacterial and anti-tumoral responses. Other cytokines (e.g. IL-4, IL-13, IL-10, and TGFβ) induce M2-like phenotype through the activation of several transcription factors, including STAT3/6 and NF-κB (p50–p50 homodimer). M2-polarized macrophages produce specific cytokines (IL-10), chemokines (CCL17, CCL18, CCL22), and other proteins (CD163, CD206, Arg1, etc), relevant in tissue repair, matrix remodelling, angiogenesis, immunosuppression, and favouring tumor growth.[191]

The M2 or alternative activated macrophage population is certainly more intricate and heterogeneous. Generally, this definition embraces both the regulatory and wound-healing patterns of activation, which partially overlaps.[187] During tissue injury one of the first innate signals to be released is thought to be IL-4 which rapidly converts resident macrophages into a population of cells that promote wound healing; IL-4 stimulates ARG1 activity in macrophages, allowing them to convert arginine to ornithine, a precursor of polyamines and collagen, thereby contributing to the production of extracellular matrix. Furthermore, the M2 activation is efficiently driven by Th2 cytokines, including IL-13, together with IL-4.[186,187,192]

Other factors inducing an alternative and immune-regulatory activation are glucocorticoids, immune complexes and anti-inflammatory cytokines, such as TGFβ and IL-10. The latter two are released by macrophages themselves following phagocytosis of apoptotic cells with the intent to dampen the inflammatory response.[186]

M1 vs M2 paradigm is challenged *in vivo*, where the co-expression of a large number of stimuli with different temporal patterns may induce mixed and opposing functional states. Therefore, M1/M2 polarization of macrophages is a simplistic, although useful, concept that emphasizes the extremes of a continuum depicting macrophage plasticity (Fig. 8) .[187]

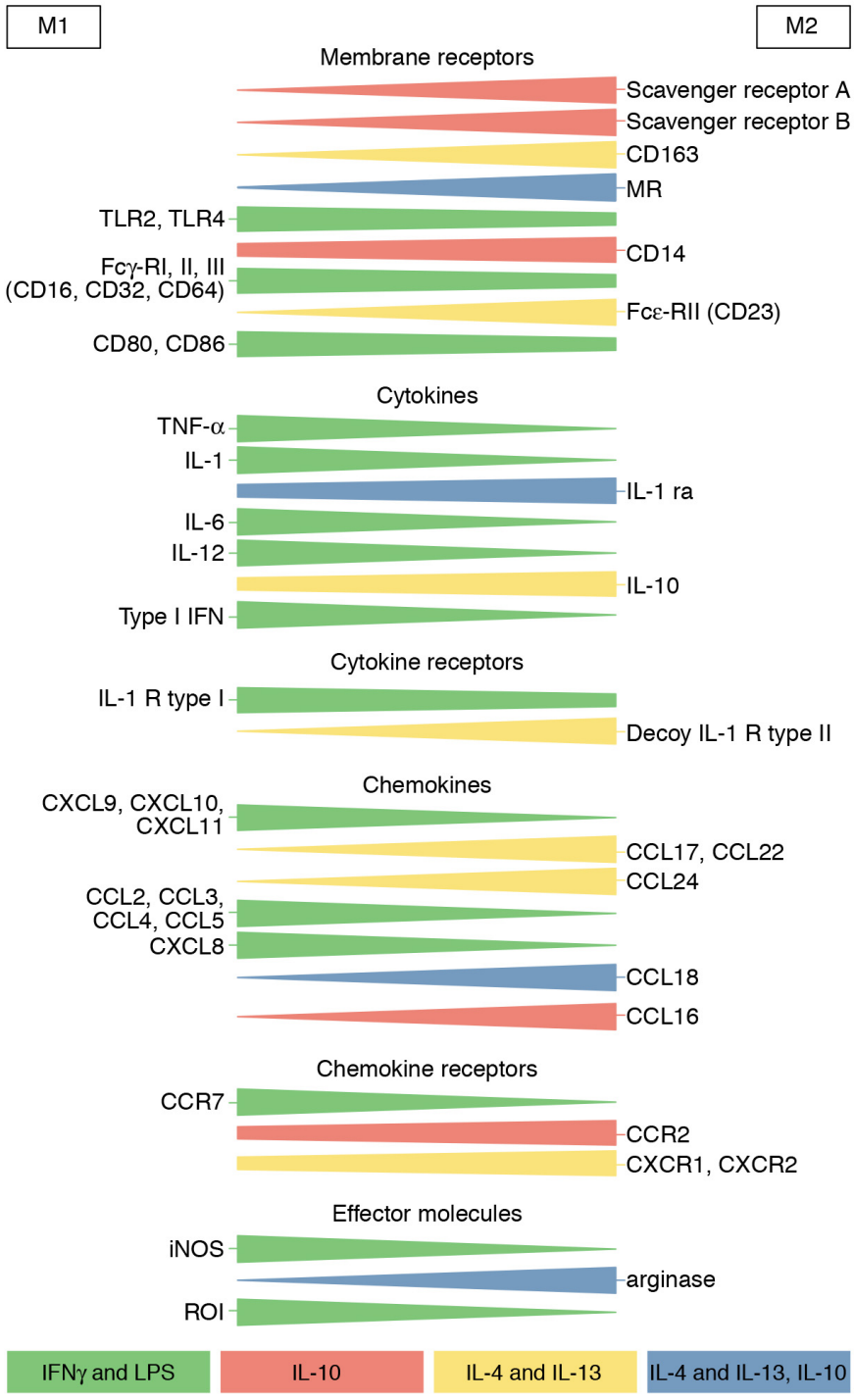

Figure 8. ***M1 and M2 macrophages, the extremes of a continuum.***[187]

M1 and M2 extremes exhibit specific expression of metabolic enzymes (iNOS vs ARG1), cytokines (IL-12^{high}IL-10^{low} vs IL-12^{low}IL-10^{high}), chemokines (CXCL9/CXCL10 vs CCL17/CCL22), as well as transcription factors (NF-κB p65-p50 vs NF-κB p50-p50; STAT1 vs STAT3/6).[189]
Generally, classically activated M1 macrophages support specific anti-tumor T cell-mediated activities. However, during tumor progression TAMs generally acquire an M2-like phenotype which dampens tumoricidal activity and promotes tissue remodelling and angiogenesis.[190]

1.4.2 Origins and recruitment of TAMs

For a long time, TAMs were considered to originate exclusively from bone marrow-derived monocytic precursors that undergoes differentiation upon tissue infiltration. However, new evidence suggests heterogeneity also in the autologous pathways.[193–195] In most cancer models, TAMs originate from circulating Ly6C^{high} CCR2^{+} monocytes. In fact, blocking the CCL2/CCR2 axis of monocyte recruitment leads to a strong decrease in TAMs abundance, suggesting that TAMs originate from bone marrow-derived monocyte CCR2^{+} precursors.[196,197] In an inducible lung carcinoma model, splenectomy resulted in a strong reduction in TAMs. These spleen-derived TAMs were shown to be also CCR2-dependent, suggesting the contribution of an extramedullary myelopoiesis in TAMs generation.[198] However, deletion of *Ccr2* did not result in full depletion of macrophages. Bone marrow transfer experiment in a model of pancreatic ductal adenocarcinoma, proved that TAMs are composed of both newly recruited bone marrow-derived macrophages (BMDM) and tissue-resident macrophages (TRMs). Fate mapping models strongly support that a significant fraction of these TRMs have an embryonic origin and actively proliferate along with tumor growth.[194]

In spontaneous models of breast carcinoma it has been identified that the major TAMs population (CD11b^{lo}F4/80^{hi}MHCII^{int}) as well as the mammary

tissue-resident macrophage population ($CD11b^{hi}F4/80^{lo}MHCII^{hi}$) originates both from $Ly6C^{hi}CCR2^{+}$ monocytes. However, the role of local non-monocytic population cannot be excluded, since TAMs also expand their population through *in situ* proliferation.[199,200]

These findings suggest that TAMs can arise from tissue-resident macrophages, originating either from embryonic precursors ($Ly6C^{-}$) or from circulating monocytes ($Ly6C^{+}$) (Fig. 9).

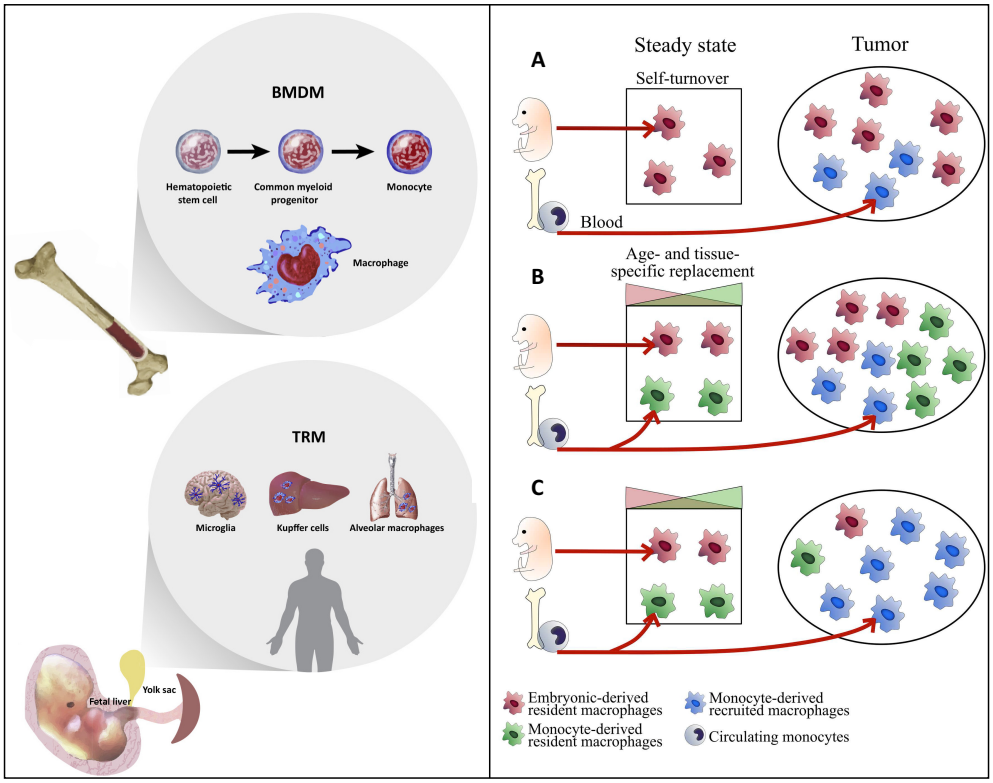

Figure 9. ***Heterogeneity of TAM origin.*** (Left panel) BMDMs develop from hematopoietic stem cells that generate common myeloid progenitors. These progenitors, then divide and mature into monocytes in the BM. Under appropriate stimuli, monocytes traffic to other sites where they can become macrophages. TRMs develop from macrophage progenitors that are seeded into tissues during embryonic development. (Right panel) Schemes represent different scenarios of TAM ontogeny. In some tissues, embryonic-derived resident macrophages self-maintain over time. A) Other subsets are progressively replaced by monocyte-derived macrophages with turnover rates depending on tissue specificity; B). At tumor onset, classical monocytes are recruited to the tumor (mainly in a CCR2-dependent manner) and differentiate into inflammatory TAM. Depending on tumor localization and the inflammatory state TRMs proliferate (A and B) or not (scenario C) and contribute more or less to the pool of TAM. For abbreviation and details, see the text.[201,202]

In addition, a smaller subset of TAMs deriving from monocytes that express the angiopoietin-2 receptor TIE2, may arise from Ly6C^{lo}CCR2^{-} monocytes. These pro-angiogenic TAMs, known as Tie2-expressing monocytes (TEMs), are recruited to the tumor by angiopoietin-2, localize preferentially in areas of angiogenesis, aligned along the abluminal surface of blood vessels, and play important non-redundant roles in tumor neovascularization.[203,204]

The monocyte-to-macrophage differentiation is normally regulated by several soluble factors.[205] Among these, M-CSF, also known as colony stimulating factor 1 (CSF1), is the major lineage regulator of macrophages, both embryonal- and bone marrow-derived, and plays as a chemotactic factor for macrophages.[206] Many cancer models showed that M-CSF inactivation (genetic deletion or neutralizing antibody) results in a reduced number of TAMs and decreased disease progression.[207,208] VEGFA is another factor recruiting macrophage progenitors that differentiate to TAMs under the influence of IL-4, and promoting tumor angiogenesis and invasion.[209] These factors collaborate with locally produced tumor-derived factors (e.g. IL-4, IL-10, IL-13, CCL2, TGFβ, PGE2, C5a) to drive macrophage differentiation and activation.[210,222–224]

Interestingly, metabolic environmental signals associated with malignant neoplasms can influence macrophage activity. The ‘Warburg effect’, a metabolic shift to a glycolytic pathway, generally occurring in highly proliferative cancer cells results in copious production of lactic acid that shifts TAMs toward an M2-like activation state.[213] Hypoxic regions of tumors are generally characterized by a high frequency of TAMs. Hypoxia shapes and induces specific macrophage phenotype that promotes immune evasion, angiogenesis, tumor cell survival, and metastatic dissemination.[214,215]

1.4.3 Pro-tumoral functions of TAMs

A pivotal hallmark of TAMs is the ability to directly influence cancer progression through several mechanisms (Fig. 10), such as promotion of tumor angiogenesis, invasion and metastasis of neoplastic cells, and immune suppression.[216]

The M2-like phenotype of TAMs includes low production of NO and ROS, low function as antigen presenting cells, suppression of cytotoxic T lymphocytes and induction of immunomodulatory Tregs.[217] In agreement with the M2 signature, TAMs were reported to express high levels of M2 markers like ARG1, YM1, FIZZ1, MSR1, MMR1, and low levels of inflammatory cytokines (e.g. IL-12, IL-1β, TNFα, IL-6).[218]

Among other events, the activation of the transcriptional factor NF-κB has a key role in the modulation of the inflammatory response of macrophages. In acute inflammation activated by M1 stimuli (e.g. LPS, TNFα), NF-κB acts as heterodimer of subunits p65-p50.[219] In advanced cancer stages, TAMs display a preferential M2 polarization, guided by the p50 NF-κB homodimer, that promotes transcriptional program with anti-inflammatory and pro-tumorigenic effects.[220,221]

TAMs recruitment into tumor microenvironment induce the production of angiogenic factors including VEGF, platelet-derived endothelial cell growth factor (EC-PDGF), thymidine phosphorylase.[222,223] TAMs also play an active role in promoting the spread of distal tumor cells, expressing extracellular matrix-degrading enzymes (e.g. MMPs, plasmin).[224]

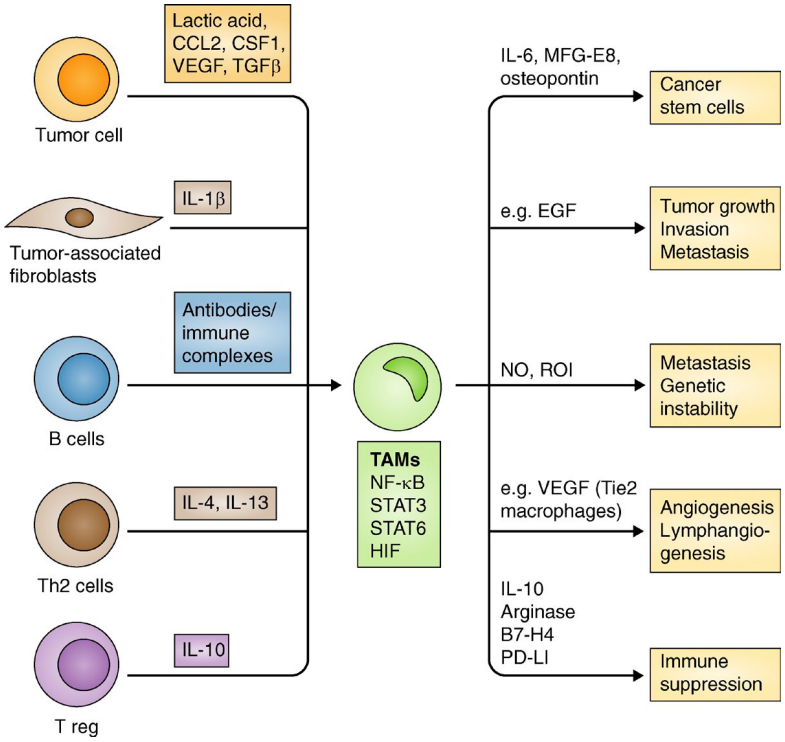

Figure 10. ***Schematic representation of cells and mediators influencing the function of TAMs***. On the left side, different cells belonging to the immunological network, as well as tumor cells and tumor-associated fibroblasts, influence the phenotype of TAMs by producing specific soluble mediators such as cytokines, chemokines, and growth factors. For instance, Th2 cytokines (IL-4/IL-13) and other cytokines such as IL-10 and TGFβ, metabolic products derived from tumor cells (lactic acid) and immune complexes, drive TAM polarization into tumor-promoting macrophages. On the right side are listed the major pro-tumor functions of TAMs. For instance, by producing survival factors TAMs protect cancer stem cells from the toxic effect of chemotherapy or directly stimulate tumor cell proliferation via the epidermal growth factor (EGF). TAM production of nitric oxide (NO) and reactive oxygen species (ROS) induces genetic instability. TAMs switch on neoangiogenesis by secreting VEGF and suppress immune responses through expression of inhibitory molecules (PD-L1 and B7-4) and production of immunosuppressive cytokines/mediators (IL-10 and ARG1). In the rectangle, selected transcription factors that orchestrate TAMs functions are highlighted.[225]

TAMs can suppress the cytotoxic functions of T cells through several mechanisms: nitrosylation of T cell receptors via ARG1, iNOS and peroxynitrite, inducing T cell apoptosis; expression of PD-L1, interacting with PD-1 on T cells and inducing their apoptosis; high levels of IL-10, which negatively regulate the production of pro-inflammatory IL-12 and induce Tregs recruitment; induction of immunomodulatory Th2 cells which produce IL4 and IL-13 reinforcing TAMs pro-tumor phenotype.[226]

1.4.4 Targeting TAMs in cancer therapy

In the last decade, the therapeutic power of immunotherapy has opened a new era in cancer medicine.[227,228] The effectivness of immune checkpoint blockade therapies (ICBs), such as anti-PD-1/PD-L1, anti-CTLA4 antibodies, which impede the activity of inhibitory receptors, and the generation of chimeric antigen T cell receptor (CAR-T) therapy are novel treatments able to increase tumor-specific cytotoxic T cells (CTLs) activity in various tumors.[227,228] Nevertheless, there are a large number of patients resistant to immunotherapy.[229] Recent evidences showed that, at least in part, this resistance can be mediated by abundant and/or immunosuppressive infiltrating myeloid cells, including tumor-associated macrophages.[230,231] Therefore, novel strategies targeting and reprogramming TAMs properties (recrutiment, polarization, survival, phagocytosis, angiogenesis) are currently in clinical development (Fig.11).[202]

As described above, CCL2/CCR2 axis is the most well characterized pathway promoting the recruitment of $CCR2^+$ myeloid cells. In preclinical models, CCR2 blockade inhibited infiltration and immunosuppressive polarization of macrophages.[232,233] A number of CCR2 inhibitors (CCX872-B, PF-04136309, MLN1202, and BMS-813160) are currently in clinical trials for the treatment of solid tumors, in combination with other therapeutic agents (i.e. FOLFIRINOX regimen), achieving at least partial response and local tumor control.[202]

Another chemoattractant target for cancer therapy is IL-1β that promotes recruitment of myeloid cells into tumors.[234,235] The IL-1 receptor antagonist antibody (anakinra) showed to suppress myeloid cell accumulation and tumor progression in mouse models, and, in combination with other agents, is currently under Phase Ib clinical investigation.[202]

As already mentioned, TAM subsets derived from proliferation of tissue-resident macrophages (TRM), that originally arise from $CX3CR1^+Kit^+$

erythromyeloid progenitors, self-maintain independently of the bone marrow.[195,199,202] TRMs have specific markers (e.g. CX3CR1^{hi}, and F4/80^{hi}) and tissue-specific transcription factors (e.g. Gata6 in peritoneal-resident macrophages), which do not appear in bone marrow-derived macrophages (BMDMs).[236] Thus, with the goal of improving cancer immunotherapy, selective therapeutic approaches targeting BMDMs and TRMs may be needed to limit macrophage content in tumors.

The M2-like immunosuppressive polarization of TAMs correlates with tumor progression. Strategies to dampen this immunosuppressive signature and acquisition of pro-inflammatory signatures in macrophages (repolarization or reprogramming) are acquiring increasingly beneficial impact in cancer therapy.[230] In preclinical studies, PI3Kγ inhibition (genetic or pharmacological) suppressed accumulation of immunosuppressive BMDMs (monocytes and granulocytes) but did not affect the accumulation of TAMs in tumors.[230] However, PI3Kγ inhibition increased expression of proinflammatory cytokines such as TNFα, IL-12, iNOS, and MHC-II, and inhibited the expression of immunosuppressive factors such as IL-10, TGFβ, ARG1 and CCL2, resulting in significant reduction of tumor progression, and increased survival in different mouse models of cancer, mediated by increasing CD8$^+$ T effector cell activity.[230,231,237] The PI3Kγ inhibitor IPI-549 is currently in clinical testing in multicenter Phase 1b trials for several advanced solid tumors in combination with anti-PD-1 nivolumab.[202]

Another strategy to repolarize macrophages include targeting Bruton's tyrosine kinase (BTK), a transcription factor acting downstream of PI3Kγ activated in TAMs. Pharmacological inhibition of BTK, ibrutinib (PCI-32765), stimulates M1 macrophage polarization, reduces myeloid cell infiltration, and increases CD8$^+$ T cells in murine pancreatic adenocarcinoma (PDAC).[238] Ibrutinib is in Phase II and III clinical trials for the treatment of different solid tumors in combination with other therapeutic agents.[202]

TMP195, a class IIa histone deacetylase (HDAC) inhibitor, has been reported to repolarize TAMs to express proinflammatory cytokines and to synergize with PD-1 inhibitors, reducing tumor burden and metastasis in an autochthonous mouse model of breast cancer.[239]

Receptor-interacting serine/threonine protein kinase 1 (RIPK1) is upregulated in TAMs, and its inhibition with the small molecule GSK547 repolarizes TAMs towards a proinflammatory state with increased MHCII, TNFα, and IFNγ expression, reducing tumor growth.[240]

Another target for macrophage repolarization is STAT3 which induces immunosuppression in myeloid cells.[155,241] The STAT3 inhibitor TTI-101 is now undergoing testing in a Phase I clinical trial in patients with advanced cancers.[202]

Toll-like receptor (TLR) agonists represent alternative strategies to reprogram macrophage activation. TLRs activation stimulates polarization of macrophages towards a proinflammatory phenotype. Intratumoral injection of 3M-052, a TLR7/TLR8 agonist, induced macrophage repolarization and tumor regression in a mouse model of subcutaneous melanoma. Although TLR agonists stimulate potent proinflammatory polarization in myeloid cells, their effects can be limited, as they develop, in continuous administration, a tolerogenic response and expression of PD-L1, dampening anti-tumor inflammation.[202,221]

Activation of CD40, a member of the TNF receptor family, promotes macrophage production of proinflammatory factors such as IL-1, IL-12, TNFα, and NO.[242] Agonistic antibodies anti-CD40 are reported to induce cytotoxic macrophage activation against tumor cells. Several anti-CD40 antibodies and recombinant CD40 ligands are currently under investigation in several clinical trials for solid tumors as single agents, or in combination with chemotherapy, immunotherapy, or tumor vaccines.[202]

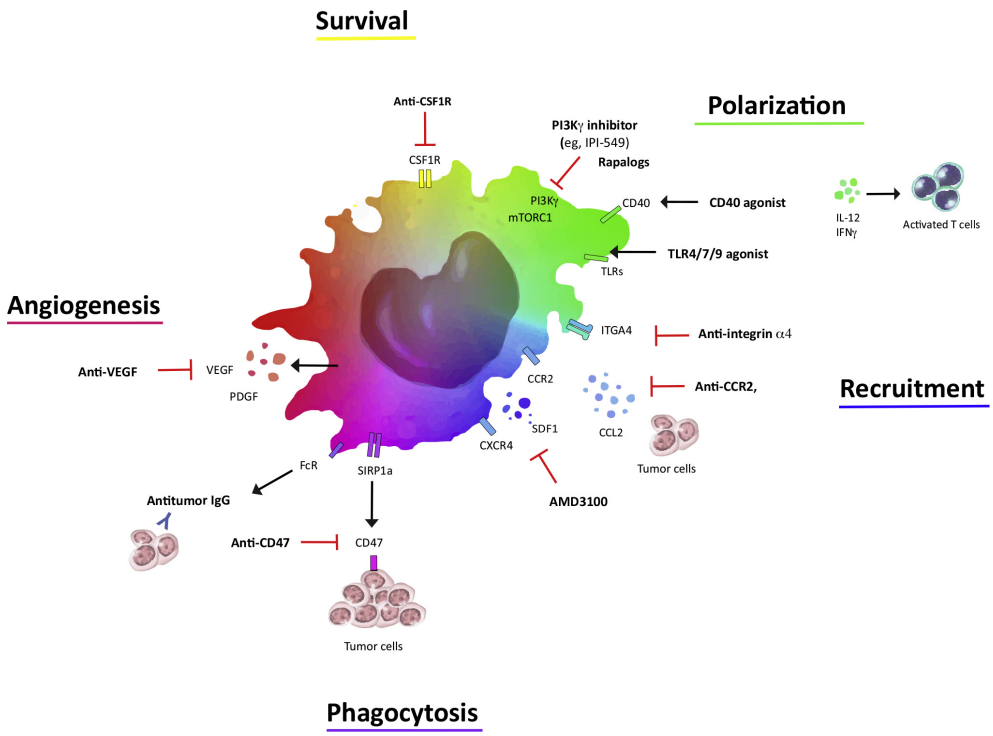

Figure 11. ***Macrophage Targeting Strategies in Cancer Therapies.*** Critical pathways that regulate the recruitment, polarization, survival, phagocytosis, and proangiogenic properties of TAMs. CSF1R inhibitors can suppress the survival of TAMs. Inhibitors of PI3Kγ/mTOR pathway (e.g. IPI-549, rapalogs-rapamycin homologs), as well as agonists of CD40, or TLR4,7,8, or 9 can repolarize TAMs towards a proinflammatory phenotype. Inhibitors of CCR2 can suppress monocyte recruitment, and thereby macrophage accumulation in tumors. Inhibition of CD47 on tumor cells together with antitumor antibodies can promote phagocytosis of tumor cells by macrophages. Additionally, inhibition of macrophage-derived VEGF can suppress tumor angiogenesis and progression. These molecular targets that are currently under clinical development to promote effective antitumor immune responses. For abbreviation and details, see the text.[202]

Although complement activation is an effective innate immune response pathway, complement-induced inflammation can also paradoxically promote neoplastic progression.[243] Indeed, TAMs upregulate the production of C5a which in turn activates its C5a receptor (C5aR) on macrophages, resulting in immunosuppressive macrophage polarization and inhibition of $CD8^+$ T cell activation.[243] Inhibiting C5aR with the peptide antagonist PMX-53 in combination with paclitaxel effectively inhibited tumor growth by repolarizing TAMs towards the proinflammatory phenotype.[202]

CSF1R plays a major role in regulating macrophage proliferation and survival. Inhibition of CSF1R reduces immunosuppressive macrophage polarization, decreases tumor cell proliferation and increases apoptosis, resulting in reduced tumor progression and prolonged survival.[244,245] Indeed, an anti-CSF1R antibody, emactuzumab, depletes TAMs and reduces tumor growth in a mouse model of subcutaneous colon carcinoma.[246] Moreover, inhibition of CSF1R in combination with paclitaxel decreases macrophage recruitment to tumors, increased CTL activation resulting in prolonged survival in a breast cancer mouse model.[202]

CD47, the '*don't eat me*' signal, is a self-molecule that protects hosts and tumor cells from destruction by macrophages, binding to the SIRP1a present on the surface of macrophages.[247] Antibody-mediated inhibition of human CD47 promotes macrophage-mediated phagocytosis of tumor cells and T cell activation.[248] Targeting CD47 with TTI-621, a human recombinant protein that blocks binding of SIRPa to CD47, stimulates phagocytosis of tumor cells, thus reducing tumor growth.[249]

1.5 The transcription factor NF-κB

The nuclear factor-κB (NF-κB) family of transcription factors was first described as a B-cell-specific transcription factor that binds the kB site in the Ig κ light chain enhancer. Soon after, NF-κB activity was found to be inducible in all cell types.[250] NF-κB is a key player in the regulation of many genes involved in various biological process, including cell growth and survival, development, and immune responses, both innate and adaptive.[251–253] Furthermore, several tumors have been shown to have a constitutive activation of NF-κB.[254]

At present, more than 150 genes under control of NF-κB have been identified, as a demonstration of its vast spectrum of biological functions.[255,256] In immune cells, NF-κB activation is triggered by the engagement of several receptors, such as B-cell receptor (BCR), T-cell receptor (TCR), Toll-like receptors (TLRs), TNF receptor and CD40, resulting in the expression of cytokines, growth factors and effector enzymes.[250]

Due to its pleiotropic and central activity, dysfunction of NF-κB system can lead to various pathological immune-related conditions, such as rheumatoid arthritis, asthma, intestinal bowel diseases (IBDs), multiple sclerosis and cancer.[257–259] Thus, exploring and defining the NF-κB pathways could provide the basis for the development of therapeutic strategies with a relevant impact on human diseases.

1.5.1 NF-κB family and activation pathways

NF-κB family is evolutionarily conserved and includes five members: NF-kB1 (p105/p50), NF-κB2 (p100/p52), RELA (p65), RELB and c-REL (Fig. 12). Each family member shares an N-terminal 300 amino acid conserved region known as the rel homology domain (RHD) through which they can

form homo- and hetero-dimers. RHD region is also responsible for the nuclear localization, binding to DNA, and interaction with IκB family members.[250,260] The RELA, RELB and c-REL proteins also have a C-terminal non-homologous transactivation domain (TD), which strongly activates transcription of target genes once the NF-κB dimers bound to specific DNA consensus sites named κB.[260] p50 and p52 are generated by proteolytic degradation of p105 and p100 precursors, respectively, and they lack TDs; therefore, p50 and p52 can activate transcription by forming heterodimers with p65, RELB and c-Rel, which contains TDs. If they form homodimers (e.g. p50/p50 homodimer) they can still bind the DNA consensus sites, but they do not activate transcription.[261,262] The main activated form of NF-κB is the heterodimer containing p65 together with p50 or p52.[253]

In most cells, NF-κB complexes are inactive, residing predominantly in the cytoplasm in a complex with inhibitory IκB proteins, and the intact NF-kB subunits p100 and 105. When signalling pathways are activated, the IκB protein, p100 and p105 are degraded and NF-κB dimers enter the nucleus to modulate target gene expression. Whereas p50 and p65 are expressed widely in various cell types, the expression of RELB is restricted to specific regions of the thymus, lymph nodes and Peyer's patches. The expression of c-REL is confined to haematopoietic cells and lymphocytes.[253]

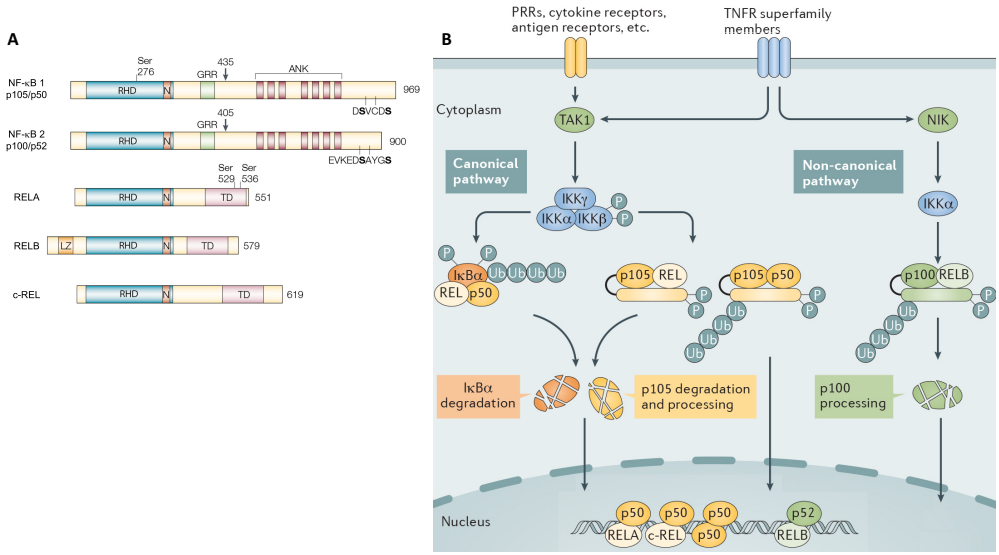

Figure 12. ***NF-κB proteins and their activation pathways.*** A) NF-κB proteins contain a well-conserved N-terminal rel-homology domain (RHD), which includes DNA-binding and dimerization domains, and a nuclear localization signal (NLS). Some proteins, such as RELA, RELB and c-Rel contain transactivation domains (TD) in addition to the RHD. B) Canonical pathway is triggered by numerous signals, including those mediated by innate and adaptive immune receptors. It involves the activation of IKK complex by TAK1, IKK-mediated IκBα phosphorylation, and subsequent degradation, resulting in rapid and transient nuclear translocation of the prototypical NF-κB heterodimer RELA p65/p50. Non-canonical NF-κB pathway relies on phosphorylation-induced p100 processing, which is triggered by signalling from a subset of TNFR members. This pathway is dependent on NIK and IKKα, but not on the trimeric IKK complex, and mediates the persistent activation of RELB/p52 complex.[253,260]

In resting conditions, IκBs exert regulatory functions binding and masking the Nuclear Localization Sequence (NLS) on NF-κB proteins, thus impeding the nuclear translocation of IκBs-NF-κB complexes, retained into the cytoplasm in inactive forms. Activation of several receptors can induce NF-κB signalling that starts upon phosphorylation of IκB proteins by IκB Kinases (IKK). IKK is a complex of three subunits: two kinases IKKα and IKKβ, and the regulatory IKKγ (or NEMO, NF-κB Essencial Modifier). Hence, upon activation of IKKβ, IκB is phosphorylated and degraded by the proteasome; the released NF-κB dimers translocate into nucleus and activate gene transcription.[250,263]

The activation of NF-κB involves two major signalling pathways, the canonical and non-canonical (or alternative) pathways (Fig. 12), both

important for regulating inflammatory responses despite their differences in signalling mechanism.[250,252,260]

The canonical NF-κB pathway is mainly mediated by Toll like receptors (TLRs), TNF receptor (TNFR) superfamily members, as well as T-cell receptor (TCR) and B-cell receptor.[264] The primary mechanism for canonical NF-κB activation is the inducible degradation of IκBα triggered through its site-specific phosphorylation by a multi-subunit IκB kinase (IKK) complex.[265] IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit named NF-κB essential modulator (NEMO) or IKKγ.[252] IKK can be activated by different stimuli, including cytokines, growth factors, mitogens, microbial components and stress agents.[265] Upon activation, IKK phosphorylates IκBα triggering ubiquitin-dependent IκBα degradation in the proteasome, thus resulting in rapid and transient nuclear translocation of canonical NF-κB members predominantly the p50/p65 and p50/c-Rel dimers.[251,265]

On the other side, the non-canonical NF-κB pathway selectively responds to a specific group of stimuli, including ligands of a subset of TNFR superfamily members, such as LTβR, CD40 and RANK.[260,266] In addition, the alternative NF-κB activation does not involve IκBα degradation but rather relies on processing of the NF-κB2 precursor protein p100.[265] A central signalling molecule for this pathway is NF-κB-inducing kinase (NIK), which activates and functionally cooperates with IKKα to mediate p100 phosphorylation, which in turn induces p100 ubiquitination, and degradation in mature NF-κB2 p52 subunit. This translocate into nucleus as p52/RELB dimer.[265]

Functionally, canonical NF-κB is involved in almost all aspects of immune responses, whereas the non-canonical NF-κB pathway appears to be evolved as a supplementary signalling axis that cooperates with canonical NF-κB pathway in the regulation of specific functions of the adaptive immune system.[266]

1.5.2 NF-κB in cancer-related inflammation

Accumulating evidences are profiling a key role of NF-κB pathway in cancer progression beyond the inflammatory-related activity.[267] Indeed, NF-κB signalling in malignant cells can modulate the transcription of genes encoding for proteins important for cell proliferation (e.g. cyclin D1, c-Myc) survival (BCL-2, c-FLIP), angiogenesis (e.g. VEGF, MMP-9), and adhesion (e.g. MMPs, VCAM1).[267–269] Nevertheless, the major role of NF-κB in tumor diseases is focused on the modulation of a wide variety of genes that control cancer-related inflammation.[254]

Indeed, NF-κB represent a master regulator of inflammation triggering the transcription of several pro-inflammatory factors, such as IL-1β, TNFα, IL-6 and IL-8, which in turn upregulate the NF-κB activation, thus generating a positive feedback pathway into the inflammatory microenvironment. This is directly involved in both early stages of the disease, when inflammation acts predominantly as anti-tumoral, and advanced disease, characterized by immunosuppressive and anti-inflammatory microenvironment favouring cell proliferation, transformation and survival, and consequently cancer development (Fig.13).[220,256,270] Moreover, the pro-inflammatory stimuli, such as bacterial products or inflammatory cytokines (e.g. TNFα and IL1β), through the respective receptors, activate NF-κB that translocates into the nucleus inducing the expression of cytokines (such as TNFα and IL6) and chemokines, which contribute to inflammation-related tissue damage. Elevated IKK/NF-κB activity may also lead to aberrant upregulation of certain tumorigenic adhesion proteins, chemokines, and inhibitors of apoptosis that promote cell survival.[271] In particular, in a breast cancer model, the potent induction of epithelial-mesenchymal transition by TNFα has been shown to be mediated by the activation of the NF-κB pathway.[272]

NF-κB was also found to promote the metastatic process in a genetic mouse model of prostate cancer, in which inactivation of IKKα was found to reduce

metastatic spread.[273] Moreover, it has been demonstrated in many cancers that NF-κB signalling is constitutively active. Although with many unclear aspects, several mechanisms regulating this activation have been proposed, such as IL-1β and TNFα production, shorter IκBα half-life or IκBα mutations.[274,275] For these reasons, NF-κB represents an ideal therapeutic target for the development of new anti-tumor therapies.

1.5.3 NF-κB1 (p105/p50) in tumor-associated myeloid cells

As described above, the NF-κB1 gene encodes for the p105 protein, which, once cleaved, gives rise to the functional form p50. The p50 subunit could generate dimers with another p50 subunit or with other NF-κB family members.[261] The role of p50 and its precursor in cell physiology and function is very complex and not fully defined. Indeed, although originally it was considered a repressor of transcription, p50 could also be a transcriptional activator, and the balance between pro- and anti- inflammatory activity of p50 depends on cell type and environmental conditions.[276,277]

The nuclear translocation of the homodimeric p50/p50 complexes deeply controls functions of myeloid cells in cancer.[270] In macrophages, under prolonged stimulation with LPS, the TLR4-mediated massive production of TNFα results down-regulated via overexpression of p50 protein, inducing a tolerant, M2-like, immunosuppressive phenotype. Genetic deletion of p50 in macrophages unable the induction of tolerance by long-term pre-treatment with LPS.[48,221,278] Moreover, our group has demonstrated that TAMs display a defective NF-κB activation in response to the M1 polarizing signals (LPS and TNFα) and that this phenotype is due to the overexpression of nuclear p50 NF-κB homodimers which inhibits the transcription of pro-inflammatory genes.[221] By contrast, LPS treatment of TAMs genetic deleted for p50 recover an M1 phenotype with increased expression of IL-12, TNFα, and decreased IL-10, restoring antitumor immunity.[279] Further, a detailed analysis of the role

of p50 NF-κB homodimer in macrophage functions revealed that its nuclear accumulation, both in TAMs and LPS-tolerant macrophages, not only mediates a status of unresponsiveness (tolerance) toward pro-inflammatory signals, but actually plays a role as key regulator of M2-driven inflammatory responses.[221] In contrast, other studies showed that inhibition of NF-κB activation in TAM correlates with impaired expression of inflammatory functions, such as expression of cytotoxic mediators (NO) and cytokines (TNFα, IL-1 and IL-12).[280] Possible explanations for this discrepancy are based on the functional feature of macrophages, which are considered plastic cells able to finely modulate their programs in response to different microenvironmental conditions.[280] It has been speculated that dynamic changes of the tumor microenvironment may occur during the transition from early neoplastic events toward advanced tumor stages.[281] These events would drive an M1 toward M2 switch of TAMs functions (Fig. 13) and are probably connected to the profound changes occurring in the tumor microenvironment.[280]

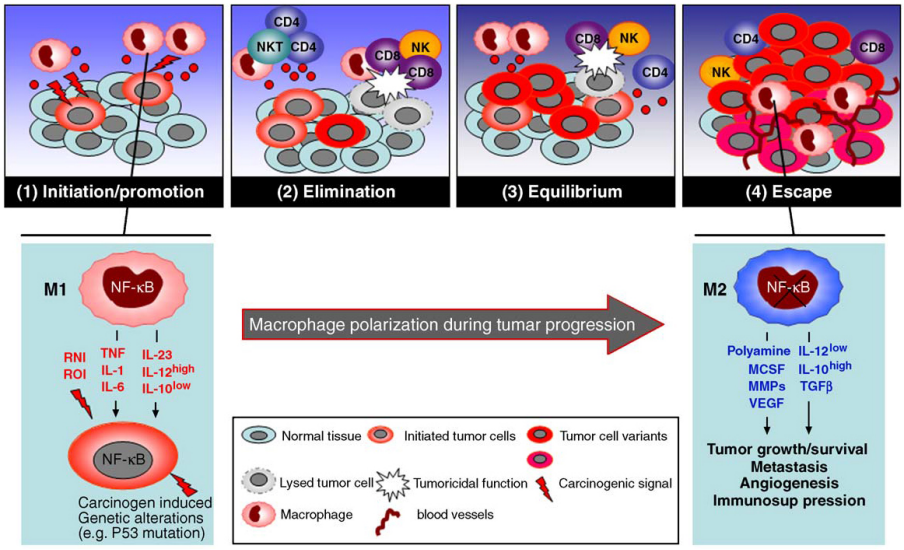

Figure 13. ***NF-κB role in TAMs reprogramming during tumor progression.*** It is proposed that a gradual switching of TAMs polarization, from M1 to M2, is paralleled by the gradual inhibition of NF-κB, during different stages of tumor progression. Early in the carcinogenesis, T cell driven M1 activated macrophages may contribute to elimination. Regulatory mechanisms result in M2 polarized TAMs that orchestrate smouldering, non-resolving tumor-promoting inflammation.[282]

Further, nuclear accumulation of p50 NF-κB promotes a tolerogenic phenotype in dendritic cells (DCs), affecting both their survival and capacity to drive activation of effector T cells. Lack of p50 subunit in murine DCs promoted increased lifespan, increased maturation and expression of the pro-inflammatory cytokines IL-1, IL-18 and IFNβ, enhanced capacity of activating and expanding effector T cells and decreased ability to induce differentiation of regulatory T cells.[283] In colorectal cancer models the p50-dependent inhibition of M1-polarized gut inflammation supported colorectal cancer development. Indeed, lack of p50 showed an enhanced activation of Th1/M1 cytokine pathway (e.g. IL-12, CXCL10), reduced frequency of TAMs at the tumor site, and increased recruitment of NK, NK-T and CD8 T cells, resulting in a marked dampening of disease progression.[284] Therefore, a profound investigation of the molecular mechanisms associated with NF-κB activation, with particular attention to p50 protein activity, represents a novel fascinating approach to identify strategies that revert the immunosuppressive phenotype of tumor-infiltrating myeloid cells and boost anti-tumor immunity.

1.6 The transcription factor RORγ(t)

The retinoic acid-related orphan receptor (ROR) gamma (t) is a member of RORs subfamily of nuclear receptors, that, by definition, function as ligand-dependent transcription factors.[285] Recent studies have demonstrated that RORs, including the other two members RORα and RORβ, have been implicated in several physiopathological processes.[286–288] Therefore, RORs represent an active area of research and are emerging as important drug targets for the treatment of various diseases, such as multiple sclerosis, rheumatoid arthritis, psoriasis, cancer and metabolic syndrome.[286,289,290]

1.6.1 RORγ(t) structure, activation and functions

The RORγ(t) encoding gene (*RORC*) was initially discovered in Jetten's lab from human pancreas poly(A)$^+$ RNA.[291] Murine *Rorc* gene and the human form (*RORC*) share an 88% sequence homology.[292] Both murine and human RORC genes encode for two different mRNA splicing variants, which produce two different protein isoform: 1) RORγ (or RORC1, RORγ1) and RORγt (or RORC2, RORγ2) (Fig.14).[293] Structurally, the RORγt isoform lacks the 24 N-terminal amino-acidic residues present in RORγ form, which are encoded by the first two exons (1γ and 2γ). These latter are replaced by a unique exon (1γt) specific for RORγt isoform, which result in 3 amino-acidic residues (Fig.14).[294]

RORγ(t) exhibits a domain structure that is typical of nuclear receptors and contain an N-terminal domain, a highly conserved DNA-binding domain (DBD) consisting of two zinc finger motifs, a ligand-binding domain (LBD), and a hinge domain spacing the DBD and LBD (Fig.14).[290] As opposed to the majority of other nuclear receptors, which act as dimers (e.g. LXRs, RXRs), RORγ(t) regulates gene transcription recognizing and binding as monomer to specific ROR response elements (ROREs) consisting of the RGGTCA

consensus preceded by an A/T-rich sequence in the regulatory regions of target genes (Fig.15).[285,295]

Another peculiarity of RORs, as compared to the other nuclear receptors, is their intrinsic constitutive capacity to activate transcription in a ligand-independent manner. Indeed, the positive and negative transcriptional regulation of the RORs occurs through the recruitment of nuclear receptor co-activators and co-repressors, proteins with intrinsic chromatin remodeling or integration activity (e.g corepressors NCoR, RIP140, SMRT, and coactivators GRIP, SRC-1/2, PGC1α).[285]

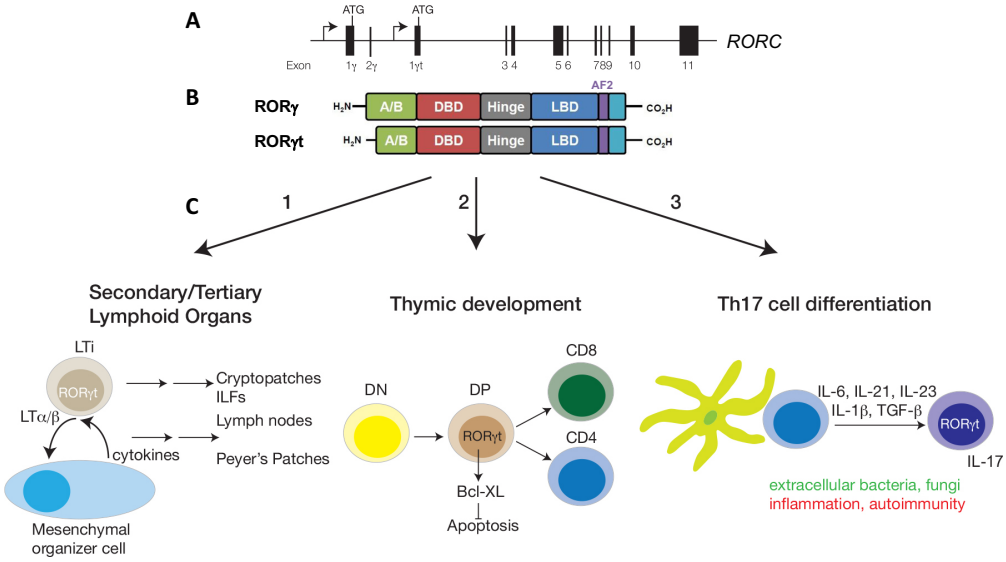

Figure 14. ***RORγ(t) structure and functions.*** (A) Organization of the *RORC* locus. The usage of exons 1γ and 2γ produces the RORγ protein. These are replaced by a unique exon (exon 1γt) to produce the immune-specific isoform RORγt. As a result, the 24 first amino-acids of RORγ are replaced by 3 residues from exon 1γt. (B) Schematic representation of the domain structure of RORγ isoforms: RORγ and RORγt. Amino-terminal domain (A/B or AF1), DNA- binding domain (DBD), hinge region, ligand-binding domain (LBD), and the activation function helix-2 (AF2). (C) Biological functions of RORγt. 1. Development of secondary/tertiary lymphoid organs. RORγt induce the expression of lymphotoxin α/β on lymphoid tissue inducer (LTi) cells, which interact with mesenchymal organizer cells, driving tissue formation. 2. During thymocyte development, RORγt is expressed in DP cells and is required for the survival of these cells by controlling the expression of the survival factor Bcl- X_L. 3. RORγt is the master transcription factor of Th17 cells, sources of IL-17. RORγt is induced in naïve $CD4^+$ T cells upon their activation by an antigen-presenting cell, in the presence of a cocktail of cytokines (IL-6, IL-21, IL-23, IL-1β, TGFβ). Th17 cells have been shown to be required for immunity against infective agents. Excessive IL-17 production has been linked with inflammation and autoimmunity.[294 (adapted)]

Conversely, the ligand-dependent transcriptional activity of RORγ remains controversial for two main issues. The first issue is –as the definition of 'orphan' receptors claims– the little-known molecular source of endogenous ligands for RORγ. Although until ten years ago the natural RORγ ligands were almost unknown or merely supposed, many recent evidences indicate **cholesterol and cholesterol derivatives** as endogenous molecules with high affinity for the LBD or RORγ protein.[286,296,297]

The second issue concerns the functional role of endogenous ligands. Indeed, as mentioned above, RORs can result constitutively active and retain the ability to interact with several co-activators also in the absence of binding any ligand.[298] It appears that some ligands induce trivial conformational changes in protein structure, but with unclear capacity to alter their functions.[298] It has been proposed that they are important to maintain the constitutive transcriptional output level of the receptor and to prevent hyperactivation via agonist or inadvertent repression by endogenous inverse agonists.[298]

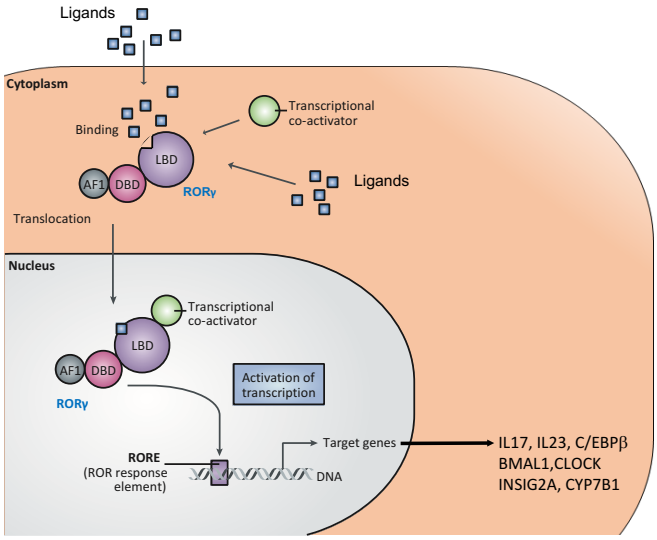

Figure 15. ***RORγ(t) activation.*** The nuclear receptor RORγ(t) acts its transcriptional activity through the DNA-binding domain (DBD) inducing expression of target genes. These are related to immune response (e.g. IL17, IL23, C/EBPβ) circadian rhythms (BMAL1, CLOCK), and lipid and glucose metabolism (e.g. INSIG2A, CYP7B1). Conversely to other NRs, RORγ(t) can function as monomer, and translocate into the nucleus exerting its transcriptional function in a ligand-independent manner. Ligands modulate the entity of RORγ activity which is strictly dependent by co-activator factors. LBD, ligand-binding domain; AF1, activation function 1.[286 (adapted)]

The tissue expression levels and functional effects of the two RORγ isoforms are quite varied. RORγt isoform is well-known for its involvement in immunological processes. Indeed, it is highly expressed in lymphoid tissues, such as thymus, gut-associated lymphoid tissue (GALT), lymph-nodes (LNs) where it plays a fundamental role during organogenesis. Mice genetic deleted for RORγt do not develop Peyer's patches, peripheral lymph node and generally develop thymic lymphoma.[287] Moreover, RORγt is expressed in various T cell subsets, including innate lymphoid cells (ILCs) (Fig.14).

RORγ, instead, is more known for its metabolic predominance. It is expressed in many tissues including skeletal muscle, kidneys, adipose tissue, and liver.[290] Indeed, it has been emerged an important involvement of RORγ in the metabolic regulation of adipogenesis and insulin sensitivity, with direct effect in the development of metabolic syndrome.[285,287,299]

In addition, RORγ isoform is involved in the regulation of circadian rhythms. Indeed, in the liver and kidneys, it can bind and activate the promoter of the ARNTL (BMAL1) gene, a transcription factor which, dimerizing with CLOCK protein, has a central role in the generation of physiological circadian rhythms, inducing or repressing the transcription of genes mainly involved in the metabolic pathway.[293]

Although RORγ isoform it has been considered for a long time not involved and/or expressed in immune cells, our group has recently identified that tumor-derived factors (e.g. CSFs) induce an overexpression of RORγ in myeloid progenitor cells, driving the cancer-associated emergency myelopoiesis.[119] Furthermore, RORγ(t) is emerging to be involved in various mechanisms of tumorigenesis. These include both immunological and metabolic patterns, and also regulate molecular pathways into tumor cells (Fig.16).[288]

1.6.2 RORγt in lymphoid cells and cancer

RORγt isoform has a T cell specific expression. In the thymus, RORγ is transiently expressed to induce the transition from $CD4^-CD8^-$ double-negative (DN) immature cells to $CD4^+CD8^+$ double-positive (DP) cells, which subsequently differentiate into single-positive $CD4^+$ or $CD8^+$ T cells (Fig.14).[293] The absence of RORγt results in a dramatic decrease in the number of DP and mature $CD4^+$ and $CD8^+$ cells, due to a reduced expression of anti-apoptotic factor Bcl-X_L thus resulting in the apoptosis of DP cells.[293]

Mature, but naïve $CD4^+$T (Th0) cells can be differently polarized to produce the cytokines characteristic of Th1, Th2 and Th17 cells. RORγt is required for the development of Th17 cells whose name reflects their ability to produce the cytokines IL-17A and IL-17F, as well as IL-21 and IL-22.[285] Th17 cells protect against extracellular pathogens, but are also associated with various autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and asthma.[300] RORγt is recruited to ROREs in several Th17 marker genes, including *Il17a*, *Il17f*, and *Irf4*. The latter is a transcription factor which in turn can induce the expression of *Rorc* gene. IL-6 and low levels of TGFβ can also induce the expression of *Rorc* in a STAT3-dependent manner.[301]
Th0 cells can also differentiate in Tregs. Specific signals, such as high levels TGFβ, IL-2 and retinoic acid, upregulate the expression of FOXP3 which interacts with and inhibits RORγt, thus promoting Treg differentiation.[302]

RORγt also plays a critical role in the generation of innate lymphoid cells (ILCs). ILCs are a heterogeneous population of cells with the typical lymphoid cell morphology, but lacking cell surface molecules typical of lymphocytes, and thus, impeding ILCs to recognize specific antigens and to respond to cytokines produced during innate immune responses.[303] ILCs have been classified into three groups, based on their cytokine production profiles and

mirroring the corresponding T helper (Th) cell types: ILC1 (Th1) producing IFNγ; ILC2 (Th2) producing IL-5 and IL-13; and ILC3 (Th17) producing IL-17. As a consequence, RORγt is required for the development of ILC3s, a heterogeneous population of cells, including lymphoid tissue inducer (LTi) cells ($CD4^+CD3^-$) required for the development of secondary lymphoid organs and Peyer's patches.[304]

In cancer, RORγt showed controversial activities (Fig.16).[288] In a study exploring the function of Th17 cells in a mouse B16 melanoma model, RORγt was found to be expressed by tumor-infiltrating Th17 cells. Th17 cells did not exhibit in vitro tumor cell killing activity, although $CD8^+$ cytotoxic T cells stimulated by Th17 cells could activate the tumor killing response.[305]

The expression of RORγt mRNA and protein was found to be downregulated in PBMCs from non-small cell lung cancer (NSCLC) patients compared with controls, suggesting a possible pro-tumor activity of RORγt.[306] Other evidence showed that RORγt is overexpressed in neoplastic cells, contributes to their proliferation and is negatively correlated NSCLC prognosis.[307] Other studies confirmed that RORγt mRNA is upregulated in the peripheral blood of patients with NSCLC compared with that of healthy controls.[308–310] Conflicting evidence in hepatic cancers showed that RORγt mRNA expression was found both increased and decreased in tumor tissues.[311,312] In colorectal cancer, $Foxp3^+IL\text{-}17^+$ cells were found to express RORγt[313], and directly correlated with the capacity of regulatory T cells to suppress anti-tumoral inflammation and with the stage of human colon cancer.[314] Another study showed that RORγt was not expressed within colorectal cancer tissues or by infiltrating $CD4^+$ T cells.[315]

The expression and regulation of RORγt in various cancers remain controversial, which makes difficult to confirm its involvement in tumorigenesis.

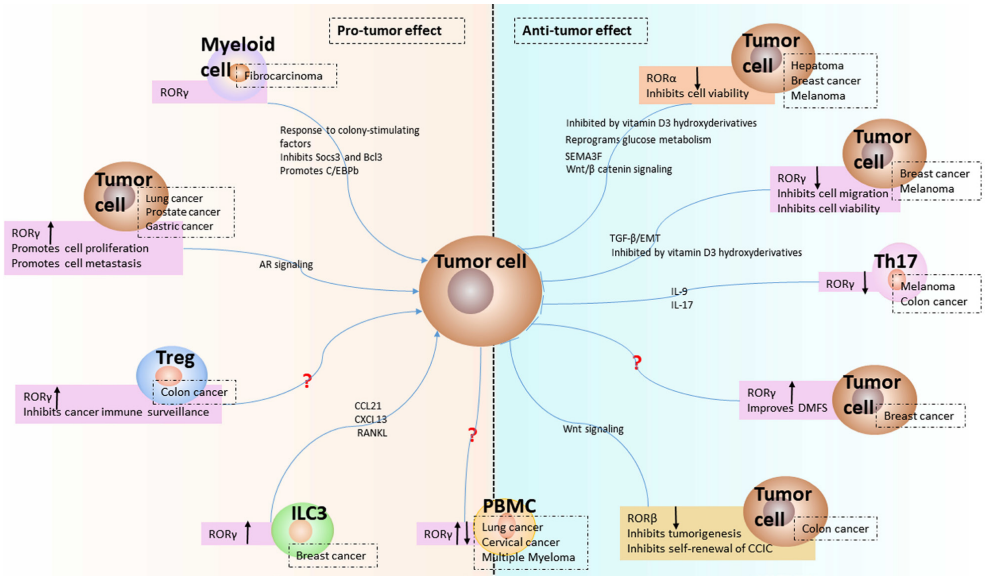

Figure 16. ***Expression and function of RORγ(t) in tumor microenvironment.*** The upregulation of RORγt in ILC3 leads to protumor effect. The downregulation of RORγt in Th17 cells indicates the antitumor effect of IL-17 in colon cancer. The upregulation of RORγt in Treg shows protumor effect in colon cancer. The expression of RORγ in myeloid cells has protumor effects via Socs3, Bcl3, and C/EBPβ. The expression of RORγ in tumor cell is either increased or decreased depending on the cancer type. Increased expression of RORγ(t) in lung cancer, prostate cancer, and gastric cancer results in protumor effects, while decreasing expression of RORγ(t) in breast cancer and melanoma induces antitumor responses via TGFβ/epithelial–mesenchymal transition (EMT) or vitamin D3 derivatives. The question mark refers to unknown mechanisms. There are indicated the expression of RORα and RORβ, other members of RORs family, whose downregulation induces antitumor effect in hepatoma, breast cancer, melanoma, and colon cancer. The up or down black arrow refers to upregulation or downregulation.[288]

1.6.3 RORγ in tumor-associated myeloid cells

Controversial evidences are described also for the RORγ isoform. Indeed, in castration-resistant prostate cancer (CRPC), RORγ was found to be overexpressed and to directly drive androgen receptor (AR) hyperactivity through binding to an exonic RORE, exacerbating tumor proliferation and growth.[316] Therefore, inhibition of RORγ may represent a possible treatment option for CRPC. In breast cancer, high expression of RORγ in cancer cells was both inversely correlated with reduced expression of PRMT2, suppressor of cell migration, and positively associated with a longer metastasis-free survival, suggesting a protective role by RORγ.[317]

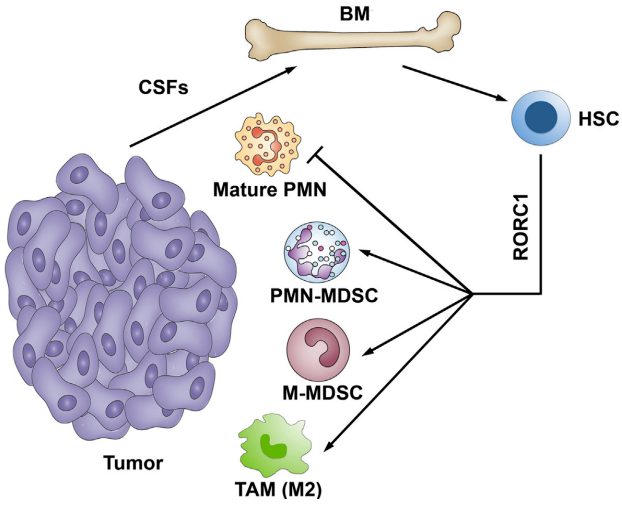

Figure 17. ***RORC1 drives tumor-promoting emergency myelopoiesis***. Tumor-derived colony-stimulating factors (CSFs) up-regulate RORγ in myeloid progenitors, driving the expansion of MDSCs, the inhibition of neutrophils maturation, and the induction of M2 phenotype in TAM, and, thus, promoting tumor growth.[119]

Accordingly, the different functions of RORγ in cancers could be due to distinct cell origins as well as isotypes. For instance, when expressed by immune cells, RORγ acts as an immune suppressor, although when produced by cancer cells, it acts as a potential survival factor. Indeed, in a recent work published by our group it has been demonstrated that RORγ expression is upregulated in myeloid progenitors by tumor-derived CSFs and drives tumor-promoting "emergency" granulo-monocytopoiesis by suppressing negative (Socs3 and Bcl3) and promoting positive (C/EBPβ) regulators of myelopoiesis. These lead to RORγ-dependent expansion of immunosuppressive MDSCs and TAMs, with marked pro-tumor functions (Fig.17).[119] Moreover, PBMCs from cervical cancer patients showed a high expression of RORγ.[318]

Additional observations are needed to elucidate the regulating mechanisms and functions of RORγ in each cell type, in different tumor microenvironments.

1.6.4 RORγ in regulation of metabolism

RORγ, together with RORα, have been implicated in the control of energy homeostasis and the regulation of several lipid and glucose metabolic genes.[285] Regulation of energy homeostasis is a complex process that involves multiple interrelated glucose and lipid metabolic pathways in many organs and is controlled by the circadian clock, gut microbiota, and by the endocrine, immune and nervous systems.[319] This has made it difficult to determine whether the metabolic changes observed in RORγ deficiency are cause or effect. In this context, most of the studies and evidences in metabolic regulation by RORs focused on the RORα protein, which is structurally and functionally similar to RORγ, and in certain cases it exerts the same transcriptional activities in alternative and/or synergistic manner.[285]

For instance, both RORα and RORγ have been shown to be induced during adipocyte differentiation.[320] RORγ plays a role in the regulation of glucose metabolism and insulin sensitivity.[285,299,321] RORγ-deficient mice were significantly more insulin sensitive and glucose tolerant than wild-type mice; instead, the overexpression of RORγ in hepatocytes led to an increase in glucose production starting from pyruvate.[299,321] It was also identified the recruitment of RORγ in the regulatory region of a number of metabolic genes involved in glycolysis and gluconeogenesis, including G6Pase, PPARδ, Glut2, which result decreased in RORγ deficient mice.[299] Together, these observations indicated that RORγ is an important modulator of hepatic gluconeogenesis and glycolysis, and, thus of insulin sensitivity. This is supported by studies showing that the level of RORγ expression positively correlates with adiposity and insulin resistance in human obese patients.[321,322]

In addition to gluconeogenesis, RORγ regulates hepatic lipid metabolism. Several studies showed that RORγ deficiency significantly decreased the expression of genes involved in lipid regulation and in fatty acids and cholesterol metabolism, including the insulin-induced gene 2a (Insig2a),

elongation of very long chain fatty acids-like (Elovl3), Cyp7b1, Cyp8b1, Sult1e1.[285] These data indicated that RORγ positively regulates the transcription of these metabolic genes by directly binding ROREs in their regulatory regions. Moreover, the dysregulated expression of these genes in RORγ-deficient mice fed with high-fat diet (HFD) were associated with reduced levels of triglycerides, cholesterol, and bile acids in liver and blood.[285] These observations suggest that RORγ antagonists might be beneficial in controlling glucose homeostasis and in the management of metabolic diseases.

1.6.5 The dual relationship between RORγ and cholesterol metabolism

Cholesterol and its metabolites are bioactive lipids that interact with and regulate the activity of various proteins and signalling pathways implicated in the control of several physiological and pathological processes.

A clear connection has been established between cholesterol intermediates/metabolites and their regulation of RORs activity. Several studies demonstrated that various intermediates of the cholesterol biosynthesis pathway, downstream to lanosterol, bind RORγ and enhance its activity, suggesting their potential function as endogenous ligands for RORγ (Fig.18). Zymosterol, 7-dehydrodesmosterol and desmosterol were among the most effective sterols activating RORγ. [297,323]

Cholesterol and other sterols can be metabolized by a number of cytochrome P450 enzymes and lead to the formation of a wide range of oxysterols (Fig.18).[324] CYP7A1 catalyzes the formation of 7-ketocholesterol (7-KC) and 7α-hydroxysterols (7α-OHC), while CYP27A1 generates 27-hydroxycholesterol (27-OHC), and CYP46A1 produces various hydroxysterols, such as 24(S)-hydroxycholesterol (24-OHC). Many of these cholesterol modifications appear concomitantly, generating 'combined' oxysterols (e.g. 7α-,27α-dihydroxystolesterol). Many of these cholesterol modifications can occur in a non-enzymatic manner, through oxidation by

reactive species (e.g. ROS) aberrantly expressed in several pathological conditions like cancers (Fig.18).[325]

A number of these oxysterols can act as ligands for RORγ, with both agonistic or antagonistic activity.[298,326] For instance, 7-ketocholesterol (7-KC) and 24(S)-hydroxycholesterol (24-OHC) function as inverse agonists for RORγ, while 25-hydroxycholesterol (25-OHC), 27-hydroxycholesterol (27-OHC), 7α,27- and 7β,27-dihydroxycholesterol act as agonists for RORγ.[326] In addition to being hydroxylated, sterols can be sulfo-conjugated by sulfotransferase SULT2A1 and SULT2B1, and generating sulfated sterols, such as cholesterol- and desmosterol sulfate, which function as RORγ agonists.[323] Therefore, alterations in sterol homeostasis and, thus, modification of the intracellular pool of RORs ligands affect RORγ transcriptional activity and, consequently, the physiological and pathological processes in which RORγ is implicated.

This hypothesis was strongly supported by studies examining the effect of disruption of cholesterol biosynthesis on RORγt-dependent regulation of Th17 differentiation and gene expression. Interestingly, during Th17 differentiation, the expression of several cholesterol biosynthetic genes (e.g., SQS, DHCR24) are enhanced, whereas genes involved in cholesterol metabolism (e.g., CYP7A1, CYP27A1) and efflux (e.g., ABCA1, ABCG1) are decreased.[323] In addition, Th17 differentiation is accompanied by an increase in the expression of the sulfotransferase SULT2B1.[323] Together, these findings are consistent with the concept that increased expression of genes involved in cholesterol biosynthesis would enhance the availability of RORγt agonists and subsequently stimulate RORγt activity. Indeed, several studies have demonstrated that disruption of the cholesterol biosynthesis pathway has a negative effect on RORγt activation and Th17 differentiation.

Statins, which inhibit HMGCR, the key enzyme in the initiation of cholesterol biosynthesis (Fig.18), and thus, lower the generation of RORγ sterol agonists,

have been shown to dampen Th17 differentiation and the expression of inflammatory cytokines.[327] Moreover, other studies of either pharmacological or genetic inactivation of sterol synthesis (e.g. azoles, CYP51A deletion), showed similar effects in inhibiting RORγ activity.[326]

Cytochrome P450 enzymes play a key role in the regulation of various aspects of cholesterol homeostasis and formation of oxysterols, several of which act as agonists of RORγ(t). CYP27A1 catalyzes the formation of 27-hydroxycholesterol, 7α,27- and 7β,27-dihydroxycholesterol. These oxysterols, which were reported to be preferentially produced by mouse Th17 cells, act as RORγt agonists and stimulate Th17 differentiation.[296] Moreover, oxysterols production and accumulation in various cells and tissues are increased in dyslipidemic conditions such as obesity and hypercholesterolemia.[324,328] Accordingly, hypercholesterolemic mice (ApoE deficient) spontaneously develop atherosclerosis and are susceptible to arthritis, pathological conditions in which Th17 pro-inflammatory cells play a key role in pathogenesis.[329] The lack of conclusive evidences correlating elevated blood levels of cholesterol, Th17- or myeloid cells-driven inflammation, and RORγ activation suggests to explore these patterns as potential targets for cardiovascular, autoimmune and cancer diseases.

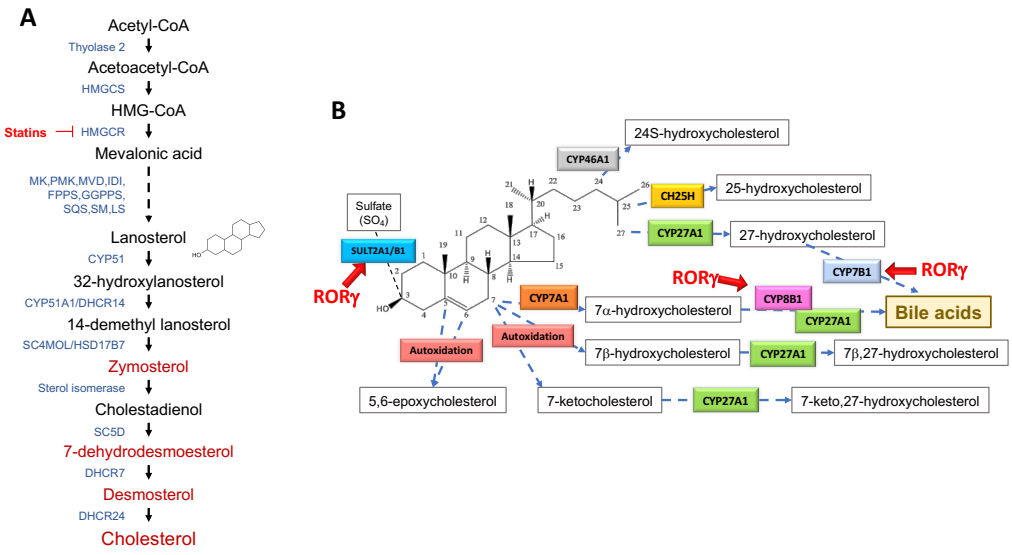

Figure 18. ***RORγ and cholesterol metabolism relationship.*** (A) Representation of cholesterol biosynthesis pathway. Acetyl-CoA molecules are joined and modified through several enzymatic steps in mevalonic acid, first, and then in squalene (mevalonate pathway). The linear structure of squalene is cyclized to give rise to lanosterol, the first molecule with the 'four ring' sterol backbone. The intermediates downstream to lanosterol (lanosterol pathway) have a certain affinity for the RORγ ligand-binding pocket. In red, cholesterol precursors with the highest affinity for RORγ. Statins inhibits HMGCR, the key enzyme of cholesterol biosynthetis. (B) Representation of oxysterols with the highest affinity for RORγ. Cholesterol is highly predisposed to enzymatic or non-enzymatic oxidation to produce oxysterols, biologically active molecules and precursors of bile acids. Cholesterol is also modified by enzymatic sulfonation, increasing its affinity for RORγ. Red arrows indicate the enzyme which expression is regulated by RORγ.[296] (adapted)

In addition to being regulated by sterols, RORγ itself regulates the expression of a number of genes involved in cholesterol transport and sterol metabolism. The hepatic expression of sterol-12α-hydroxylase, CYP8B1, and 7β-hydroxylase, CYP7B1, which are involved in the conversion of cholesterol into bile acids, and in the regulation of hepatic and serum cholesterol levels, have been reported to be directly regulated by RORγ.[326] Moreover, RORγ has been implicated in the circadian regulation of sterol sulfotransferases SULT2A1 and SULT2B1.[330] Regulation of sterol metabolic genes by RORγ may enhance or reduce the synthesis of agonists, thereby modulating RORγ activity and creating a positive or negative feedback loop. Whether the regulation of sterol metabolic genes by RORγ affects the availability of RORγ (inverse) agonists and therefore its activation, needs further studies.

1.7 Obesity, cholesterol metabolism and cancer-related inflammation

Obesity incidence is continuously growing and reaching pandemic proportions; population data link obesity to the increased incidence of cancers.[331–333] Cancer is predicted to overtake heart disease as the leading cause of death across all age groups in the U.S. by 2030, and moreover it is expected an epidemiologic shift from previously known modifiable risk factors, such as smoking and alcohol consumption, toward obesity as the main risk factor for malignancies.[334,335] Obesity also portends worse cancer-specific outcomes after diagnosis in several tumor types including those of the breast, esophagus, colon, prostate, kidney, ovary, uterus, liver, tongue, and others.[332] Additionally, obesity is a poor prognostic factor for both adenocarcinoma and squamous cell carcinoma histologies.[335]

Obesity is defined conventionally as a body mass index (BMI) $\geq$ 30 kg/m^2 [(weight in kg)/(height in m)^2], typically as a consequence of excess adipose tissue.[335] The primary function of white adipose tissue (WAT) is to store energy as lipid and to maintain energy homeostasis. Uncontrolled hyperadiposity as a result of excess caloric intake or reduced caloric expenditure leads to expansion of adipose compartments via hyperplasia and/or adipocyte hypertrophy. These conditions commonly lead to a number of pathophysiologic effects that have been associated with tumor development and progression.[336]

Moreover, in the most recent review of the cancer hallmarks, the alteration of energy metabolism into tumor microenvironment has been highlighted to fuel neoplastic cell growth and division, as well as cancer-related inflammation and immune escape (Fig. 2).[337,338] In this scenario, obesity-associated metabolic syndrome, inducing a systemic deregulation of energy metabolism, could play a fundamental role in cancer development.

Currently, the mechanisms by which obesity is postulated to promote cancer include: (1) increased levels and bioavailability of growth factors, such as insulin and insulin-like growth factor (IGF-1); (2) increased sex steroid hormones such as estrogen and factors affecting their metabolism; (3) altered adipokine levels such as leptin, adiponectin, and visfatin, all originally thought to primarily affect energy balance, but known to have growth, immune, and tumor-regulatory functions; (4) low-grade inflammation and oxidative stress affecting growth-promoting cytokines and immune-modulation; and more recently, (5) altered microbiomes, especially in the intestinal flora.[332]

Obesity is associated with a form of non-resolving inflammation, referred to as chronic low-grade sterile inflammation or "metaflammation" (inflammation in metabolic tissues) and characterized by a modest increase in circulating proinflammatory factors and the absence of clinical signs of inflammation (hence the term 'subclinical inflammation').[11,339]

Obesity-related inflammation is usually simplified by elevated levels of IL-6 and TNFα but a number of evidences highlighted the role of different molecular mechanisms associated with alterations of the immune system, especially of myeloid compartment, and predisposing to cancer devolpment and progression.[335] For instance, elevated circulating levels of TNFα and IL-6 occur in obese women and have been associated with the development and progression of breast tumors.[336] Additionally, systemic inflammation characterized by elevated levels of prostaglandin E2 (PGE2) metabolite in the urine is associated with increased risk of developing postmenopausal breast cancer.[340,341] Free fatty acids (FFAs) released from the entrapped adipocyte and from other sources can activate toll-like receptor (TLR)-4 on the macrophage plasma membrane, leading to increased NF-κB-dependent expression of proinflammatory genes, including TNFα, IL-1β, and cyclooxygenase-2 (COX-2).[342]

Circulating chemokines, including monocyte chemoattractant protein-1 (MCP-1), promote the recruitment of monocytes to adipose tissue, where the cells differentiate and become the so-called adipose tissue macrophages (ATMs). In addition to playing a role in recruitment, MCP-1 was recently found to stimulate ATMs proliferation in situ.[343] Moreover, adipose tissue produces alarmins S100A8/A9, which in turn stimulate ATMs to express high levels of IL-1β via NLRP3 inflammasome. The elevated levels of IL-1β in circulation are able to reach bone marrow niche and to stimulate myelopoiesis.[344,345]
A recent study shows that obesity induces an increase of IL-5 and GM-CSF production by adipose tissue, which in turn promotes the expansion and recruitment of granulocytic subsets that increase breast cancer metastasis.[346]

The increase of adipose tissue associated with obesity correlates with enhanced leptin levels and reductions in adiponectin levels. Leptin regulates appetite and energy balance through activation of a negative feedback loop between the adipose tissue and the CNS. Leptin has been shown to activate cell proliferation and survival in cancer cell lines, including those of the prostate, breast, endometrium, and colon. Leptin can also induce IL-6 production, suggesting that changes in leptin levels may contribute to the systemic inflammation observed in the obese conditions.[347] Further, leptin overexpression can drive the accumulation of immune suppressive MDSCs that promote tumor progression but also provide some protection against the metabolic dysfunction that accompanies obesity.[348] Adiponectin balances many of the pro-tumorigenic effects of leptin; it inhibits cell growth in models of breast, endometrial, prostate, and colon cancers.[347]

Steroid hormones, in particular sex hormones androgens and estrogens, have been shown to be directly linked to obesity, inflammation and cancer development. Androgens are converted in estrogens by aromatase CYP19A1.[349] The rate of conversion of androgens to estrogens is elevated in

postmenopausal women with obesity, and increased levels of estrogens in these women are associated with increased risk of breast cancer.[350] Other evidences indicate that several proinflammatory mediators associated with WAT inflammation, including TNFα, IL-1β, and COX-2–derived prostaglandin E2, can induce aromatase expression in breast adipose tissues.[351] Moreover, steroid hormones are biosynthetic products which derive from cholesterol and oxysterol molecules by several enzymatic steps.[349] The availability of adequate cholesterol substrate is a critical requirement for the optimal steroid hormone production.[349]

Obesity is a condition generally characterized by dyslipidemias, namely abnormal amount in blood triglycerides and cholesterol.[352] The recent growing interest on the comprehension of the mechanisms connecting and balancing obesity, cholesterol, hormones, inflammation, and cancer is opening new and hopeful perspectives for the treatment of both metabolic syndrome and cancer.

1.7.1 Cholesterol metabolism and cancer development

Cholesterol metabolism

Cholesterol is an essential lipid constituent of cell membranes, and is a precursor of steroid hormones, bile acids and Vitamin D.[353] Cholesterol sources are both endogenous and exogenous; in both cases, liver plays a central role in the regulation of cholesterol management (Fig.19).[354]

Exogenous cholesterol is obtained directly from the diet (significant amounts of cholesterol only occur in meat, eggs, and milk products). Free cholesterol, triglycerides and free fatty acids arriving in the intestine in emulsified form, are mixed with bile salts released by the liver and/or gallbladder to form micelles. These particles are able to mediate the absorption of cholesterol and fatty acids across brush border membranes and into enterocytes of the small intestine. While details of the mechanism are still unclear, recent studies have identified NPC1L1 as a putative transporter of cholesterol expressed on the

brush border membranes of enterocytes. In addition to NPC1L1, the ATP binding cassette G5 and G8 proteins (ABCG5/8) appear to negatively regulate cholesterol transport into enterocytes.[355]

Once into the enterocytes, cholesterol and triglycerides are complexed with ApoB48 and a series of other apolipoproteins to form chylomicrons.[356] Chylomicrons are drained by lymphatic vessels (lacteals), reach the blood circulation and, interacting trough lipoprotein lipase on the surface of endothelium, release triglycerides and relatively small quantities of cholesterol to peripheral tissue. Chylomicrons remnants arrive to the liver where they are absorbed via several LDL receptor-related proteins (LRPs).[356] Cholesterol esters and remnant triglycerides are now catabolized, excreted or re-used to form VLDL lipoproteins.[353]

The endogenous cholesterol is synthetized starting from Acetyl-CoA, derived from glycolysis, and from beta-oxidation of fatty acids, through several enzymatic steps and transformation (see Fig.18).[353] The key enzyme of cholesterol metabolism HMGCR (target of statins) is subjected to feedback inhibition by cholesterol itself, directly on the enzymatic activity or at the transcriptional level, through a sophisticated mechanism.[357]

The sterol response element (SRE)-binding proteins (SREBPs) are the downstream inducer of transcription of *HMGCR* gene. SREBP in the endoplasmic reticulum is bound to a second protein, namely SREBP cleavage activating protein (SCAP). This protein is the cholesterol sensor, adopting two different conformations, depending on cholesterol content.[357]

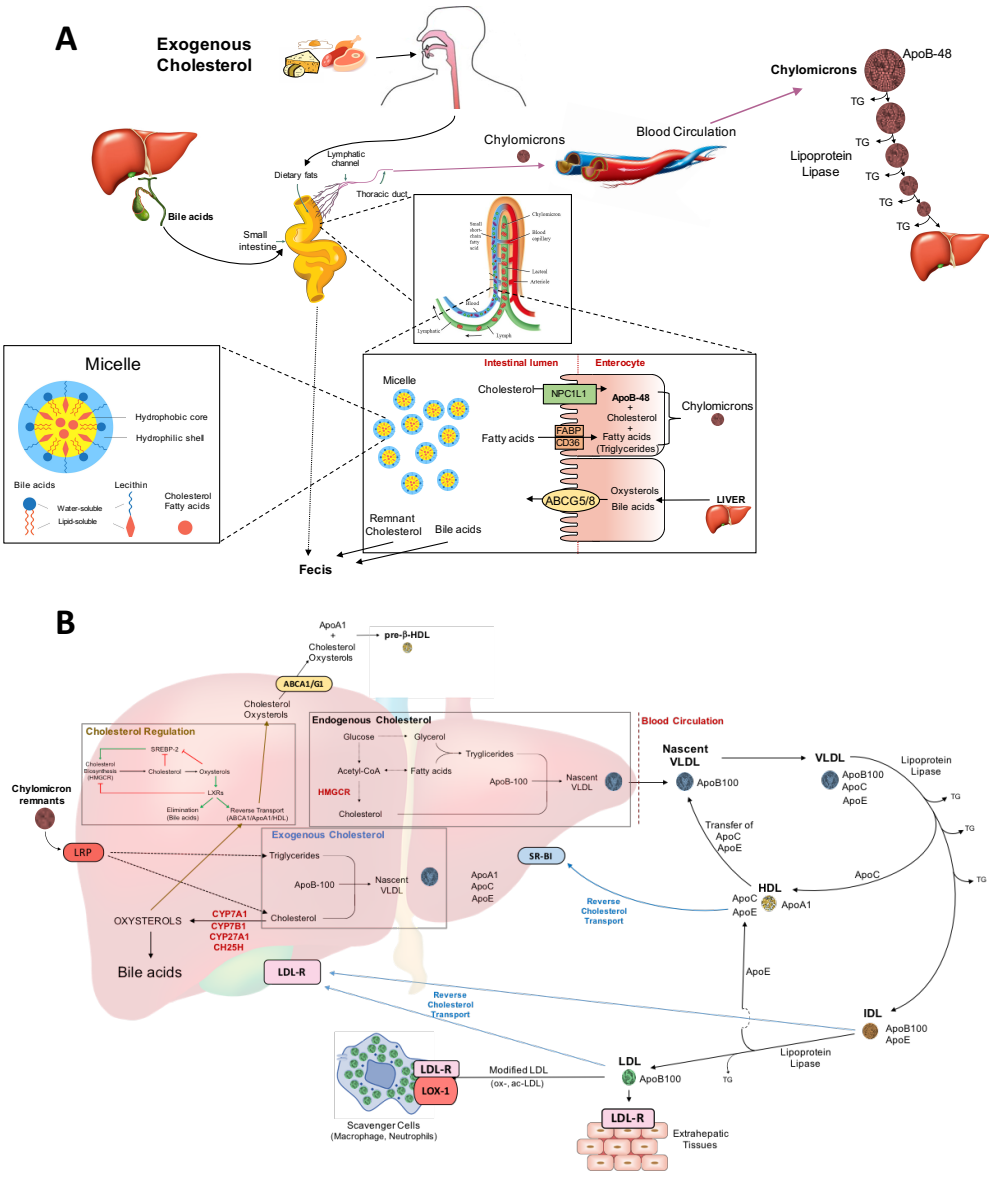

Figure 19. ***Schematic representation of the critical step of cholesterol metabolism***. (A) Dietary cholesterol is absorbed by enterocytes, complexed in chylomicrons, drained by lacteals. Once arrived in blood chylomicrons release via endothelial lipase triacylglycerol, and then reach the liver to re-use or excrete cholesterol. (B) Remnant cholesterol from chylomicrons or endogenous cholesterol synthesized in the liver are complexed with ApoB100 and other apolipoproteins to form VLDL that are secreted into circulation distributing triacylglycerol and cholesterol to peripheral tissue, and thus becoming LDL, rich in cholesterol. Remnants LDL cholesterol is either picked up by the liver and excreted into the intestine in the form of bile acids and eliminated by fecis, or, in case of hypercholesterolemia, captured by macrophages via LDL receptors or in case of modified-LDL by other scavenger receptors (e.g. LOX-1, SR-A1). For abbreviations and more details, see the text.

At high cholesterol levels SCAP bind to a third protein, INSIG. When this ternary complex form, it is rapidly targeted toward proteolytic degradation. At lower cholesterol concentrations, SCAP binds to COPII, another membrane protein, creating vesicles which reach Golgi apparatus. Once SREBP reaches the Golgi, it is cleaved realising the DNA-binding domain of the protein that enters into the nucleus and binds to sterol response elements (SRE) sequence, increasing the expression of HMGCR and also of various other enzymes from cholesterol metabolism (e.g. LDLR).[357]

Like other lipids, cholesterol has low water solubility and therefore requires special mechanisms and vehicles for transport. In the bloodstream, both cholesterol and triacylglycerol are transported within lipoproteins. These contain diverse apolipoproteins which mainly mediate the interaction with target molecules and cells, such as lipoprotein lipase on endothelial cells (ApoC2) or the LDL receptor (ApoE).[354,358]

The cholesterol pool in liver cells derived from endogenous biosynthesis and/or chylomicron remnants, is esterified and packaged together with triacylglycerol and apolipoproteins into particles of very low-density lipoprotein (VLDL), released into the blood circulation to distribute lipid to peripheral tissues. Like chylomicrons, VLDL interacts with lipoprotein lipase and thereby turns into intermediate (IDL) and then low-density lipoprotein (LDL). LDL is taken up by cells in the periphery through endocytosis mediated by the LDL receptor.[358]

Excess cholesterol is sensed into cells through several mechanisms such as LXR nuclear receptors, which are activated by cholesterol precursors and oxysterols, and then induce the transcription of genes involved in reverse cholesterol transport (RCT). Among these, membrane cells efflux transporters ABCA1 and ABCG1 mediate the efflux of cholesterol delivering it to high density lipoprotein (HDL), which then carries it back to the liver via scavenger receptor B1 (SR-BI) to be excreted through bile acids.[353,354,359] Bile acids are

the quantitatively most important derivatives of cholesterol. Aside from their essential role in fat digestion, they are also required to keep hydrophobic constituents of the bile in solution, such as unconjugated bilirubin and cholesterol itself.[360]

Cholesterol in the liver is catabolized through a series of cytochrome P450 enzymes and pathways into oxysterols which are then transformed and conjugated with other molecules (e.g. taurine, glycine) in bile acids. Cholic acid, taurocholate, glycocholate are some examples of bile acids produced through conjugation with glycine.[360]

In macrophages, as for other cell types, uptake of native LDL via the LDL-receptor is regulated by negative feedback, which avoids intracellular cholesterol overload. In contrast to other cells, however, macrophages also have a number of so-called *scavenger receptors*, through which they bind and ingest various kinds of debris. Scavenger receptors do not take up native LDL and are not subject to cholesterol-dependent regulation. Oxidized or otherwise modified LDL, however, does enter via the scavenger receptors, which induces cholesterol overload and transforms the macrophages to foam cells. This is a crucial step in the pathogenesis of atherosclerosis.[361]

The role of cholesterol in cancer cells

Generally, for hypercholesterolemia is intended an imbalance of serum cholesterol counterparts with high levels of total and LDL cholesterol (the 'bad' cholesterol), often accompanied by decreased levels of HDL cholesterol (the 'good' cholesterol).[362]

In absence of genetic predisposition (e.g. LDL receptor deficiency)[363], the most common cause of hypercholesterolemia in industrialized societies is the consumption of high-fat, high-cholesterol diets, known as Western-type diets (WDs).[359] In addition to cardiovascular diseases (CVDs), increased serum cholesterol levels have been reported to be positively correlated with higher

risk of developing cancers, such as colon, rectal, prostate, testicular and breast cancer.[364–366]

Elevated serum LDL levels and an opposed decrease of HDL were associated with increased development of metastasis and with a poor prognosis in initially metastatic colorectal cancer patients and in esophageal squamous cell carcinoma patients.[367–369] Further, two meta-analysis demonstrated that high serum HDL cholesterol levels are associated with increased overall survival and disease-free survival in patients with different solid tumors, proposing HDL levels as a prognostic factor for the outcome of patients.[366,370]

Observations based on cancer models further support the positive correlation between hypercholesterolemia and carcinogenesis. Using the murine MMTV-PyMT breast cancer model, it was found that a high cholesterol diet enhanced the growth and metastasis of tumors.[371] Statins treatment, the gold standard for lowering high cholesterol levels, is associated with reduced cancer-related mortality.[372]

Despite these positive correlations between hypercholesterolemia and carcinogenesis, some epidemiologic observations suggest that no association exists. A recent meta-analysis found that five years of statins treatment had no effect on the risk of cancer-related death.[373] More surprisingly, a clinical study involving patients with bladder cancer found that the tumors became more aggressive in more than half of patients treated with statins.[374] Further, nine cohort studies involving healthy individuals performed in 1980s showed that low cholesterol was associated with colon and lung cancer, yielding the opposite conclusion.[375]

While the contradictory epidemiologic studies fuel the controversy regarding a role for cholesterol in cancer, preclinical studies more consistently suggest involvement. The clearest evidences arise from deregulation of intracellular molecular mechanisms of cholesterol homeostasis in cancer cells, more than serum cholesterol.[376]

A gene expression profiling study identified 7 cholesterol synthesis' genes upregulated in different cancers (sarcoma, acute myeloid leukemia, melanoma). The increased activity of these genes was directly correlated with decreased patient survival.[376] A direct involvement in the regulation of cholesterol biosynthesis in cancer cells was shown by PI3K/AKT/mTOR signalling, also responsible of intracellular oncogenic signals.[376,377] Further, in several cancer types, elevated mitochondrial cholesterol levels induced resistance to apoptotic signals.[378]

Another example of cholesterol homeostasis' gene deregulated in cancer cells is ABCA1 transporter which mediates cholesterol efflux. Decreased activity of ABCA1 promoted cancer cell survival by increasing mitochondrial cholesterol levels. ABCA1 activity is reduced in colorectal cancer cells, either through loss-of-function mutations or gene downregulation.[379]

Cholesterol metabolites (steroids and oxysterols) have also been associated with the development of various cancers.[376,380] Oxysterols play an essential role in cholesterol homeostasis, inhibiting cholesterol synthesis and enhancing its export by activating LXRs.[381] Certain oxysterols have shown anti-proliferative effects on various cancer types.[376] However, 27-hydroxycholesterol (27-OHC) has recently been shown to act as an estrogen receptor agonist in breast cancer, inducing tumor growth and metastasis.[380] In breast cancer, decreased expression of CYP7B1 triggers accumulation of 27-OHC.[382]

As an important component of the cell membrane, cholesterol may be closely related to membrane receptors through which cholesterol could directly activate oncogenic signalling, such as the Hedgehog pathway and Wnt/β-catenin pathway.[383] Cholesterol is a vital structure of lipid rafts, necessary platforms for cellular transduction and cancer signalling. Changes in membrane cholesterol and cholesterol-rich membranes have been shown to affect cancer progression and invasion, also providing a signal transduction

platform for oncogenic signalling pathways. AKT is the major example of intracellular transducer interacting with membrane lipid components.[384]
Overall, hypercholesterolemia may be an important factor in some types of cancer, such as breast and prostate cancer, which is supported by clinical and preclinical studies. However, because of the discrepant observations, another level of interaction should be considered, such as the tissue of origin of cancer and/or the immune response influence.

1.7.2 Cholesterol metabolism and tumor-associated myeloid cells

The association and interaction of hypercholesterolemia, inflammation and myeloid cells is widely characterized in the context of cardiovascular diseases and atherosclerosis.[359,385,386] Indeed, the accumulation of absorbed LDL cholesterol into the intima of arterial wall generates inflammatory signals (e.g. IL-6, TNFα, IL-1β) inducing the increase in the hematopoietic output and the recruitment of myeloid cells, especially monocytes which infiltrate the artery wall and differentiate into macrophages. Herein, macrophages up-take massive quantities of LDL and oxidized LDL (ox-LDL) through several scavenger receptors and, thus, transforming in foam cells and contributing to atherosclerotic plaque formation and to subsequent pathological processes (Fig.20).[359,387]
It has been well described that hypercholesterolemia is associated with a prominent expansion of immature monocytes, marked as Ly6C^{hi} monocytes, which share some phenotypical features of monocytic Ly6C^{hi} myeloid-derived suppressor cells.[388] Once infiltrated into tissue, monocytes differentiate into macrophages, which, beyond their activity of 'LDL scavenging' cells, exert dynamic inflammatory patterns, ranging between M1 and M2 activities, at different stages and in different tissue sites along the pathological process.[389]

Moreover, it was demonstrated that hypercholesterolemia, specifically elevated LDL cholesterol, promotes hematopoietic stem cells (HSCs) proliferation, leading to increased production of GMP in the blood, as well as myelomonocytic differentiation, resulting in increased frequency of monocytes and neutrophils in the bone marrow and blood. In addition, elevated LDL cholesterol generates an increase of plasma levels of CXCL12 which elevates the mobilization of $CXCR4^{+}$ bone marrow progenitor cells. These effects of LDL appear to be counteracted by HDL.[390,391]

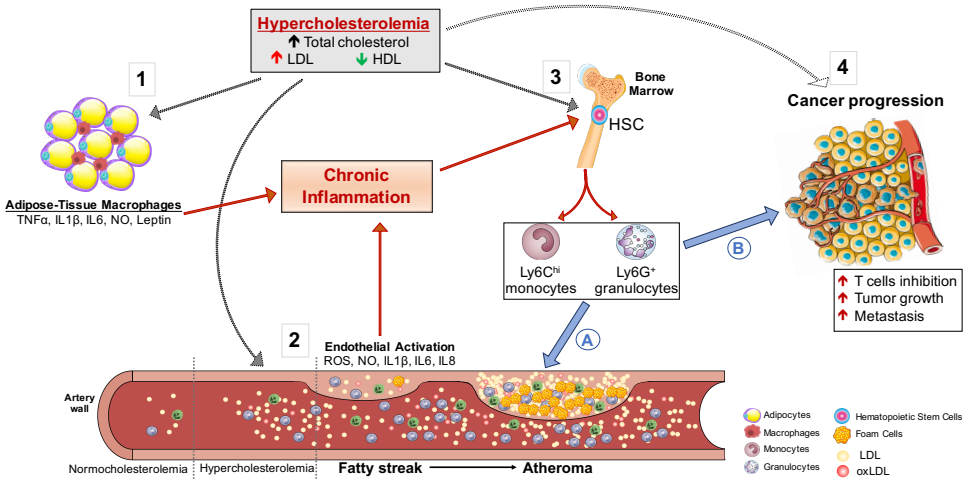

Figure 20. ***Schematic representation of how hypercholesterolemia influences the functions of myeloid cells in cancer and atherosclerosis.*** Increased levels of serum LDL cholesterol and decreased HDL, contribute to: 1) hyperplasia and hypertrophy of adipose tissue, resulting in recruitment of macrophages and production of inflammatory factors (e.g. TNFα, IL-1β, IL-6); 2) activation of endothelial cells with the production of nitric oxide (NO) and cytokines; 3) proliferation, mobilization and differentiation of hematopoietic/myelopoietic progenitor cells; 4) fuel tumor microenvironment and cancerous cells to proliferate. The results is a chronic inflammatory state with an expansion of myeloid cells, which: A) differentiating in macrophages and scavenging LDL and ox-LDL contribute to formation (M1-like) and stabilization (M2-like) of atherosclerotic lesions; B) skewing to immunosuppressive phenotype (MDSCs), that inhibits cytotoxic T cells and promote tumor growth and metastasis.

Recent evidences demonstrated that obesity in both mice and human increases the frequency of MDSCs.[348,392] In particular, obese mice, both genetic (leptin deficient mice, *ob/ob*) and high-fat diet-induced models, together with higher frequency of MDSCs, showed an increased promotion of tumor growth and

metastasis in different cancer models (breast, kidney, melanoma, prostate). Although some molecular mechanisms have been defined to drive obesity/MDSCs regulation (e.g. IL5, GM-CSF, CCL2/MCP-1, leptin), the role of metabolic components influencing this scenario remain still poor clarified.[346,348,393–395]

In a recent characterization of granulocytic subsets in cancer patients, it has been unravelled that LOX-1, scavenger receptor for oxidized LDL, is a specific marker which distinguish the immunosuppressive PMN-MDSC subset from mature and pro-inflammatory neutrophils.[396]

Oxysterols, biological active cholesterol derivatives, are highly represented in obesity and hypercholesterolemia.[324,328] In the most common estrogen receptor-positive breast cancer, CYP27A1 enzyme converts cholesterol in 27-hydroxycholesterol (27-OHC), which in turn, promotes primary tumor growth through the binding of estrogen receptor and increases metastatic process via liver X receptor (LXR).[382,397] Recently, it has been shown that 27-OHC acts directly on HSC via ERα, leading to increased cell mobilization.[398]
25-hydroxycholesterol (25-OHC) is generated directly from cholesterol by CH25H, and it is further converted into 7α,25-dihydroxycholesterol (7α,25-OHC) by CYP7B1. Expression of *Ch25h* is upregulated in macrophages and dendritic cells after stimulation with various inflammatory mediators and, its product 25-OHC, in turn, augments the production of inflammatory cytokines in macrophages.[399] Moreover, human monocytes treated in vitro with constant levels of oxysterols (i.e. 27-hydroxycholesterol) showed a prominent macrophage differentiation and polarization toward an M2 immunomodulatory functional phenotype.[400]

As already mentioned, 25-, 27-OHC as well as cholesterol precursors and other oxysterols interact with LXR nuclear receptors. LXRα and LXRβ are two isoforms involved in cholesterol homeostasis, regulating synthesis, uptake

and efflux of cholesterol. LXRs and their ligands exert an anti-inflammatory role in macrophages and dendritic cells (DCs).[401]
LXRs/oxysterols signalling affects also tumor-associated immune cells. Indeed, oxysterols produced by tumor cells impair antigen presentation by inhibiting chemokine receptor 7 (CCR7) expression on DCs.[402] Oxysterols promote tumor immunosuppression also by recruiting pro-tumor neutrophils in a LXRs-independent, C-C motif chemokine receptor 2 (CXCR2)-dependent manner.[403]

The epigenetic reprogramming of innate immune cells induces a long-lasting pro-inflammatory phenotype, a phenomenon known as 'innate immune memory' or 'trained immunity'. Emerging evidences indicate that not only pathogens, but also hypercholesterolemia and ox-LDL are capable to induce 'trained immunity' via epigenetic modification of monocytes, and that this imprinting persists after treatment with statins despite normalization of circulating cholesterol.[404,405]

Mice deficient in ABC transporters (ABCA1/ABCG1) show hyper-proliferation of hematopoietic progenitor cells, resulting in monocytosis and neutrophilia and activation of inflammatory gene expression.[406] The anti-inflammatory capacities of ABCA1 and ABCG1 are probably because of their role in cholesterol efflux.[407]
Hitherto, the role of reverse cholesterol transport in cancer-related immune response is controversial. ApoA1 and HDL were reported to exert an antitumor role in B16 melanoma mouse model, by skewing TAMs toward an M1 phenotype.[408]
In contrast, the genetic absence of ABCG1, which, together with ABCA1, mediated cholesterol efflux and complexing it with ApoA1 in HDL –thus reducing reverse cholesterol transport–, is associated with an anti-tumor M1 phenotype of macrophages, increased NF-κB activation and inhibition of tumor growth in high-cholesterol diet-fed mice.[409]

These findings have been recently confirmed by another study where the block of cholesterol efflux in myeloid cells, by deletion of ABCA1 gene or ABCA1 and ABCG1 genes, resulted in a significant reduction of tumor growth, associated with a reduction of MDSCs.[410] More recently, in line with these evidences, it has been identified that membrane cholesterol efflux of macrophages via ABCA1/G1 enhances their pro-tumoral activation in response to the M2 polarizing IL-4 stimulus.[411]

Characterizing the obese state by systemic and tissue-specific measures could provide more reliable identification of high-risk populations and a mechanistically informed approach for the development of effective prevention and treatment strategies that target cancers. This thesis aims to discuss the mechanisms through which adipose tissue inflammation fosters a tumor-supportive microenvironment through local and systemic effects.

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Chapter 2

2. Outline of the thesis

During the last decade cancer immunotherapy has witnessed remarkable advances in the treatment of a number of malignancies. However, in some cancers and some patients, the success of cancer immunotherapy has been limited to partial or modest clinical responses, as well as to severe adverse effects. One of the great challenges to improve the efficacy of immunotherapy is deciphering the molecular network controlling cancer-related immune alterations.

Cancer growth provides an immunological stress perceived by hematopietic progenitors and resulting in modifications over the magnitude and composition of hematopoietic output ("emergency" hematopoiesis). Myeloid cells, in particular, abundantly expand in tumor bearers and infiltrate solid cancers where they are exposed to microenvironmental signals (e.g. cytokines, growth factors) and micro-pathophysiological conditions (e.g. hypoxia, pH, glucose levels) that reprogram their genetic programs towards a tumor-promoting phenotype.

New evidence indicates that the metabolic instruction of immune response (immunometabolism) relates to the influence of metabolic pathways, expressed either by immune cells or systemically, on the differentiation and functions of immune cells. This metabolic/immune interplay is strongly affected by nutrition, microbioma metabolites as well as cancer-driven metabolic alterations.

Although dynamic changes in myeloid cell heterogeneity and functions have been reported to parallel tumor progression, a large gap remains in our understanding of the molecular inflammatory, epigenetic and metabolic pathways guiding this cancer-driven "emergency" myelopoiesis.

Tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) are two major myeloid populations associated with cancer development and are considered to have a major impact on the orchestration

of cancer-related inflammation, promoting the construction of a pro-tumor microenvironment that unleashes tumor growth and metastasis formation.

Tumor-associated myeloid cells result from pathological expansions, differentiation and mobilization of bone marrow hematopoietic progenitors in response to inflammatory cytokines and growth factors (e.g. TGF-β, VEGF, IL-1β, IL-6, CSFs) secreted by growing cancers. Myeloid cell plasticity is exemplified in the M1 vs. M2 extremes of macrophage polarization, which respectively express pro- vs. anti- inflammatory functions and whose balance is a main determinant of cancer growth. Epigenetic reprogramming of regions encoding for receptors and transcription factors involved in myelopoiesis represents a key event in the pathological myelopoiesis of cancer bearers. While in last years, several advances have been achieved in the understanding of the molecular players regulating this pathologic process, several open questions remain. Hence, the common thread of our works is to decipher the driving mechanisms of differentiation and recruitment of myeloid cells in cancer disease.

We previously reported that, during cancer progression, massive nuclear accumulation of p50 NF-κB drives the M2 polarization of tumor-associated macrophages (TAM), which share common gene signature with monocytic-MDSC (Biswas KS et al Blood 2006). p50 NF-κB is a key intracellular transcription factor regulating positively (heterodimer p50/p65) or negatively (homodimer p50/p50) the expression of a multitude of genes involved in immunity and inflammation. However, little is still known about the involvement of p50 NF-κB in MDSCs expansion and functions. Hence, we investigated the role of p50 NF-κB in differentiation, recruitment and immunosuppressive functions of M-MDSCs.

Another intriguing aspect we investigated is the interplay between immunity and metabolism (immunometabolism) in cancer berarers, pointing to obesity-related metabolic events as potential inducers of inflammation and

myelopoietic alterations. Our lab has recently identified RORC1/RORγ as a pivotal transcription factor driving cancer-associated emergency myelopoiesis (Strauss L. et al Cancer Cell 2015). RORγ is a nuclear receptor whose activity has been recently described at the molecular level to be enhanced by binding of cholesterol derivatives. However, the relevance of this molecular interaction has not been yet investigated, neither in tumor biology nor in innate immunity. Furthermore, high circulating cholesterol levels are known to promote expansion of the myelomonocytic lineage. Given this premise, we determined the impact of cholesterol metabolism on cancer-related inflammation and emergency myelopoiesis observed in cancer bearers.

Chapter 3

Tumor-derived prostaglandin E2 promotes p50 NF-κB-dependent differentiation of monocytic MDSCs

Submitted for publication

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Summary

Tumor-derived PGE2 induces nuclear p50 NF-κB, that epigenetically reprograms the response of monocytic cells to IFNγ towards an immunosuppressive ($NOS2^{high}/TNF\alpha^{low}$) phenotype. Inhibition of the PGE2/p50 axis in vivo prevents the differentiation of suppressive MDSCs, retrieving the anticancer properties of IFNγ.

Abstract

Myeloid-derived suppressor cells (MDSCs) include immature monocytic (M-MDSCs) and granulocytic (PMN-MDSCs) cells that share the ability to suppress adaptive immunity and hinder the effectiveness of anti-cancer treatments. Of note, in response to interferon-γ (IFNγ) M-MDSCs release the tumor-promoting and immunosuppressive molecule nitric oxide (NO) whereas, in contrast, macrophages largely express anti-tumor properties. Investigating these opposing activities, we found that tumor-derived prostaglandin E2 (PGE2) induces nuclear accumulation of p50 NF-κB in M-MDSCs, diverting their response to IFNγ towards NO-mediated immunosuppression and reducing TNFα expression. At genome level, p50 NF-κB promoted binding of STAT1 to regulatory regions of selected IFNγ-dependent genes, including inducible nitric oxide synthase (*Nos2*). In agreement, ablation of p50, as well as the pharmacological inhibition of either the PGE2 receptor EP2 or NO production, reprogrammed M-MDSCs towards an $NOS2^{high}/TNF\alpha^{low}$ phenotype, restoring the *in vivo* antitumor activity of IFNγ. Our results indicate that inhibition of the PGE2/p50/NO axis prevents MDSCs suppressive functions and retrieve the efficacy of anticancer immunotherapy.

Introduction

Microenvironmental signals are sensed by myeloid cells through cytokine and/or innate immune receptors, whose differential engagement leads different polarized programs.[1,2] This functional plasticity is exemplified in the M1 vs M2 extremes of macrophage polarization[1–3] and has major impacts in the orchestration of cancer-related inflammation, immunosuppression[4–6] and clinical responses.[2,7] Of note, a time-dependent M1 to M2 transcriptional reprogramming of myeloid cells was reported in response to prolonged exposure to Toll-like receptors (TLR) ligands (e.g. lipopolisaccharide) (i.e. LPS tolerance)[8,9], which requires the nuclear accumulation of p50 NF-κB and results in altered responsiveness to polarizing cytokines, such as IFNγ and IL-4.[8] Cancer fuels myeloid cells heterogeneity also by sustaining altered myelopoiesis[10–13] that supports resistance to anticancer immunotherapy.[14] Of relevance, divergent outcomes have been reported in response to immunotherapy, either with cytokines[15–17] or checkpoint inhibitors.[18,19] In particular, IFNγ, originally termed "macrophage activating factor" [20], was paradoxically shown to be equally necessary for melanoma development and rejection.[17] Indeed, IFNγ has pleiotropic and contrasting effects in the tumor microenvironment. On one hand it exerts anti-angiogenic activities, suppression of pro-tumorigenic properties, enhancement of tumoricidal activity of macrophages and of processing and presentation of tumor antigens to T lymphocytes.[15,21] On the other hand, IFNγ promotes immunosuppressive functions in myeloid cells[22] inducing expression of the immunosuppressive enzymes indoleamine 2,3 dioxygenase (*Ido*) and inducible nitric oxide synthase (*Nos2*), involved in the catabolism of L-tryptophan[23] and L-arginine[12], as well as the ligand programmed-death receptor-ligand 1 (PD-L1, B7-H1)[24], whose interaction with the co-inhibitory receptor Programmed Death-1 (PD-1) can be blocked to restore antitumor immunity.[25] Mixed responses to IFNγ were also reported in different human malignancies.[15,16,26]

We have previously reported that accumulation of nuclear p50 NF-κB plays an essential role in the M2 orientation of tumor-associated macrophages (TAM)[8,10], which share common myeloid precursors and a similar M2-like gene signature with M-MDSC.[10] Here, we investigated how tumor-derived signals affect transcriptional activities and myeloid cell functions in response to immune stimulatory cytokines and the impact of these events on cytokine-mediated cancer immunotherapy.

Results

p50 NF-κB controls both M-MDSC suppressive functions and differentiation of myeloid precursors.

Nuclear accumulation of p50 NF-κB, as occurring in TAMs and LPS-tolerant macrophages, impairs both M1 polarization and antitumor activities.[8,10] Similar to TAMs, confocal microscopy analysis showed selective nuclear accumulation of p50 NF-κB in splenic and tumor-infiltrating monocytic (M)–MDSCs ($CD11b^+Ly6G^-Ly6C^+$ cells) from fibrosarcoma (MN/MCA1) bearing mice, at advanced stages of tumor development (day 21; Fig.1A, Supplementary Fig.1A). As inhibition of T cells proliferation validated their suppressive activity (Supplementary Fig.1B), we explored whether p50 NF-κB could be actually involved in accumulation of suppressive M-MDSC occurring during tumor development. According to our previous findings[10,27], C57BL/6 $p50^{-/-}$ mice displayed both reduced tumor growth and metastasis formation, as compared to WT mice (Fig.1B), but, paradoxically, displayed increased accumulation of splenic myeloid cells expressing the M-MDSCs phenotype $CD11b^+Ly6G^-Ly6C^+$ (Fig.1C and Supplementary Fig.1C), which coincided temporally with the nuclear accumulation of p50 (21 days) (Fig.1A). Hence, we analyzed whether these cells expressed genes encoding for immunosuppressive activities.[28,29] Noteworthy, in the absence of p50, magnetically sorted splenic $CD11b^+Ly6G^-Ly6C^+$ cells (Supplementary

Fig.1D) showed a drastic reduction of both *Nos2* gene expression (Fig.1D) and NO production (Fig.1E), in response to IFNγ treatment. Accordingly, p50 depletion impaired in vivo NO production in tumor tissues (Supplementary Fig.1E). In contrast, expression of both arginase I (*Arg1*)[22] and indoleamine-2,3-dioxygenase (*Ido1*), Programmed Cell Death 1 Ligand 1 (*Pd- l1*) were poorly affected (data not shown) and not further investigated. To establish the actual role of p50 NF-κB in the suppressive activity of M- MDSCs, splenic $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells isolated from tumor bearing mice were activated with IFNγ and tested for antigen-specific suppressive activity. In keeping with the data above, $p50^{-/-}$ M-MDSC displayed reduced suppressive activity (Fig.1F, left), and NO levels in the co-culture supernatants (Fig.1F, right). The M-MDSC suppressive activity was NO-dependent, as addition of the NOS inhibitor L-NMMA to the co-culture abolished both T cell suppression (Fig.1F, left) and NO production (Fig.1F, right) and by the similar death rate of T cells observed in the presence or absence of MDSCs (data not shown). These data suggested that the NO-dependent suppressive capacity of M-MDSCs in response to IFNγ, relies on p50 nuclear accumulation.

We next determined whether the nuclear accumulation of p50 was also observable in blood M-MDSCs[22,30] from colorectal carcinoma patients (CRC). Compared to healthy donors, the frequency of M-MDSC ($CD33^{+}CD14^{+}HLA\text{-}DR^{low/-}$) was increased (Fig.1G, left) and these cells showed higher nuclear levels of p50 (Fig.1G, center and right), along with increased NOS2 (Fig.1H, Supplementary Fig.2A). To confirm the role of p50 in human myeloid cells, circulating $CD14^{+}HLA\text{-}DR^{+}$ mononuclear cells from healthy donors were transfected with a small interfering RNA against p50 (p50 siRNA). Silencing of p50 (Supplementary Fig.2B), significantly inhibited NOS2 mRNA expression in response to IFNγ treatment (Supplementary Fig.2C).

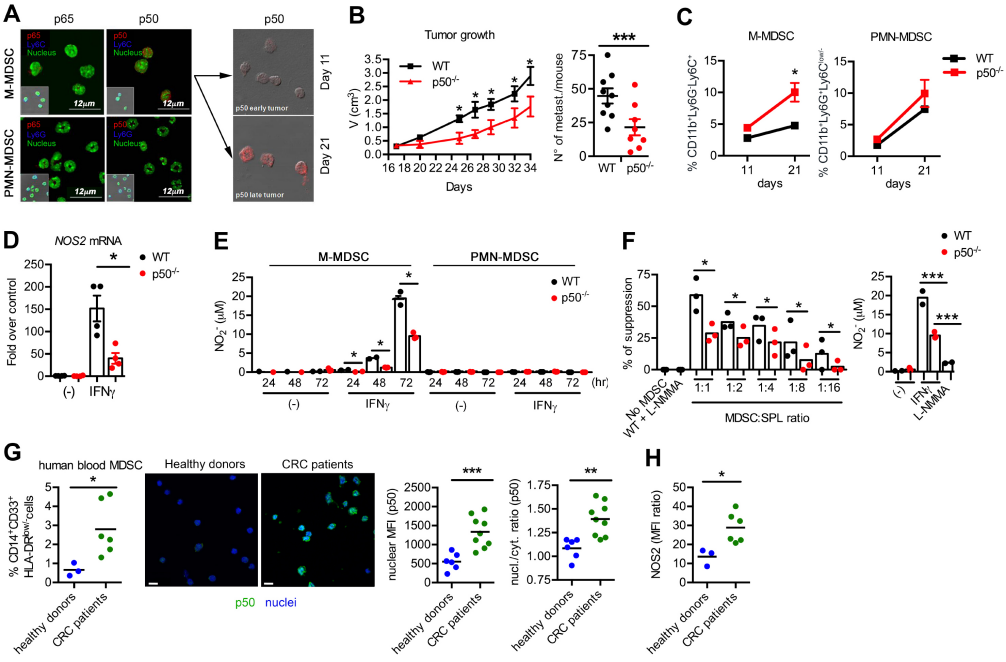

Figure 1. ***Role of p50 NF-κB in IFNγ-induced NO-dependent suppressive activity of M-MDSC.*** (A) Confocal microscopy showing selective up-regulation of nuclear p50 NF-κB in the splenic M-MDSC subset during tumor growth (day 21). (B) Inhibition of both tumor growth (MN/MCA1) (data shown are mean±SD *p<0.01 by two-tailed two-way ANOVA, n=9 WT and n= 8 $p50^{-/-}$ mice) and metastasis formation in p50 NF-κB–deficient mice (data shown are mean±SEM ***p<0.001 by two-tailed t-test, n=9 WT and n= 8 $p50^{-/-}$ mice). (C) Increased number of M-MDSC ($CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells) in the spleen of $p50^{-/-}$ tumor-bearing mice (data shown are mean±SEM *p<0.05 by two-tailed two-way ANOVA, n=5 WT and n= 5 $p50^{-/-}$ mice). (D) Lack of p50 in M-MDSC impairs Nos2 mRNA expression in response to IFNγ data shown are mean±SEM ***p<0.001, by two-tailed t-test n=4 WT and n=4 $p50^{-/-}$ biological replicates each run in triplicate). (E) Reduced IFNγ-mediated NO production in $p50^{-/-}$ M-MDSC (data shown are from a representative experiment *p<0.05 by two-tailed t-test, n=3 WT and n=3 $p50^{-/-}$ technical replicates). (F) Left, decreased antigen-specific suppressive activity of $p50^{-/-}$ M-MDSC in response to IFNγ, at different MDSC:OT1 splenocytes ratio (data shown are from a representative experiment *p<0.05, ***p<0.001 by two-tailed t-test, n=3 WT and n=3 $p50^{-/-}$ technical replicates). Right, decreased NO production in the co-culture supernatants of IFNγ-treated $p50^{-/-}$ M-MDSC, as compared to WT M-MDSC. L-NMMA, nitric oxide synthase inhibitor. (data shown are from a representative experiment *p<0.05, ***p<0.001 by two-tailed t-test, n=2 technical replicates). (G), Left, Increased number of $CD33^{+}CD14^{+}HLA\text{-}DR^{low/-}$ cells in blood from CRC patients (*p<0.05 two-tailed t-test; n=3 healthy donors n=6 CRC patients). Center, Representative confocal microscopy on nuclear p50 in peripheral blood $CD14^{+}HLA\text{-}DR^{low/-}$ cells from CRC patients, as compared to peripheral blood mononuclear $CD14^{+}$ cells from healthy donors (scale bars are 10μm). Right, MFI of nuclear and nuclear vs cytoplasmic ratio of p50 in $CD14^{+}HLA\text{-}DR^{low/-}$ cells from CRC patients (**p<0.01; ***p<0.001 by two-tailed t-test, n=6 healthy donors n=9 CRC patients). (H), NOS2 expression from $CD14^{+}$ and $CD14^{+}HLA\text{-}DR^{low/-}CD33^{++}$ cells in blood from healthy donors and CRC patients (*p<0.05 by two-tailed t-test n=3 healthy donors n=6 CRC patients).

The predominant increase in the M-MDSCs subset within the spleen of tumor-bearing mice might result from its accelerated proliferation rate or preferential skewing of precursors. To study the implication of p50 in normal hematopoiesis, we evaluated the BM for composition in hematopoietic stem cells (HSC), along with their proliferation and differentiation potential. HSCs are immunophenotypically defined as cells lacking lineage specific markers (Lin^-) but expressing Sca-1 and c-Kit ($Lin^-Sca\text{-}1^+c\text{-}kit^+$, LSK), while the methylcellulose-based colony-forming unit (CFU) assay allows to quantify their derived progeny in vitro. We observed a significant enrichment of LSK progenitors ($Lin^-Sca\text{-}1^+c\text{-}kit^+$) in BM of $p50^{-/-}$ mice (Fig.2A-B), associated with higher clonogenic potential of $p50^{-/-}$ HSCs in both GM-CFU and M-CFU progenitors, but not in G-CFU (Fig.2C). These results indicate that lack of p50 results in a preferential skewing of HSC towards the monocytic branch at the myeloid/granulocytic bifurcation. Accordingly, histopathological analysis showed a severe impairment of terminal granulopoiesis in the BM of $p50^{-/-}$ mice, which was associated with an increased number of immature myeloid precursors (Fig.2D). Consistently, the hematopoietic parenchyma of $p50^{-/-}$ mice was characterized by the marked reduction of mature segmented granulocytes and by the expansion of myeloid blasts showing abnormal interstitial localization and aggregation in clusters (Fig.2D). Monocytic differentiation (Fig.2E), as well as erythropoiesis and megakaryocitopoiesis (data not shown) was preserved in the BM of $p50^{-/-}$ mice. Consistent with BM histopathology, flow cytometry revealed a neat decrease in the $Gr1^+c\text{-}Kit^-$ granulocytic population and a paralleled increase in $Gr1^+c\text{-}kit^+$ myeloblasts in $p50^{-/-}$, as compared to control WT BM (Fig.2F). Accordingly, blood granulocytes from $p50^{-/-}$ mice were enriched in immature and blast-like forms compared with circulating granulocytes from WT controls (Fig.2G).

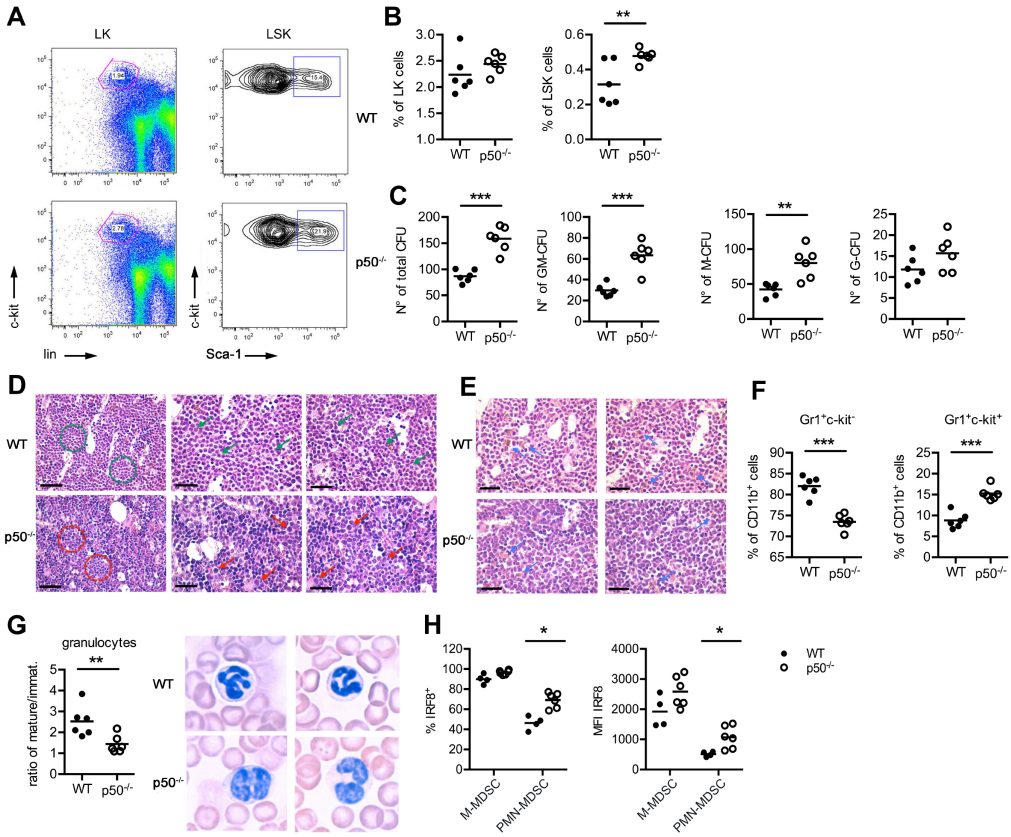

Figure 2. ***p50 deficiency in the BM stroma associates with enhanced myelopoiesis***. BM cells from WT and p50$^{-/-}$ mice were stained with mAb to c- Kit, Sca-1 and lineage-specific markers (CD3, CD11b, CD11c, Gr-1, B220, ter119). LSK progenitors were defined as Sca1^{+} cells within the gate of lin^{-}c Kit^{+} cells. (A) Representative FACS analysis of LSK progenitors in WT and p50$^{-/-}$ BM. (B) Collective data showing that the fraction of LSK progenitors is increased in p50$^{-/-}$ mice (*p<0.05 by two-tailed t-test n=6 WT and n=6 p50$^{-/-}$ mice). (C) Hematopoiesis was analyzed using a clonogenic colony culture assay. The relative number of total BM-CFU, GM-CFU and M-CFU myeloid colonies was significantly increased in p50$^{-/-}$ mice compared to the WT counterpart. (**p<0.01,***p<0.001 by two-tailed t-test n=6 WT and n=6 p50$^{-/-}$ mice). (D) Histopathological analysis (H&E) of the BM of p50$^{-/-}$ and control WT mice. The BM hematopoietic parenchyma of p50$^{-/-}$ mice is characterized by the marked impairment of terminal granulopoiesis and by the increase in the density of immature myeloid precursors and blasts that show aggregation in clusters. Red arrows indicate myeloid blasts. Original magnifications: upper panels, scale bars are 100µm; lower panels, scale bars are 50µm. (E) Monocytic differentiation is preserved in the BM of p50$^{-/-}$ mice, as testified by the presence of cells with mature monocytic morphology and by the normal density of hemosiderin-laden macrophages. scale bars are 50µm. (F) Fraction of mature Gr-1^{+}c-kit^{-} and immature Gr-1^{+}c-Kit^{+} granulocytes in BM from WT and p50$^{-/-}$ mice. The fraction of immature granulocytes is increased in the absence of p50 in comparison to the WT counterpart (***p<0.001 by two- tailed t-test n=6 WT and n=6 p50$^{-/-}$ mice). (G) Morphologic analysis (left) and quantification (right) of Giemsa-stained peripheral blood smears from 12 weeks-old p50$^{-/-}$ and WT mice showing that circulating granulocytes from p50$^{-/-}$ mice are enriched in immature and blast-like forms (*p<0.05 by two-tailed t- test n=6 WT and n=6 p50$^{-/-}$ mice). (H) Cytofluorimetric analysis of IRF8 in M- (CD11b^{+}Ly6G^{-}Ly6C^{+} cells) and PMN-MDSC (CD11b^{+}Ly6G^{+}Ly6C$^{low/-}$ cells). (*p<0.05 by two-tailed t-test WT n=4, p50$^{-/-}$ n=6 mice).

Overall, these results confirm previous observations[31] and demonstrate that p50 NF-κB deficiency is associated with defective granulocytic differentiation in favor of the monocytic lineage. Of relevance cytofluorimetric analysis of tumor infiltrating MDSC revealed a significant increase of the negative regulator of MDSC expansion IRF8[32] expression by the p50-deficient PMN-MDSC subset ($CD11b^{+}Ly6G^{+}$ cells) (Fig.2H), which also acts as promoter of terminal monocytic maturation.[33] This result is in agreement with the preferential expansion of non-suppressive $Ly6C^{+}$ monocytic cells (M-MDSC-like) observed in tumor bearing p50-deficient mice.

Hematopoietic deletion of p50 NF-κB abolishes NO production by M-MDSCs and impairs metastasis formation

To establish whether the expression of p50 NF-κB in the hematopoietic compartment was uniquely responsible for the suppressive activity of M-MDSCs, we generated chimeric mice by transplanting WT or $p50^{-/-}$ BM cells into sub-lethally irradiated WT and $p50^{-/-}$ mice, which were subsequently implanted with the MN/MCA1 fibrosarcoma. Splenic $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells isolated from mice receiving $p50^{-/-}$ BM cells, and subsequently activated with IFNγ, were strongly impaired in their capacity to suppress both T cell proliferation and NO production (Fig.3A). To investigate further the in vivo importance of p50 in the hematopoietic compartment, we used the deleter strain B6.Cg-Tg(Tek-Cre)1Ywa to ablate p50 in all hematopoietic lineage cells ($p50^{Tie2}$ mice).[34] In agreement with Figure 1A and B, we observed inhibition of lung metastasis formation in tumor bearing $p50^{Tie2}$ mice, which was paralleled by an increased number of splenic and tumor-infiltrating $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells, while an increase of $CD11b^{+}Ly6C^{low/-}Ly6G^{+}$ cells was observed at the tumor site (Fig.3B and Supplementary Fig.3A). Further, splenic and tumor-associated $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells from $p50^{Tie2}$ mice (Fig.3C) showed decreased NOS2 but higher TNFα expression, associated with increased number of both splenic and tumor-infiltrating IFNγ

expressing CD4$^+$ and CD8$^+$ T cells (Fig.3D), these latter also expressing high levels of the cytotoxic molecule Granzyme B (GZMB). Of note, transplantation of B16 melanoma cells[35] in p50^{Tie2} mice fully recapitulated the phenotype of the MN-MCA1 fibrosarcoma, both in terms of tumor growth (Supplementary Fig.3B) and accumulation of splenic and tumor-infiltrating immune cells (Supplementary Fig.3C-E).

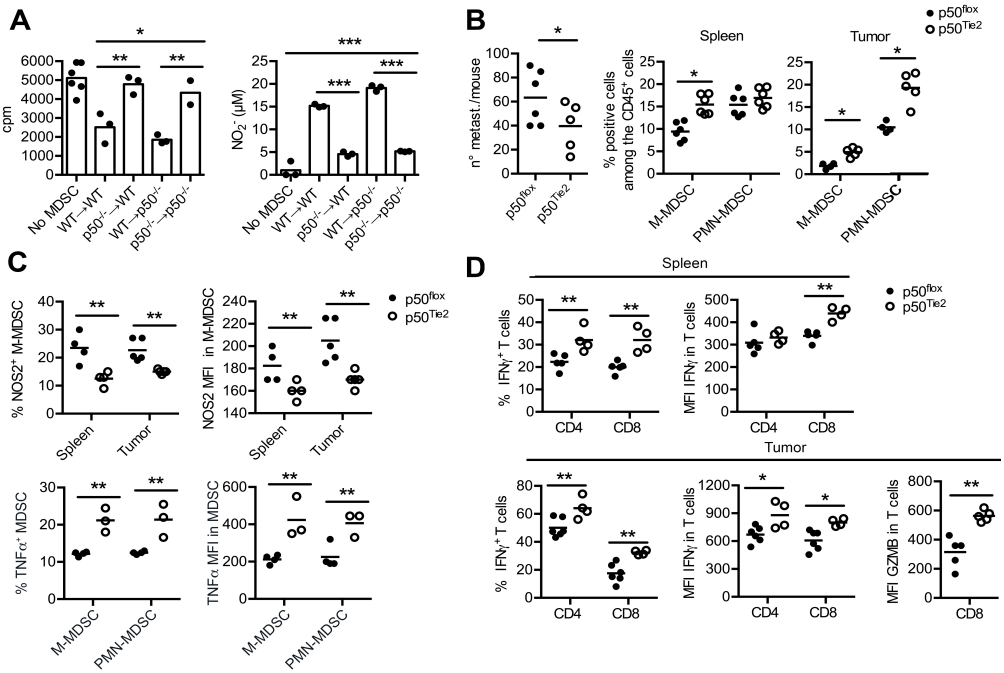

Figure 3. ***Myeloid-specific p50 NF-κB reprograms innate ad adaptive immune functions.*** (A) Splenic M-MDSC from p50$^{-/-}$ BM-transplanted mice display reduced suppressive activity (left) and NO production (right) in response to IFNγ. Proliferation was assessed by ^{3}H-thymidine incorporation (*p<0.05; **p<0.01; ***p<0.001 by two-tailed one-way ANOVA n=6 technical replicates of T cells only, n=3 technical replicates of pooled WT mice with WT BM, n=3 technical replicates of pooled WT mice with p50$^{-/-}$ BM, n=3 technical replicates of pooled p50$^{-/-}$ mice with WT BM, n=2 technical replicates of pooled p50$^{-/-}$ mice with p50$^{-/-}$ BM). NO production by a technical triplicate (n=3) for each group (***p<0.001 by two-tailed one-way ANOVA) (B) Left, metastasis formation in control p50^{flox} and p50^{Tie2} mice (*p<0.05 by one-tailed t-test; p50^{flox} n=6; p50^{Tie2} n=5). Center and right, increased number of splenic and tumor infiltrating M-MDSC (CD11b$^+$Ly6G$^-$Ly6C$^+$) and PMN-MDSC (CD11b$^+$Ly6G$^+$Ly6C$^{low/-}$) respectively in p50^{Tie2} tumor-bearing mice (*p<0.05 by two-tailed t-test; spleen n=6 p50^{flox} and n=6 p50^{Tie2} tumor-bearing mice, tumor n=4 p50^{flox} and n=5 p50^{Tie2} mice). (C) Top, flow cytometry analysis of NOS2 in M-MDSC from both spleen (**p<0.01 by two-tailed t-test; n=4 p50^{flox} and n=4 p50^{Tie2} tumor-bearing mice) and tumor of p50^{flox} and p50^{Tie2} tumor- bearing mice (**p<0.01 by two-tailed t-test; n=5 p50^{flox} and n=5 p50^{Tie2} mice). Bottom, flow cytometry analysis of TNFα in tumor-infiltrating M- and PMN-MDSC from p50^{flox} and p50^{Tie2} tumor-bearing mice (**p<0.01 by two-tailed t- test; n=4 p50^{flox} and n=3 p50^{Tie2} mice). (D) Flow cytometry analysis of IFNγ and GZMB-expressing CD4$^+$ and CD8$^+$ T cells in spleen and tumor from p50^{flox} and p50^{Tie2} mice (*p<0.05, **p<0.01 by two-tailed t-test; spleen n=5 p50^{flox} and n=4 p50^{Tie2} tumor-bearing mice; IFNγ tumor n=6 p50^{flox} and n=4 p50^{Tie2} mice; GZMB tumor n=5 p50^{flox} and n=5 p50^{Tie2} mice).

p50 influences IFNγ-induced Stat1 recruitment to a subset of p50-dependent genes

Since the absence of p50 has strongly altered the response of $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells to IFNγ, we further investigated this aspect. IFNγ-induced Stat1 phosphorylation[36] was not reduced in the absence of p50 (Fig.4A) suggesting that downstream events occurring in $p50^{-/-}$ cells could be responsible for defective IFNγ-mediated gene expression. To directly assess this hypothesis, we generated mRNA sequencing (mRNA-Seq) data sets of wt and $p50^{-/-}$ $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells,
isolated from the spleen of tumor bearing mice, treated or not with IFNγ. $p50^{-/-}$ $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells showed selective gene expression defects in response to IFNγ stimulation as compared to WT controls (Fig.4B and Supplementary Table 1). Using as cut-offs a log2(fold change) ≥ 1 and a FDR ≤ 0.05, 15 (2.4%, cluster 2) of the 628 genes induced by IFNγ in WT $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells 15 (2.38%) were down-regulated in $p50^{-/-}$ cells, whereas 16 (2.5%) were up-regulated. Analysis of the generated gene profiles confirmed that p50 deficiency results in a significant impaired induction of *Nos2* (Fig.4C) along with an increased induction of some interferon inducible genes, such as Oas1g and Pyhin1/Ifix, (Supplementary Table 1). Collectively, our data highlight a specific function of p50 in controlling a subset of functionally relevant genes in response to IFNγ stimulation. Since transcriptional responses to IFNγ predominantly rely on the Stat1 transcription factor, we evaluated the recruitment of Stat1 on the *Nos2* gene using Chromatin Immunoprecipitation (ChIP) with a validated antibody directed against Stat1.[37] Lack of p50 reduced binding of Stat1 on the Nos2 gene in $CD11b^{+}Ly6C^{high}$ cells under both steady state and IFNγ-treatment (Fig.4D), therefore indicating that p50 controls IFNγ-dependent responses at epigenetic level.

Along with MDSCs, macrophages produce NO in response to IFNγ, which mediate either their immunosuppressive[5,22] or tumoricidal capacity[38]. Similar to M-MDSCs, in thioglycollate elicited macrophages (PECs) lack of p50 decreased *Nos2* mRNA expression and NO production in response to IFNγ (Supplementary Fig.4A), without reducing Stat1 phosphorylation (Supplementary Fig.4B). In further analogy with MDSCs, PECs primed with the tumor supernatant (TSN) produced higher level of NO in response to IFNγ, in a p50-dependent manner (Supplementary Fig.4C). Moreover, transcriptional analysis (mRNA-Seq) of WT and p50$^{-/-}$ PECs, confirmed that

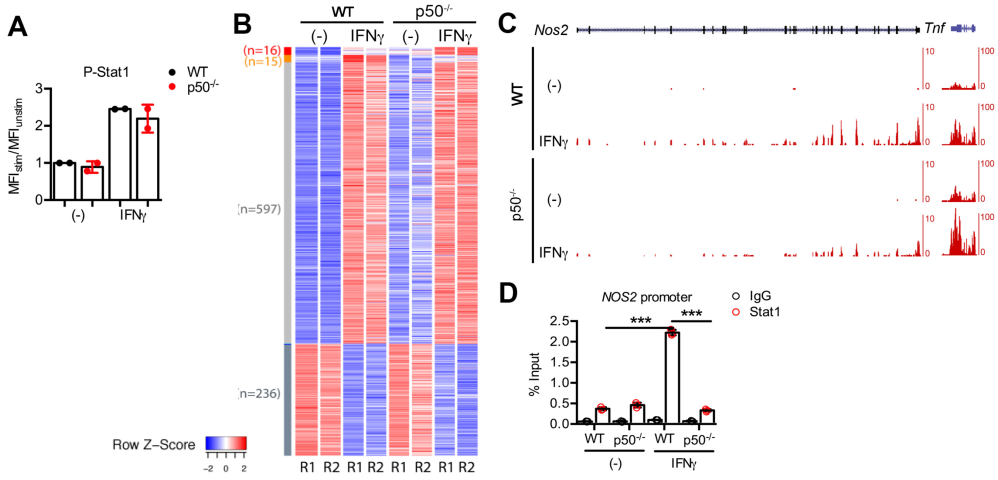

Figure 4. ***Effects of p50 deficiency on gene expression and STAT1 binding in splenic M-MDSC, from tumor bearing mice, treated with IFNγ.*** (A) M-MDSC from WT and p50$^{-/-}$ tumor-bearing mice were isolated from spleen and stimulated with IFNγ for 15'. Cells were stained with an anti-P- Stat1 antibody and analyzed by FACS (*p<0.05 by two-tailed t-test n=2 WT and n=2 p50$^{-/-}$ biological replicates) (B) Heatmap showing selective gene expression alterations in p50$^{-/-}$ M-MDSC stimulated with IFNγ for 4 hours (n=2 WT and n=2 p50$^{-/-}$ biological replicates). Data obtained with two biological replicates were highly correlated (R^2>0.97) indicating high reproducibility between samples. For each transcript, the expression level (log2-transformed FPKM) is z-score scaled (red: highly expressed; blue: lowly expressed). In the vertical bar, colors indicate six groups of genes (Red: IFNγ-induced genes up-regulated in p50$^{-/-}$ M-MDSC; orange: IFNγ induced genes down-regulated in p50$^{-/-}$ M-MDSC; light grey: IFNγ induced genes not affected by p50 ablation; blue: IFNγ repressed genes down-regulated in p50$^{-/-}$ M-MDSC; light blue: IFNγ repressed genes up-regulated in p50$^{-/-}$ M-MDSC; grey: IFNγ repressed genes not affected by p50 ablation. The number of genes belonging to each group is indicated on the left. (C) A representative snapshot of RNAseq expression data for the *Nos2* and *Tnf* genes. (D) Chromatin immunoprecipitation showing impaired recruitment of STAT1 on regulatory regions of the NOS2 promoter, in splenic M-MDSC from p50$^{-/-}$ tumor bearing mice mice treated with IFNγ (***p<0.001 by two-tailed one-way ANOVA n=3 WT and n=3 p50$^{-/-}$ technical replicates).

p50$^{-/-}$ PECs showed selective gene expression defects in response to IFNγ (Supplementary Fig.4D and Supplementary Table 2), including *Nos2*. On the other hand, in agreement with Figure 2H, p50 deficiency resulted in an increased induction of the monopoiesis-inducing transcription factor *Irf8* [33](Supplementary Table 2). We then performed ChIP coupled to next-generation sequencing (ChIP-Seq). Almost all DNA binding events occurred only after Stat1 activation (Supplementary Table 3) and positively correlated with IFNγ-induced gene expression in a statistically significant manner (Supplementary Fig.4E), highlighting the prominent role of Stat1 as a transcriptional activator. A discrete fraction of the Stat1 cistrome was selectively affected by p50 deficiency, with an abrogation or reduction of Stat1 occupancy at 2571 sites (8.3% of all inducible peaks) in p50$^{-/-}$ PEC relative to WT controls. For instance, we confirmed that Stat1 was not efficiently recruited to either promoters or enhancers of *Nos2* gene in p50$^{-/-}$ macrophages, and this was associated with its reduced induction in response to IFNγ (Supplementary Fig.4F). These observations were then validated at a genomic scale by computationally integrating our ChIP-Seq and mRNA-Seq datasets (Supplementary Table 4). As shown in Supplemental Fig.4G, p50-dependent genes were located at shorter distances from p50-dependent Stat1 peaks than p50-independent genes. Conversely, p50-independent genes were closer to p50-independent Stat1 peaks.

These findings identify a role for p50 in controlling IFNγ-induced Nos2 gene expression in myeloid cells, and are consistent with a model of p50- dependent assistance of Stat1 recruitment to selected p50-dependent genes in response to IFNγ treatment.

Ablation of p50 NF-κB in M-MDSCs restores the in vivo antitumor activity of IFNγ

We next investigated whether increased nuclear p50 NF-κB in M-MDSCs limits cytokine-mediated immunotherapy *in vivo*. While IFNγ treatment of WT tumor-bearing mice was ineffective, the same treatment significantly reduced tumor development in $p50^{-/-}$ mice (Fig.5A). Noteworthy, in vivo depletion of $CD4^+$ and $CD8^+$ T cells in p50 NF-κB-deficient tumor bearing mice restored tumor growth (Fig.5A, center) and metastasis formation (Fig.5A, right), indicating that myeloid-specific p50 NF-κB is suppressing specific anti-tumor immunity. Hence, to test the specific contribution of p50 to the immunosuppressive activity of M-MDSCs, $p50^{-/-}$ tumor-bearing mice were adoptively transferred with WT M-MDSCs ($1x10^6$) and treated daily with IFNγ. Such M-MDSCs transfer restored tumor growth in $p50^{-/-}$ mice (Fig.5B) and decreased both IFNγ production and GZMB expression by $CD8^+$ T cells, both in the spleen and the primary tumor (Fig.5C-D). Of note, transfer of $p50^{-/-}$ $CD11b^+Ly6G^-Ly6C^+$ cells did not affect tumor growth, as well as the phenotype of $CD4^+$ and $CD8^+$ T cells. These data demonstrate that accumulation of nuclear p50 in M-MDSCs promotes their suppressive functions in blunting the efficacy of cytokine-mediated immunotherapy (i.e. IFNγ). This conclusion was further strengthened observing that ablation of p50 in tumor bearing mice improved the antitumor and anti-metastatic activity of the immunostimulatory cytokine IL-12 (Supplementary Fig.5).

Tumor-derived PGE2 primes M-MDSCs for higher IFNγ-induced NO-mediated suppressive activity

In the attempt to identify tumor-derived signals controlling nuclear p50 accumulation, we used BM-MDSC[39] to test molecules detected in the tumor supernatants (TSN) (Fig.6A) and reported to either induce nuclear accumulation of p50 NF-κB homodimers in macrophages (i.e. PGE2, IL-10, TGFβ)[10] or myeloid cell differentiation (GM- CSF, G-CSF and M-CSF).

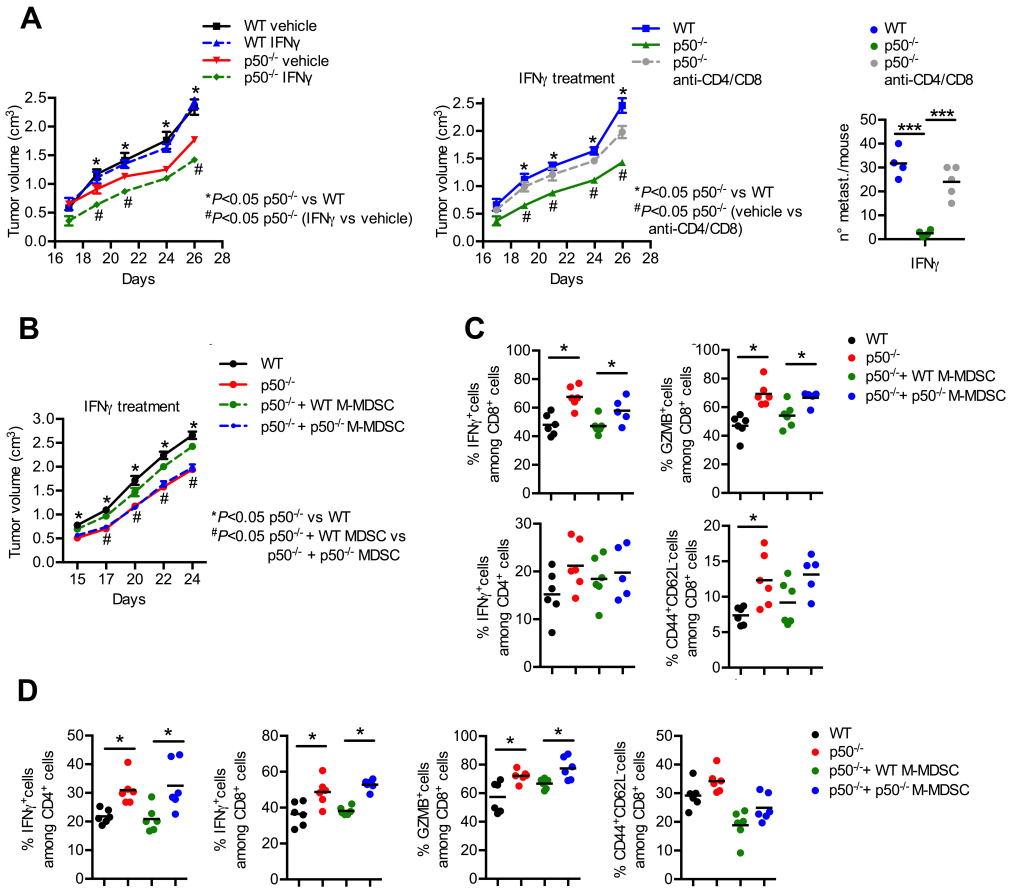

Figure 5. ***Ablation of p50 NF-κB in M-MDSC restores IFNγ-mediated antitumor activity in vivo.*** (A) Left, antitumor effects of IFNγ in WT vs $p50^{-/-}$ mice. Center and right, in vivo depletion of $CD4^+$ and $CD8^+$ T cells in p50 NF-κB-deficient tumor bearing mice abolishes the antitumor activity of IFNγ in terms of tumor growth (data shown are mean±SEM P value was calculated by two-tailed two-way ANOVA, n=5 WT vehicle treated mice, n=5 $p50^{-/-}$ vehicle treated mice n=5 WT IFNγ treated mice, n=6 $p50^{-/-}$ IFNγ treated mice, n=6 $p50^{-/-}$ anti-CD4/CD8 and IFNγ-treated mice) and metastasis formation (***p<0.001 by two-tailed one-way ANOVA n=4 WT IFNγ treated mice, n=4 $p50^{-/-}$ IFNγ treated mice, n=5 $p50^{-/-}$ anti-CD4/CD8 and IFNγ treated mice). (B) Adoptive transfer of WT M-MDSC in $p50^{-/-}$ tumor-bearing mice abolishes the antitumor activity of IFNγ in vivo, restoring tumor growth. (data shown are mean±SEM, P value was calculated by two-tailed two-way ANOVA n=8 WT mice, n=8 $p50^{-/-}$ mice, n=7 $p50^{-/-}$ + WT M-MDSC mice, n=6 $p50^{-/-}$ + $p50^{-/-}$ M-MDSC mice). (C-D) FACS analysis of $CD4^+$ and $CD8^+$ T cells for IFNγ and GZMB expression and effector memory phenotype ($CD62L^-$ $CD44^+CD8^+$ T cells) in both (C) spleen (*p<0.05, by two-tailed one-way ANOVA n=6 WT mice, n=6 $p50^{-/-}$ mice, n=6 $p50^{-/-}$ + WT M-MDSC mice, n=5 $p50^{-/-}$ + $p50^{-/-}$ M-MDSC mice) and (D) tumor (P<0.05, by two-tailed one-way ANOVA n=6 WT mice, n=6 $p50^{-/-}$ mice, n=6 $p50^{-/-}$ + WT M-MDSC mice, n=6 $p50^{-/-}$ + $p50^{-/-}$ M- MDSC mice) of tumor bearing mice. WT, WT mice; $p50^{-/-}$, $p50^{-/-}$ mice; $p50^{-/-}$ + WT M-MDSC, $p50^{-/-}$ mice adoptively transferred with WT M-MDSC; $p50^{-/-}$ + $p50^{-/-}$ M-MDSC, $p50^{-/-}$ mice adoptively transferred with $p50^{-/-}$ M-MDSC.

Confocal microscopy analysis showed that neither M-, GM- nor G-CSF induced p50 accumulation (Supplementary Fig.6A). In contrast, PGE2 efficiently induced nuclear p50, whereas the PGE2 receptor antagonists EP2 (AH6809) and EP4 (AH23848)[40] inhibited the TSN-induced nuclear accumulation of p50 (Fig.6B). Furthermore, BM-MDSCs primed with TSN showed higher levels of both *Nos2* mRNA and NO production in response to IFNγ (Fig.6C), which was prevented by AH6809 (Fig.6D). In agreement, pre-treatment with Butaprost, a selective agonist of EP2, enhanced IFNγ-mediated NO production in a p50-dependent manner (Fig.6D). Nuclear p50 accumulation was also promoted by IL-10, however neutralization of TSN-derived IL-10 only marginally reduced its priming activity (not shown).

We next evaluated the capacity of AH6809 to interfere with the MN/MCA1 fibrosarcoma *in vivo*. Daily treatment with AH6809 inhibited tumor growth in WT but not in $p50^{-/-}$ mice (Fig.6E, left) and reduced the number of lung metastasis in WT mice (Fig.6E, right). In agreement with the phenotype of $p50^{-/-}$ mice (Fig.3C-D), flow cytometry of tumor tissue (MN/MCA1) from tumor-bearing WT mice, showed that AH6809 treatment significantly reduced the expression of NOS2 (Fig.6F, left) by $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells, while increasing the expression of TNFα (Fig.6F, center), as well as the $IFN\gamma^{+}CD4^{+}$ and $IFN\gamma^{+}CD8^{+}$ T cells (Fig.6F, right). Similar results were obtained in splenic M-MDSCs (Fig.6G). Further supporting the pro-tumoral activity of the PGE2-p50-NO axis, *in vivo* treatment with AH6809 displayed an antitumor effects comparable with an antibody against the PD-1 immune checkpoint inhibitor (anti-PD-1), both in terms of primary tumor growth (MN/MCA1) and lung metastasis formation (Supplementary Fig.6B), while the expression of NOS2 in $CD11b^{+}Ly6C^{+}$ WT M-MDSCs cells was reduced to levels comparable with $p50^{-/-}$ M-MDSC (Supplementary Fig.6C).

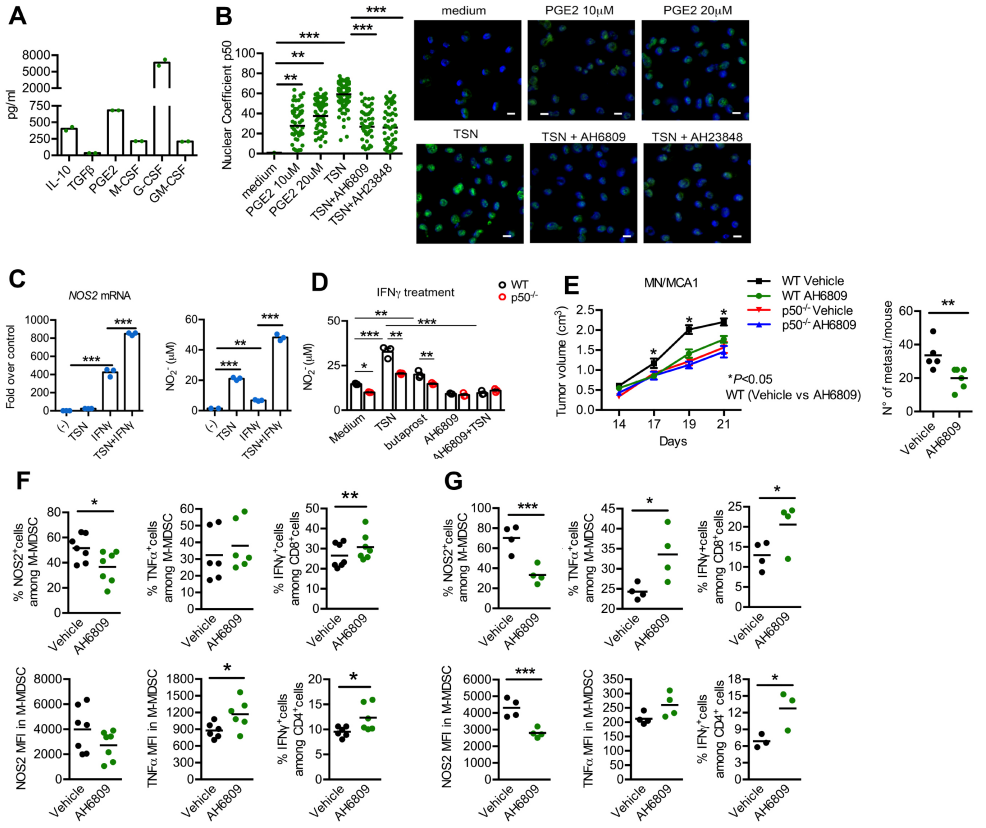

Figure 6. ***Tumor-derived PGE2 promotes p50 NF-κB mediated suppressive M-MDSC functions.*** (A) Levels of IL-10, TGFβ, PGE2, GM-CSF, G-CSF and M-CSF in n=2 independent TSN, estimated by ELISA. (B) Right, confocal microscopy of p50 NF-κB in BM-derived M-MDSC conditioned with either TSN, PGE2 (10μM and 20 μM) or TSN plus the EP2 antagonist AH6809 or the EP4 antagonist AH23848 (scale bars: 10 μm). Post 48 hrs of in vitro activation, cells were stained with anti-p50 NF-κB antibody (green) and nuclei were counterstained with DAPI (blue). Left, quantification of p50 NF-κB nuclear fluorescence intensity (**p<0.01, ***p<0.001, by two tailed one-way ANOVA, medium n=34 nuclei, PGE2 10 μM n=62 nuclei, PGE2 20 μM n=71 nuclei, TSN n=96 nuclei, TSN+AH6809 n=70 nuclei, TSN+AH23848 n=61 nuclei). (C) TSN primes IFNγ-treated MDSC for enhanced expression of NOS2 mRNA and NO production (***p<0.001 by two-tailed one-way ANOVA, n=3 technical replicates of a representative experiment). (D) Inhibition of NO production by the EP2 receptor antagonist AH6809, in TSN-primed and IFNγ-treated WT and p50-deficient BM-MDSC (**p<0.01, ***p<0.001 by two-tailed one-way ANOVA, n=3 technical replicates of a representative experiment). (E) Left, AH6809 inhibits fibrosarcoma growth (data shown are mean±SEM; *p<0.05 by two-tailed two-way ANOVA: n=6 WT vehicle mice, n=6 WT AH6809 treated mice, n=6 $p50^{-/-}$ vehicle mice, n=6 $p50^{-/-}$ AH6809 treated mice) and lung metastasis formation in tumor-bearing WT mice (**p<0.01 by two-tailed t-test n=5 WT vehicle treated mice, n=6 WT AH6809 treated mice). (F-G) FACS analysis of infiltrating immune cells in tumor (F) and spleen (G) of fibrosarcoma (MN/MCA1) bearing WT mice treated with AH6809 or untreated (vehicle), and expression evaluation of NOS2 and TNFα in M-MDSC and IFNγ in $CD4^+$ and $CD8^+$ T cells. Data pooled from two different experiments (*p<0.05, **p<0.01 by two-tailed t-test. Tumor, TNFα in M-MDSC n=6 WT vehicle and n=6 WT AH6809 treated mice, NOS2 in M-MDSC n=7 WT vehicle and n=7 WT AH6809 treated mice; IFNγ in $CD4^+$ T cells n=6 WT vehicle and n=6 WT AH6809 treated mice; IFNγ in $CD8^+$ T cells n=7 WT vehicle and n=7 WT AH6809 treated mice. Spleen, NOS2 and TNFα in M-MDSC and IFNγ in $CD8^+$ T cells n=4 WT vehicle and n=4 WT AH6809 treated mice, IFNγ in $CD4^+$ T cells n=3 WT vehicle and n=3 WT AH6809 treated mice).

In agreement, treatment of B16 bearing WT mice with AH6809 reduced tumor growth and NOS2 expression by CD11b^{+}Ly6C^{+} M-MDSC, mimicking p50 NF-κB ablation (Supplementary Fig.6D).

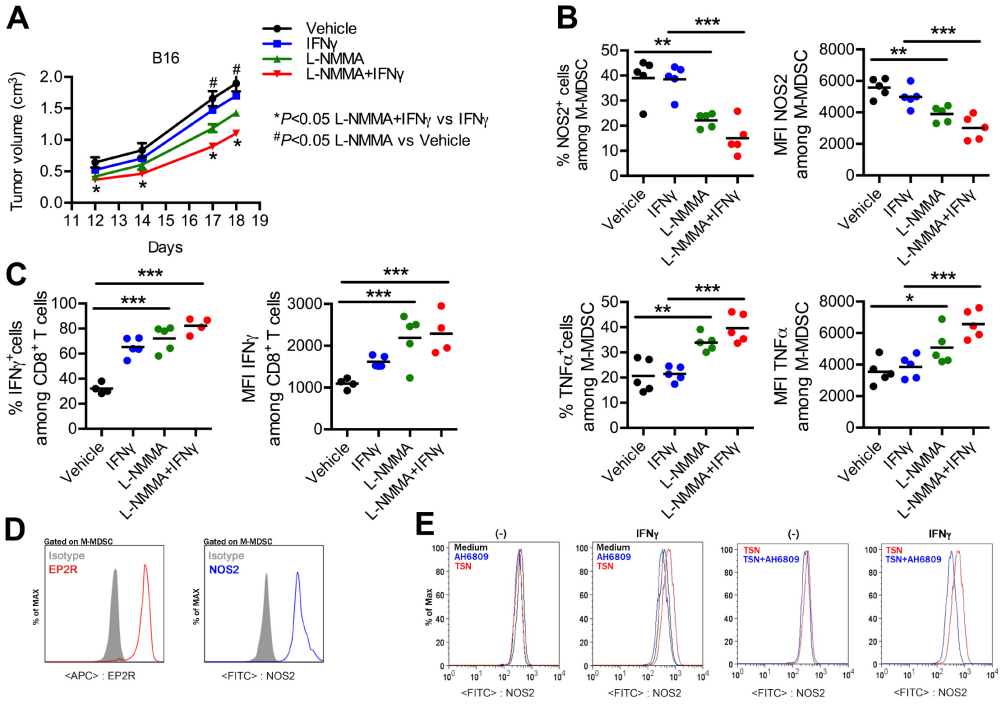

Figure 7. ***Blocking NO production restores the anti-tumor activity of IFNγ in vivo.*** (A) L-NMMA treatment reduces melanoma (B16) growth in WT mice and releases the antitumor activity of IFNγ (data shown are mean±SEM P value was calculated by two-tailed two-way ANOVA n=5 WT Vehicle treated mice, n=5 IFNγ treated mice, n=5 L-NMMA treated mice, n=5 IFNγ + L-NMMA treated mice). (B) L-NMMA reduces the expression of NOS2 (Top) and increases the expression of TNFα (Bottom), in tumor-infiltrating M-MDSC (*p<0.05 by two-tailed one-way ANOVA n=5 WT Vehicle treated mice, n=5 IFNγ treated mice, n=5 L-NMMA treated mice, n=5 IFNγ+L-NMMA treated mice). (C) L-NMMA enhances tumor infiltration of IFNγ-expressing CD8^{+} T cells (*p<0.05 by two-tailed one-way ANOVA n=4 WT Vehicle treated mice, n=5 IFNγ treated mice, n=5 L-NMMA treated mice, n=4 IFNγ+L-NMMA treated mice). (D) Representative flow cytometry of EP2 receptor and NOS2 expression in peripheral blood M-MDSC from CRC patients (n=5 CRC patients). (E) Flow cytometry analysis of NOS2 in IFNγ-treated human peripheral blood monocytes primed with TSN from the human pancreatic carcinoma cell line PANC1, in the presence or absence of the EP2 antagonist AH6809.

Finally, we investigated the effect of NO on the antitumor activity of IFNγ in vivo. WT B16-bearing mice were then treated with IFNγ and/or with L-NMMA. While treatment with IFNγ elicited poor effects on tumor growth, L-NMMA alone displayed a significant antitumor effect, which was enhanced in combination with IFNγ (Fig.7A). Moreover, L-NMMA, and even more its combination with IFNγ, reduced the expression of NOS2 by tumor M-MDSC (Fig.7B, top) and enhanced the expression of TNFα (Fig.7B, bottom), while increasing the number of tumor-infiltrating $IFN\gamma^{+}CD8^{+}$ T cells (Fig.7C). These results indicate NO as a terminal mediator of suppressive myeloid cell functions and suggest that blocking the PGE2-p50-NO axis either upstream (i.e. EP2 inhibitors) or downstream (i.e. NOS2 inhibitor L-NMMA) may restore antitumor immunity.

To corroborate this finding in cancer patients, we analyzed the expression of both the PGE2 receptor EP2 and NOS2 in PBMCs from patients with advanced colorectal cancer (CRC; stage T2/T3; n=5). In agreement with mouse data, human M-MDSCs ($HLA\text{-}DR^{low/-}CD14^{+}CD33^{high}$) (Supplementary Fig.2A) expressed the PGE2 receptor EP2 (Fig.7D). Moreover, human peripheral blood monocytes primed with TSN from the human pancreatic carcinoma cell line PANC1, containing 923 μg/ml of PGE2, and subsequently treated with IFNγ showed higher levels of intracellular NOS2 protein, that were reduced by treatment with AH6809 (Fig.7E). While confirming the suppressive activity of PGE2 in cancer bearers[41], our results are the first to identify the PGE2-driven nuclear accumulation of p50 NF-κB as leading event in the differentiation of suppressive M-MDSCs.

Discussion

Cancers support a pathological generation of immunosuppressive and tumor-promoting MDSC populations[22,29], that share phenotypic traits with TAMs, including the expression of anti-inflammatory M2 polarized genes.[10] Here, we demonstrate that tumor-derived PGE2 drives the suppressive phenotype of M-MDSCs through upregulation of nuclear p50 NF-κB, a key player in the resolution of the inflammatory response, that links unresolved cancer-inflammation with tolerance.[8,42]

Accumulation of nuclear p50 NF-κB, in both mouse ($CD11b^{+}Ly6G^{-}Ly6C^{+}$) and human (CRC patients) ($CD14^{+}HLA\text{-}DR^{low/-}$) M-MDSCs, diverts their transcriptional responses to IFNγ towards enhanced production of the immunosuppressive molecule NO. Consistently, ablation of p50 NF-κB reactivated specific antitumor immunity and reinstated the *in vivo* antitumor activity of IL-12 and IFNγ, two immunostimulatory cytokines evaluated in clinical trials against various human tumors (e.g. CRC, soft tissue sarcoma, melanoma and plasma cell neoplasms).[16] This observation provides new insights into the ambivalent action of IFNγ [21,43,44], which in clinical protocols has shown either moderate or poor efficacy.[15,17,45] This ambiguity is reminiscent of the bimodal immunological activities of IFNγ, which promotes transcription of genes involved in the activation of immune responses (e.g. MHC class I and class II, IL-12)[46] and simultaneously induces immunosuppressive pathways, including B7-H1[47,48] and the immunosuppressive enzymes IDO[23] and iNOS/NOS2[22,29]. The biological activity of IFNγ requires the nuclear translocation and the DNA binding of the STAT1 homodimer to gamma activated sequence (GAS) sites on the promoters of downstream target genes[49], including Nos2[50]. Accumulation of p50 NF-κB in myeloid cells does not affect IFNγ-dependent STAT1 phosphorylation, but rather controls their chromatin landscape to promote binding of STAT1 onto specific gene regulatory elements of IFNγ-responsive

genes, including Nos2. We also observed that lack of p50 results in increased $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells in the spleen of tumor-bearing mice, with low NO production and suppressive activity, as well as in the preferential skewing of HSCs towards the monocytic branch in the bone marrow. This hematopoietic output is in agreement with the $IRF8^{high}$ feature we observed in p50-deficient PMN-MDSCs, since IRF8 is a cell fate switching factor driving terminal differentiation of the monocytic lineage[33] and preventing MDSC expansion[32].

COX2-stimulated production of PGE2 enhances the expansion of MDSCs and promotes suppression of adaptive immunity[51]. We show that antagonists of the PGE2 receptors EP1/EP2 reprogram M-MDSC functions in response to IFNγ towards an inflammatory phenotype (i.e. $NOS2^{low}/TNF^{high}$), associated with increased specific anti-tumor immunity. Of relevance, in analogy with the results obtained both with the inhibition of PGE2 receptors and with the ablation of the NF-κB p50 gene, the NOS L-NMMA inhibitor reprogrammed the M-MDSCs towards an $NOS2^{low}/TNF^{high}$ phenotype, associated with an increased antitumoral effect of IFNγ. This evidence indicates the gasotransmitter NO [52] as the final effector of the pro-tumoral reprogramming of myeloid cells, guided by the PGE2/p50 axis.

COX inhibitors (e.g. aspirin) were suggested for prevention and treatment of cancer[41]. Nevertheless, due to their severe side effects, targeting specific prostanoid receptors endowed with immunosuppressive properties, such as EP2 [53–55], might provide an alternative selective and safer approach to burst specific immunity in cancer patients. Our work indicates that p50 NF-κB accumulation diverts the differentiation and activation status of myeloid cells, leading to the resolution of immune and inflammatory responses. It further demonstrates that in cancer an accumulation of p50 NF-κB, induced by PGE2, acts as an epigenetic hacker of M-MDSC functions, which establishes their suppressive phenotype, suggesting the use of PGE2 receptor antagonists as potential adjuvants for anticancer immunotherapy.

Materials and Methods

Mice and ethics statement. The study was designed in compliance with: Italian Governing Law (Legislative Decree 116 of Jan. 27, 1992); EU directives and guidelines (EEC Council Directive 86/609, OJ L 358, 12/12/1986); Legislative Decree September 19, 1994, n. 626 (89/391/CEE, 89/654/CEE, 89/655/CEE, 89/656/CEE, 90/269/CEE, 90/270/CEE, 90/394/CEE, 90/679/CEE); the NIH Guide for the Care and Use of Laboratory Animals (1996 edition). All experiments involving animals described in this study were approved by the Ministry of Health (authorization numbers 160/2012-B and 25/2018-PR). The study was approved by the scientific board of Humanitas Clinical and Research Center. Mice have been monitored daily and euthanized when displaying excessive discomfort. p50 NF-κB deficient mice on the C57BL/6J background were available in the laboratory.[27] The NFKB1flox/flox ($p50^{flox}$) mice was recently generated.[42] $p50^{flox}$ mice were crossed with B6.Cg-Tg(Tek-Cre)1Ywa mice (Jackson Laboratories, Bar Harbor, Maine, USA) to generate $p50^{flox}$; Tie2Cre mice ($p50^{Tie2}$ mice). OT-I mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA).

Tumor models. 8 weeks old mice were injected intramuscularly in the left leg with 10^5 cells of murine fibrosarcoma (MN/MCA1) or subcutaneously with $5x10^5$ cells of murine melanoma (B16). Tumor growth was monitored 3 times a week with a caliper.

MDSC purification. MDSC were purified from the spleens of tumor-bearing mice by magnetic separation (MACS Miltenyi). In detail, $CD11b^+GR1^+$ cells were obtained by staining splenocytes with a purified rat anti-mouse GR1 antibody (BD pharmingen) followed by positive selection with anti-rat IgG microbeads (MACS, Miltenyi). M-MDSCs ($CD11b^+Ly6C^+Ly6G^-$) and PMN-MDSC ($CD11b^+Ly6G^+Ly6C^{low}$) populations were first enriched by consequent serial negative selections with CD19 and CD11c microbeads, according to manufacturer's instruction. Then $Ly6G^+$ cells were positively selected with Ly6G microbeads kit. Remaining cells were positively selected with $CD11b^+$ microbeads, which all stained positive for the Ly6C marker. Cell population purity was assessed by flow cytometry.

Cell culture and reagents. Bone marrow MDSC (BM-MDSC) were derived from bone marrows (BM) cells isolated from C57BL/6 mice as previously described.[39] Mouse BM cells were obtained from the femurs and tibias. After blood cells lysis, BM cells were cultured for 4 days in RPMI- 1640 containing 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin- streptomycin, 10mM HEPES and 20 μM beta-mercaptoethanol and supplemented with 40 ng/ml of murine recombinant IL6, GM-CSF and G-CSF (Peprotech). If needed, cells were washed with PBS and detached with EDTA 2mM and plated for the experiments. Peritoneal Exudate Macrophages (PEC) were obtained as previously described.[10] Human monocytes were isolated

from peripheral blood as previously described[8] and cultured in RPMI-1640 containing 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin-streptomycin. The concentration for the different treatments were as follows: MN/MCA1 tumor supernatant, dilution ratio 1:1 to medium, human pancreatic carcinoma cell line PANC1 supernatant (30%), PGE2 (Sigma) 10^{-5} mol/L, PGE2 receptor (EP2) agonist Butaprost (Sigma) 10^{-5} mol/L, PGE2 receptor (EP2/EP1) antagonist AH6809 (Tocris) 10^{-5} mol/L, murine IFNγ (Peprotech) 200U/ml, M-CSF (Peprotech) 20ng/ml, GM-CSF (Peprotech) 20ng/ml, GCSF (Peprotech) 20ng/ml, LPS (ALX-581-009, Enzo Life Sciences) 100ng/ml, human IFN (Peprotech) 20ng/ml.

Real-time PCR. Real-time PCR was performed as previously described (Biswas et al., 2006). The sequences of the murine NOS2 gene-specific primers are: (Fw: gccaccaacaatggcaaca; Rev: cgtaccggatgagctgtgaatt).

Nitrite production. $2*10^5$ cells were plated and stimulated with IFNγ (Peprotech 200U/ml) for indicated time points. Nitric oxide production was evaluated in culture supernatant using the Griess Reagent System (Promega). Equal volumes of supernatant (50 µl) and 1% sulfanilamide in 5% phosphoric acid were incubated at RT for 10 min, followed by addition of 0.1% N-1-naphthylethylenediamine dihydrochloride in water. After 10 min at RT, absorbance at 550 nm was measured. Nitrite concentrations were determined by comparing absorbance values to a standard curve generated by serial dilution of 0.1 mM sodium nitrite.

Suppression assay. M-MDSCs ($CD11b^+Ly6C^+Ly6G^-$ cells) isolated from the spleen of WT MN-MCA1 tumor-bearing mice were plated in 96 wells at a final concentration of 24% and 12% of total cells and co-cultured with splenocytes from naive C57Bl6 mice, labelled with 1µM CFSE, in the presence of anti-CD3 (3µg/ml, 2C11, Biolegend) and anti-CD28 (2µg/ml, 37.5, BD). After 3 days of co-colture, cells were stained and CFSE signal of gated lymphocytes was used to analyze cell proliferation. For antigen-specific suppression assay Mixed-lymphocyte reaction was performed as previously reported (Strauss et al., 2015) M-MDSC isolated from the spleen of tumor bearing mice were then stimulated with IFNγ (200U/ml), in the presence or absence of 500µM of L- NG-monomethylarginine (L-NMMA, Calbiochem). At day 3, 50 µl of supernatant were tested for NO production (as control) and $2*10^5$ splenocytes from OT-I mice were added for additional 72h in the presence of 250 µg/ml of ovalbumin peptide (OVA257–264) (Sigma). [3H]-thymidine was added for the last 16 hours of culture and its incorporation was analyzed by MicroBeta plate counter (Perkin Elmer). As controls OT-1 splenocytes alone were pulsed with OVA peptide (250 µg/mL) or kept in culture media. All conditions were evaluated in triplicates.

Isolation of MDSCs from colorectal cancer (CRC) patients. The study was approved by the Institute Ethical Committee, and written informed consent was obtained from patients. 20 ml of peripheral blood were collected from healthy donors and T2 or T3 CRC patients. CRC patients did not receive radiation or chemotherapy before sample collection. Blood was stratified on Ficoll gradient to separate peripheral blood mononuclear cells (PBMC). All blood samples were analysed within 3 hr after collection by FACS-analysis. Briefly, $1*10^6$ cells were re-suspended in HBSS (Hank's balanced salt solution, Lonza) supplemented with 0.5% BSA (Sigma). Staining was performed at 4°C for 30 minutes, with a cocktail of mAbs to HLA-DR-Pacific Blue (clone L243); CD14-PE, -APC, -FITC (clone M5E2); CD33-PerCp-Cy5.5 (clone WM53); iNOS/NOS Type II-FITC (Clone 6/iNOS/NOS Type II), from BD Bioscience or BioLegend. Further, we used unconjugated rabbit monoclonal anti human EP2R (clone EPR8030(B); Abcam) followed by incubation with secondary goat anti rabbit Alexa Fluor® 488 conjugated antibody (Life Technologies). For intra-cellular staining, Foxp3/Transcription Factor Staining Buffer Set (eBioscience) were used according to the manufacturer's instruction. Cells were detected using the BD FACSCantoTM II or BD LSRFortessaTM and analyzed with BD FACSDiva and FlowJo (9.3.2) software. When needed, cells were stained, sorted using a BD FACSAriaTM-III cell sorter and subsequently analyzed by confocal microscopy.

Bone marrow (BM) colony formation. BM cells were isolated from the tibias and femurs of WT or $p50^{-/-}$ mice and the colony formation capacity was measured by detection and quantification using MethoCultTM GF M3434, that supports optimal growth of granulocyte-macrophage progenitors (CFU-GM, CFU-M, CFU-G), of erythroid progenitors (BFU-E) and and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitors (CFU-GEMM). 12 days after seeded, colonies were counted independently by two separate operators.

Histopathological analysis of mice BM. Sections of WT and $p50^{-/-}$ BM were routinely stained with Haematoxylin and Eosin (H&E) and analyzed by an expert pathologist (CT) using a Leica DM2000 optical microscope (x400 and x630 magnification) and microphotographs were collected with a Leica DFC320 digital camera using the Leica IM50 imaging software.

Quantification of circulating granulocytes in peripheral blood smears. Cell counts were visually performed on five May-Grunwald Giemsa-stained smears on high-power microscopic fields (x400 magnification) and the average number of total and immature granulocytes was determined by averaging the counts.

BM transfer. $5*10^6$ CD45.1 WT and $p50^{-/-}$ BM cells were injected intravenously in sub-lethally irradiated (900cGy) CD45.2 $p50^{-/-}$ mice and littermate controls. BM reconstitution was evaluated 8 weeks after transplantation by flow cytometry on peripheral blood and was over 90%.

Flow cytometry and sorting. Splenocytes were collected from spleen after disaggregation and filtration through Falcon strainers (70 μm). Primary tumors were cut into small pieces, disaggregated with 0.5 mg ml^{-1} collagenase IV and 150 U ml^{-1} DNase I in RPMI-1640 for 30 min at 37°C and filtered through strainers. Cells (10^6) were re-suspended in HBSS (Hank's balanced salt solution, Lonza) supplemented with 0.5% BSA (Sigma) and the staining was performed at 4°C for 20 minutes with specific antibodies. The following antibodies were used: CD45-FITC, -PerCP (Clone 30-F11); CD11b-PerCp- Cy5.5, -APC, -PE-Cy7, -FITC (Clone M1/70); Gr1-PE (Clone RB6-8C5); Ly6G-PE, -APC, -FITC, BV570 (Clone 1A8); Ly6C-PE, -FITC,-APC-Cy7, -PE-Cy7, -APC (Clone HK1.4); MHCII-PE (Clone M5/114.15.2); CD8-APC, (Clone 53-6.7); CD3-PerCp, -PE, -BV650 (Clone 145-2C11); CD4-APC, PE-Cy7 (Clone GK1.5); IFNγ-PE, -FITC (Clone XMG1.2); lineage markers (PE-CD3, -CD4, -CD8, –Mac-1, –Gr-1, -Ter119, and -B220); hematopoietic progenitor markers (Sca-1, c- Kit/CD117); CD44-FITC (Clone IM7); CD62L-APC, -BV570 (Clone MEL-14); TNFα-APC, -PE-Cy7 (Clone MP6-XT22); CD31-PE, (Clone MEC 13.3); PDL1-PE, (Clone 10F.9G2); Granzyme B-PE, -AF700 (Clone GB11); IRF8-PE, (Clone V3GYWCH), from BD Bioscience, eBioscience, BioLegend or Miltenyi Biotech. Further we used unconjugated we used unconjugated rabbit polyclonal anti-mouse iNOS (ab 15323, Abcam) followed by incubation with secondary goat anti rabbit Alexa Fluor® 488 conjugated antibody (Life Technologies). Cell viability was determined by LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermo Fisher), negative cells were considered viable. For intra-cellular staining Foxp3/Transcription Factor Staining Buffer Set (eBioscience) were used according to the manufacturer's instruction. Cells were detected using the BD FACSCanto™ II or BD LSRFortessa™ and analyzed with BD FACSDiva and FlowJo (9.3.2) software.

mRNA Sequencing (mRNA-Seq). Total RNA was extracted from 1-5*10^6 M-MDSC (RNeasy kit, Qiagen), and 2-5 μg were used to generate sequencing libraries with a Truseq RNA Sample Prep Kit V2 (Illumina) according to the manufacturer's instructions. Sequencing was performed on a HiSeq2000 (Illumina).

Chromatin Immunoprecipitation (ChIP). ChIP was carried out with a previously described high-throughput protocol.[37,56] Briefly, immunoprecipitation was performed by incubating the chromatin at 4°C overnight with Stat1 p84/p91 (M-22) antibody (Santa Cruz Biotechnology) or normal rabbit IgG (Millipore) as negative control. Then, ChIP DNA fragments and input DNA were analyzed by qPCR using the following NOS2 gene specific primers (Fw: tgagctaacttgcacacccaactgg; Rev: tgtaaagttgtgaccctggcagca). ChIP-enriched DNA was compared with input DNA, according to the formula $100 \times 2^{(\text{Input Ct} - \text{sample Ct})}$.

In vivo treatments. MN/MCA1-bearing C57BL6 mice were injected daily with 10U of murine recombinant IFNγ (Peprotech) or vehicle intramuscularly, from day 3 after tumor injection. When specified, starting from the day before tumor injection, mice received an intraperitoneal (i.p.) injection of 0,3 mg anti-mouse CD4 (Rat Anti-Mouse CD4 Monoclonal Antibody, Unconjugated, Clone GK1.5, BioXcell) and 0,3 mg anti-mouse CD8 (Rat Anti-Mouse CD8a Monoclonal Antibody, Unconjugated, Clone 2.43, BioXcell), once a week for the entire experimental period. FACS analysis of peripheral blood samples confirmed $CD4^+$ and $CD8^+$ cells depletion for 7 days. Murine recombinant IL-12 (Peprotech) were administrated i.p. twice a week (100ng/mouse), starting from day 7 after tumor injection. Anti-PD-1 antibody (clone RPM1-14, BioXcell) were administrated i.p. (200μg/mouse) twice a week, starting 10 days after tumor cells injection. MN/MCA1- and B16-bearing C57BL6 mice were i.p. injected daily with 5mg/kg of the PGE2 receptor antagonist AH6809 (Tocris) or vehicle, starting from day 10 after tumor injection.

Adoptive cell transfer. 8 weeks old C57BL/6 WT and $p50^{-/-}$ mice were injected intramuscularly in the left leg with 10^5 cells of MN/MCA1 and treated with IFNγ as described above. One week after tumor injection $p50^{-/-}$ mice were adoptively transferred, via i.v. injection, with $1*10^6$ WT or $p50^{-/-}$ M-MDSC, that were immune magnetically purified from the spleen of tumor-bearing mice.

Enzyme-linked Immunosorbent Assay (ELISA). Murine GM-CSF, G-CSF, M-CSF, IL6, IL-10, IL1β and TGF-β (R&D Systems) and PGE2 (Cayman) were tested by ELISA kits according to the manufacturers' instruction.

Silencing of p50 in human monocytes. Human monocytes were isolated from peripheral blood as previously described.[8] $3x10^6$ cells were transfected with 30pmoli of p50-specific Stealth RNAi siRNA (NFKB1VHS40876) or scramble (Invitrogen) by Amaxa® Human Monocyte Nucleofector® Kit according with manufacturers' instruction (Lonza). After 24h cells were left untreated or stimulated with 200ng/ml of human IFNγ (Peprotech) for 4h. Cell extracts were analyzed by SDS-PAGE (10% acrylamide) as described.[27] Immunoblotting was performed with rabbit anti-p50 (no. 1141) antisera[57] and anti-vinculin antibody (Santa Cruz Biotechnologies). Real-time PCR was performed as previously described[27] with the following gene-specific primers: NOS2 (Fw: aaagaccaggctgtcgttga; Rev: acgggaccggtattcattct), NFKB1/p50 (Fw: gcttaggagggagagcccac; Rev: tctgccattctgaagccggg) and actin (Fw: cccaaggccaaccgcgagaagat; Rev: gtcccggccagccaggtccag).

Confocal microscopy on tumor MDSC. Murine fibrosarcoma (MN/MCA1) were processed as previously described[10] than, MDSC were enriched by positive selection with CD11b microbeads, according to manufacturer's instruction (MACS, Miltenyi). $CD11b^+$ cells were plated on poly-Lys 12 well and fixed with PFA 4%, than unspecific binding sites were blocked with Donkey Serum % + BSA 2% + Triton X-

100 0,1% + NP-40 0,2% in PBS-Tween20 0,05% for 1h. Cells were stained with the following primary antibodies: rat monoclonal [ER-MP20] anti-Ly6C (#ab15627 Abcam), rat anti- mouse Ly-6G (#551459 BD Bioscience), rabbit monoclonal anti-p50 (clone E381, Abcam). After 1h of incubation at RT, goat anti-rabbit AlexaFluor-647 and goat anti-rat AlexaFluor-488 conjugated were used as secondary antibodies. Nuclei were stained with DAPI (#D1306 Life Technologies) and then mounted with ProLong Antifade Gold Reagent (P-36931 Life Technologies). The slides were analyzed with an Olympus Fluoview FV1000 laser scanning confocal microscope. Confocal images were processed with a Gaussian filter and intracellular mean fluorescence intensity was quantified using FluoView software TV10-ASW application software for spectral analysis (version 3.1a; Olympus).

Confocal microscopy on BM-MDSC and PEC. Cells were seeded on Poly-L-Lysine (Sigma-Aldrich) coated sterile rounded glasses at $2x10^5$ cells/ml in medium and fixed with 4% PFA for 10 minutes at room temperature. Cell permeabilization was obtained after 1 h incubation with PBS 0.1% Triton-X100 (Sigma-Aldrich) plus 5% normal goat serum (Dako Cytomation, Carpinteria, CA USA) and 2% BSA, (Amersham Biosciences, Piscataway Township, NJ USA). Cells were then incubated with rabbit anti-mouse p50 NF-κB (NLS, sc-114; Santa Cruz) or rabbit anti-mouse p65 NF-κB (c-20, sc-372; Santa Cruz). After 1 h of incubation at RT, goat anti-rabbit AlexaFluor-647 or goat anti rabbit AlexaFluor-488 conjugated (Life Technologies) were used as secondary antibodies. Nuclei were counterstained with DAPI (Invitrogen, Molecular Probes). Samples were mounted with FluorPreserve Reagent (Calbiochem San Diego, CA USA) and analyzed with an Olympus Fluoview FV1000 laser scanning confocal microscope using a fine focusing oil immersion lens (40X, N.A. 1.3) at 1 Airy Unit resolution and operating in channel mode with 405 and 633 nm excitations. (40×1.30 NA Plan-Apochromat; Olympus). The mean fluorescence intensity of the nucleus was quantified after a freehand drawing considering the nucleus as regions of interest using FluoView software (version 3.1a; Olympus).

NO detection in vivo. For in situ NO quantification, murine fibrosarcomas were harvested in PBS containing Glucose 1%, then included in Low Melting Point Agarose 6% and cut with the vibratome (LEICA) in slides 200mm wide. Next, slides were stained with Diaminorhodamine-4M AM (DAR- 4M- AM)[58] solution (#D9194 SIGMA-ALDRICH) 2,5mM for 2 h at 37°C in RPMI with FBS 10%, Gln 1% and Pen/Strep 1% then fixed with PFA 4%. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (#D1306 Life Technologies) and then mounted with ProLong Antifade Gold Reagent (P-36931 Life Technologies). Analysis was performed with an Olympus Fluoview FV1000 laser scanning confocal microscope. Samples were acquired with fine focusing oil immersion lens (60X, N.A. 1.35) at 1 Airy Unit resolution and operating in channel mode with 633 nm excitations. The

resulting fluorescence emissions were collected using 425-to-475 nm (for DAPI) and 655-to-750 nm (for DAR-4M-AM) band-pass filters. Confocal images were processed with a Gaussian filter and intracellular mean fluorescence intensity was quantified using FluoView software TV10-ASW application software for spectral analysis (version 3.1a; Olympus).

Statistics. Statistical significance between two groups was determined by two-tailed Student's t test or two-way ANOVA corrected for multiple comparison by Sidak's test and among more than two groups by one-way or two-way ANOVA corrected for multiple comparison by Tukey's test (Prism 6). *p<0.05; **p<0.01; ***p<0.001.

Computational Methods.

RNA-SEQ. After quality filtering according to the Illumina pipeline, paired-end reads (76 bp for M-MDSC and 51 bp for PECs) were mapped to the mm9 reference genome and to the *Mus musculus* transcriptome (Illumina's iGenomes reference annotation downloaded from UCSC http://support.illumina.com/sequencing/sequencing_software/igenome.html). We used TopHat v1.3.1[59] allowing up to two mismatches and specifying a mean distance between pairs (-r) of 150 bp for M-MDSC and of 250 for PEC. Differential gene expression was evaluated with an exact test for the negative binomially distributed counts using edgeR v3.10.5 with limma v3.24.15 (Bioconductor package, https://bioconductor.org/packages/release/bioc/html/edgeR.html)[60–63]. Read counts for edgeR analysis were obtained with the software htseq-count v. 0.5.3p9[64]. Only genes with a sum of reads of at least 10 between all 8 conditions were considered. To reduce the amount of bias in the experiments, we adjusted the standard Counts Per Million (CPM) for all 8 conditions with the Trimmed Mean of M-values (TMM) using the calcNormFactors function from the edgeR Bioconductor package. With respect to standard normalization, TMM normalization have the advantage to reduce deeply the false positive rate[63]. We modelled the data variability estimating the dispersion of the negative binomial model using the quantile-adjusted Conditional Maximum Likelihood (qCML) method. We first used the (qCML) estimateCommonDisp function and then the (qCML) estimateTagwiseDisp function, both from the edgeR Bioconductor package. We finally computed exact p-values for the negative binomial distribution using the function exactTest making a pairwise comparisons between the following groups:

- WT samples stimulated with IFNγ versus WT untreated samples;
- $p50^{-/-}$ samples stimulated with IFNγ versus WT samples stimulated with IFNγ.

Only coding genes according to the Ensembl release-67 annotation (http://ftp.ensembl.org/pub/release-67/gtf/mus_musculus/) were considered. Inducible genes were selected according to the adjusted p-value (FDR<0.05, using the Benjamini-Hochberg (B-H) correction) and according to the intensity of signal

(2-fold). In order to compare expression between genes and to compensate for different gene lengths, also the Fragment Per Kilobase of exon Per Million (FPKM>1.5 in at least one replicate condition) were considered. We selected inducible genes also according to the adjusted counts (TMM-CPM>0.1), therefore, in the WT samples, we eliminated very low expressed genes which are, erroneously detected as induced or repressed because of bias in the experiment. Inducible genes whose intensity of expression either decreased or increased after IFNγ stimulus in absence of $p50^{-/-}$ are respectively named *down*- or *up*-regulated p50-dependent genes (FDR<0.05, two-fold). The group of inducible genes whose intensity of expression is not affected by p50 is defined p50-indep genes. Selective gene expression alterations in both $p50^{-/-}$ M-MDSC and PEC stimulated with IFNγ for 4 h (two biological replicates) are shown in heatmaps. Gene expression levels (log2-transformed FPKM) are z-score scaled. Raw data sets are available at the Gene Expression Omnibus data repository (GSE84468): http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=obkjowgcpbelrkx&acc=GSE84468.

CHIP-SEQ. After quality filtering according to the Illumina pipeline, short reads (51 bp), were mapped to the mm9 genome using Bowtie v0.12.7 (Langmead et al., 2009). Only uniquely mapping reads with two or fewer mismatches (-m1 –v2) were retained.

STAT1 genomic occupancy in WT and $p50^{-/-}$ PEC. Peak calling was performed using MACS v1.4[65] with default parameters and bw=100. Each ChIP was compared to input DNA derived from BM-derived macrophages (GEO accession: GSM499415). We defined "IFNγ-inducible" the peaks that were 1) induced in treated vs untreated samples, 2) repressed in untreated vs treated samples (threshold of 1e−10 for peak calling) and 3) enriched with respect to the input genomic DNA (threshold of 1e−5). We used a threshold of 1e−5 to define p50-dependent STAT1 peaks.

p50 influences IFNγ-induced STAT1 recruitment to a group of p50-dependent genes in PEC. Each induced gene was assigned to the nearest STAT1 peak according to the distance between the TSS (Transcription Start Site) of each gene and the summit of STAT1 induced peaks. The genomic distances (log2 base pair) between genes and the nearest Stat1 peak are represented with box plots. Box plots of the genomic distances (log2 base pair) between p50-dependent (or p50-independent) genes and p50-dependent (or p50-independent) Stat1 peaks are also shown. In order to evaluate significant differences between specific groups, a Wilcoxon signed-rank test was applied (p<0.01).

Track visualization. Tracks for the UCSC genome browser[66] were generated using the unique mapping reads and were linearly rescaled to the same sequencing depth.

Figures. All graphical representations were generated with R packages (https://cran.r-project.org/).

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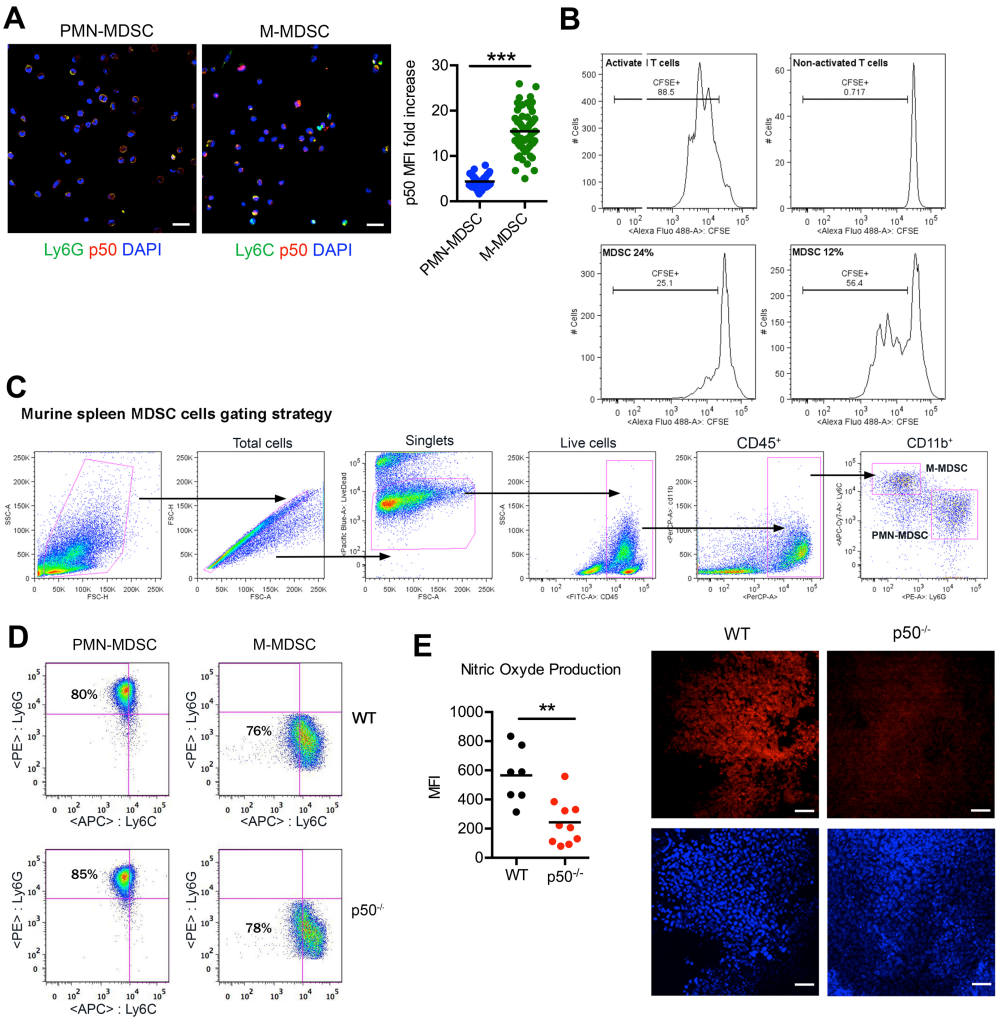
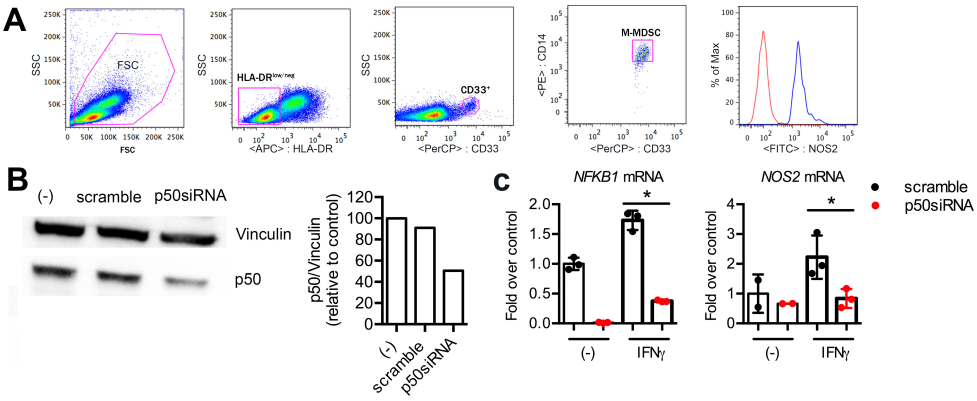
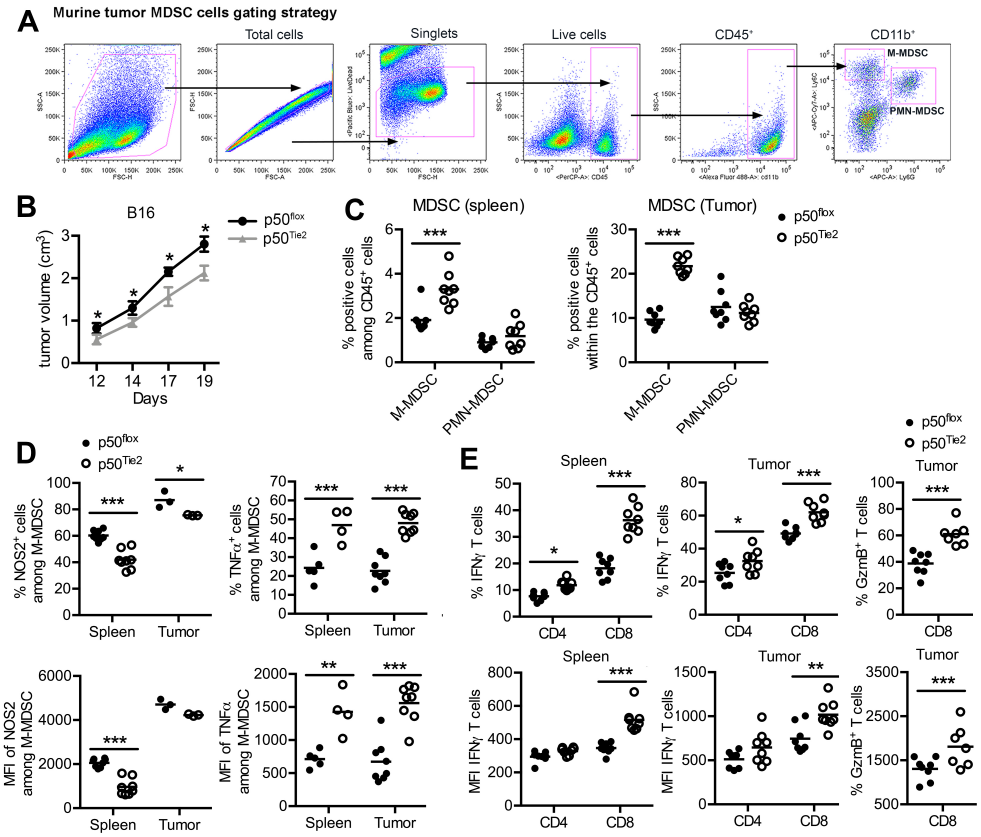
SUPPLEMENTARY MATERIAL



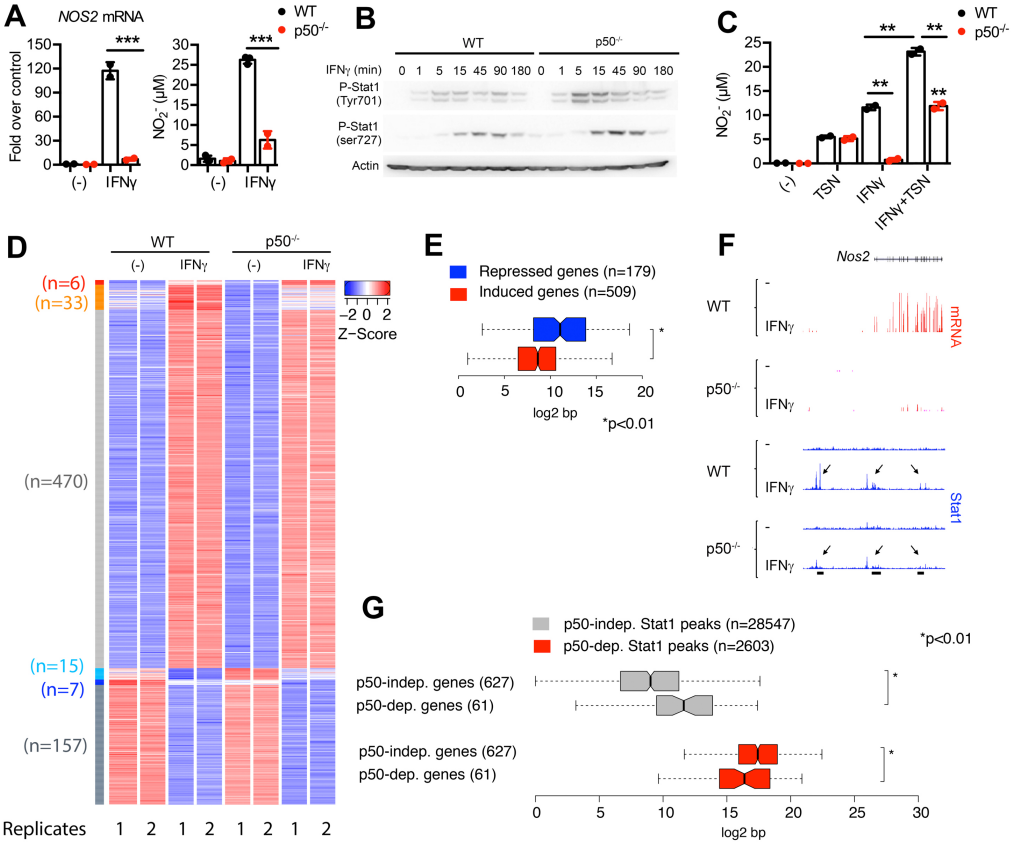
Supplementary Fig.1. Related to Fig.1. (**A**) Confocal microscopy showing up- regulation of nuclear p50 NF-κB in tumor-infiltrating M-MDSC (***p<0.001 by two-tailed t- test n=49 nuclei of PMN-MDSCs n=65 nuclei of M-MDSC). (**B**) M-MDSCs (Ly6Chi Ly6G- cells) isolated from the spleen of WT MN-MCA1 tumor-bearing mice were plated in 96 wells at a final concentration of 24% and 12% of total cells and co-cultured with splenocytes from naive C57Bl6 mice, labelled with 1uM CFSE, in the presence of anti- CD3/CD28. After 3 days of co-colture, cells were stained and CFSE signal of gated lymphocytes was used to analyse cell proliferation. (**D**) The scatterplot shows the purity of the monocytic (M-) and granulocytic (PMN-) MDSC subsets, following purification by magnetic cell sorting from the spleen of tumor-bearing mice. (**C**) Gating strategy used to analyse murine spleen MDSCs of tumor bearing mice. (**E**) NO production in tumor tissues from WT and $p50^{-/-}$ mice. NO was detected with Diaminorhodamine-4M AM (DAR-4M-AM). Scale bars are 20 µm. (**p<0.01 by two-tailed t-test n=7 fields WT tumor n=10 fields $p50^{-/-}$ tumor).



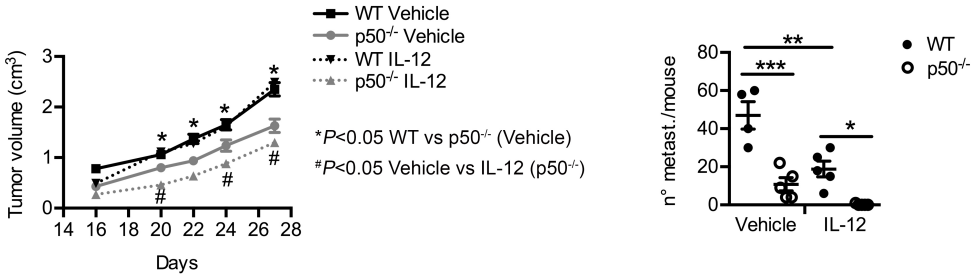
Supplementary Fig.2. Related to Fig.2. (A) Representative gating strategy of NOS2 expressing M-MDSCs in PBMC from CRC patients. (B) p50 siRNA-mediated silencing of p50 NF-κB in circulating $CD14^{+}HLA\text{-}DR^{+}$ mononuclear cells from healthy donors. (C) Effects of p50 silencing on IFNγ-induced NOS2 mRNA expression levels (mean±SD of one out of two independent experiments with similar results. *$p<0.05$ by two-tailed t-test n=3 technical replicates for each group except NOS2 mRNA expression by untreated groups for which data shown are from n=2 technical replicates).



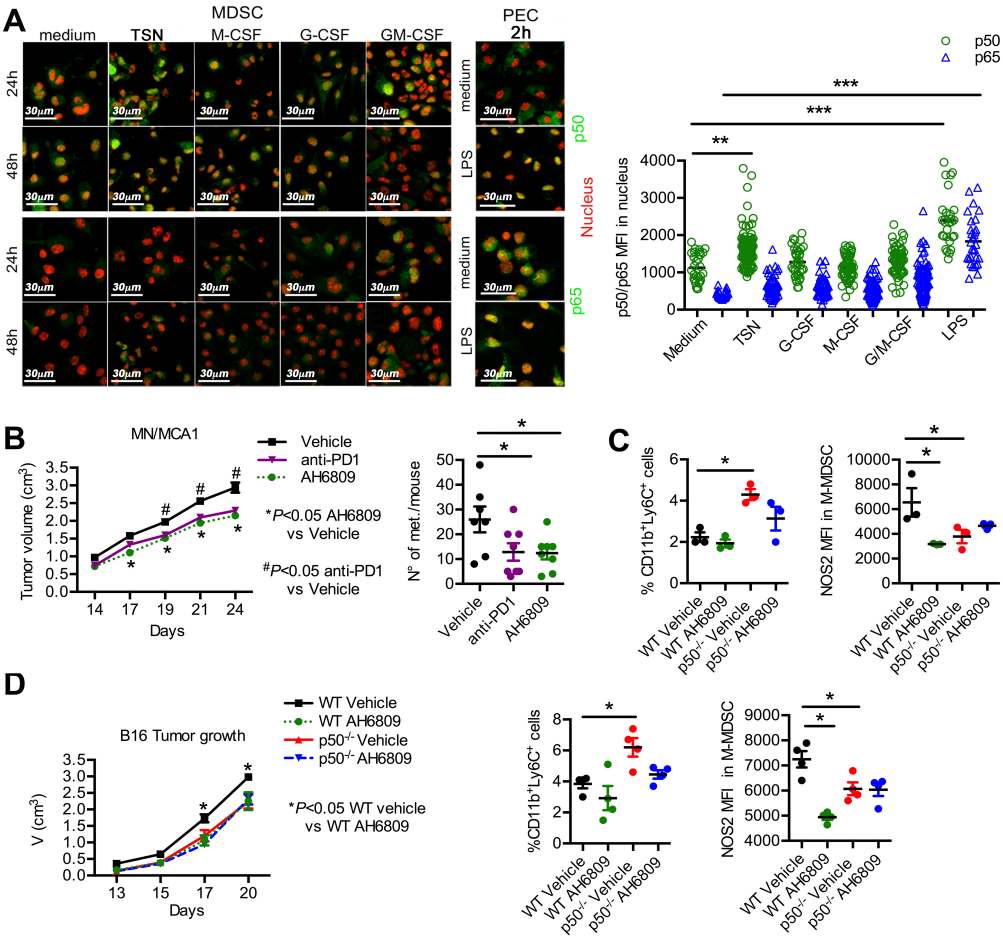
Supplementary Fig.3. Related to Fig.3. (A) Gating strategy used to analyze murine tumor infiltrating MDSCs. (B) B16 tumor growth in $p50^{flox}$ and $p50^{Tie2}$ mice (*p<0.05 by two-tailed two-way ANOVA, n=7 $p50^{flox}$ and n=7 $p50^{Tie2}$ mice). (C) Increased number of spleen and tumor-infiltrating M-MDSCs ($CD11b^{+}Ly6G^{-}Ly6C^{+}$) in $p50^{Tie2}$ tumor-bearing mice (*p<0.05 by two-tailed t-test spleen, n=6 $p50^{flox}$ mice and n=8 $p50^{Tie2}$ mice; tumor, n=8 $p50^{flox}$ mice and n=8 $p50^{Tie2}$ mice). (D) Flow cytometry analysis of NOS2 and TNFα expression in splenic M-MDSC, from $p50^{flox}$ and $p50^{Tie2}$ tumor bearing mice. (*p<0.05, **p<0.01, ***p<0.001 by two-tailed t-test, spleen NOS2 n=6 $p50^{flox}$ mice and n=8 $p50^{Tie2}$ mice, tumor NOS2 n=3 $p50^{flox}$ mice and n=3 $p50^{Tie2}$ mice; spleen TNFα n=5 $p50^{flox}$ mice and n=4 $p50^{Tie2}$ mice, tumor TNFα n=8 $p50^{flox}$ mice and n=8 $p50^{Tie2}$ mice). (E) Flow cytometry analysis of IFNγ and GzmB in $CD4^{+}$ and $CD8^{+}$ T cells, from $p50^{flox}$ and $p50^{Tie2}$ tumor bearing mice (*p<0.05, **p<0.01, ***p<0.001 by two-tailed t-test, spleen IFNγ n=8 $p50^{flox}$ mice and n=8 $p50^{Tie2}$ mice; tumor IFNγ n=8 $p50^{flox}$ mice and n=8 $p50^{Tie2}$ mice; GzmB n=8 $p50^{flox}$ mice and n=7 $p50^{Tie2}$ mice).



Supplementary Fig.4. Related to Fig.4. Effects of p50 deficiency on gene expression and Stat1 binding in macrophages treated with IFNγ. (A) Decreased Nos2 mRNA expression (left) and NO production (right) by IFNγ-treated p50$^{-/-}$ PEC. (**p<0.01, ***p<0.001 by two-tailed t-test, mRNA expression data shown are mean±SD n=2 WT and n=2 p50$^{-/-}$ biological replicates; NO production data are from one representative experiment, data shown are mean±SD n=3 WT and n=2 p50$^{-/-}$ technical replicates). (B) TSN primes IFNγ-treated PEC for enhanced NO production. (**p<0.01, by two-tailed one- way ANOVA, data are from one representative experiment, data shown are mean±SD n=2 WT and n=2 p50$^{-/-}$ technical replicates). (C) Stat1 phosphorylation in WT vs p50$^{-/-}$ PEC in response to IFNγ treatment. Data are from one representative experiment. (D) Heatmap showing selective gene expression defects in p50$^{-/-}$ macrophages (PEC) stimulated with IFNγ for 4 hours (n=2 biological replicates). Data obtained with two biological replicates were highly correlated (r2>0.96). For each gene, the expression level (log2-transformed FPKM) is z-score scaled (red: highly expressed; blue: lowly expressed). In the vertical bar, colors indicate six groups of inducible genes, according to the response to p50$^{-/-}$ (red: IFNγ-induced genes up-regulated in p50$^{-/-}$ vs WT PEC; orange: IFNγ-induced genes down- regulated in p50-/- vs WT PEC; light grey: IFNγ-induced genes not affected by p50; blue: IFNγ-repressed genes down-regulated in p50$^{-/-}$ vs WT PEC; light blue: IFNγ-repressed genes up-regulated in p50$^{-/-}$ vs WT PEC; grey: IFNγ-repressed genes not affected by p50). The number of genes belonging to each of the six clusters is indicated on the left. (E) Box plot shows the genomic distances (log2 bp) between genes induced and repressed by IFNγ and the nearest induced Stat1 peak. (F) A representative snapshot of Nos2 gene with impaired mRNA induction and Stat1 recruitment at their regulatory elements in p50$^{-/-}$ PEC stimulated with IFNγ. Arrows and black bars indicate sites of defective Stat1 occupancy. (G) Box plot of the genomic distances (log2 bp) between p50-dependent (or p50-independent) inducible genes and p50-dependent (or p50-independent) Stat1 induced peaks, showing a direct correlation between transcriptional defects and reduced Stat1 binding in p50$^{-/-}$ macrophages treated with IFNγ. *Wilcoxon signed-rank test* was used to calculate p values in E and G (p<0.01).



Supplementary Fig.5. Related to Fig.5. Ablation of p50 NF-κB restores IL-12-mediated antitumor activity *in vivo*. Left, tumor growth (MN/MCA1) in WT vs $p50^{-/-}$ mice (data shown are ±SEM P value was calculated by two-way ANOVA Vehicle n=5 WT mice, Vehicle n=5 $p50^{-/-}$ mice, IL-12 n=4 WT mice, IL-12 n=5 $p50^{-/-}$ mice). Right, metastasis formation in WT vs $p50^{-/-}$ tumor bearing mice. (data shown are ±SEM ***p<0.001 **p<0.01, *p<0.05 by two-tailed one-way ANOVA Vehicle n=4 WT mice, Vehicle n=5 $p50^{-/-}$ mice, IL-12 n=4 WT mice, IL-12 n=5 $p50^{-/-}$ mice).



Supplementary Fig.6. Related to Fig.6. Tumor supernatant (TSN) induces nuclear p50 NF-κB in BM-MDSCs. (A) Left, confocal microscopy of p50 and p65/RelA NF-κB in BM-derived M-MDSCs conditioned with either TSN, M-CSF, G-CSF or M-CSF plus G-CSF (M/G-CSF). Right, mean fluorescence intensity (M.F.I.) of nuclear p50 and p65 in BM- derived M-MDSCs conditioned with either TSN, M-CSF, G-CSF, M-CSF plus G-CSF (M/G- CSF), as indicated. PEC stimulated for 2h with LPS were used as positive controls. (**p<0.01 ***p<0.001 by two-tailed one-way ANOVA n=29 p50 nuclei and n=36 p65 nuclei medium, n= 119 p50 nuclei and n=71 p65 nuclei TSN, n=64 p50 nuclei and n=71 p65 nuclei G-CSF, n=38 p50 nuclei and n=56 p65 nuclei M-CSF, n=67 p50 nuclei and n=101 p65 nuclei M/G-CSF, n=28 p50 nuclei and n=31 p65 nuclei LPS). (B) The EP2 antagonist AH6809 and anti-PD1 antibody similarly reduce fibrosarcoma growth (data shown are mean±SEM *p<0.05 by two-tailed two-way ANOVA n=7 Vehicle treated mice, n=8 AH6809 treated mice, n=8 anti-PD1 treated mice) and lung metastasis formation in WT mice (p value was calculated by two-tailed one-way ANOVA; n=7 Vehicle treated mice, n=8 AH6809 treated mice, n=8 anti-PD1 treated mice). (C) AH6809 inhibits NOS2 expression by splenic M-MDSCs from fibrosarcoma (MN/MCA1) bearing WT mice (data shown are mean±SEM *p<0.05, **p<0.01 by two-tailed one-way ANOVA n=3 pools of WT Vehicle treated mice, n=3 pools of $p50^{-/-}$ Vehicle treated mice, n=3 pools of WT AH6809 treated mice, n=3 pools of $p50^{-/-}$ AH6809 treated mice). (D) Left, the EP2 antagonist AH6809 inhibits melanoma B16 growth only in tumor bearing WT mice, but not in the p50- deficient counterpart (*p<0.05 by two-tailed two-way ANOVA n=7). Right, AH6809 inhibits NOS2 expression by splenic M-MDSCs from melanoma B16 bearing WT mice, but not in the p50-deficient counterpart (data shown are mean±SEM *p<0.05 by two-tailed one-way ANOVA n=4 pools of WT Vehicle treated mice, n=4 pools $p50^{-/-}$ Vehicle treated mice, n=4 pools of WT AH6809 treated mice, n=4 $p50^{-/-}$ AH6809 treated mice).

Chapter 4

Interplay between RORC1 and cholesterol metabolism in cancer

Unpublished data

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Summary

Hypercholesterolemia induces RORγ up-regulation in both monocytic-MDSCs and TAMs. Disruption of RORγ expression prevents hypercholesterolemia-induced myelopoiesis disabling cancer dissemination and metastasis formation.

Abstract

Cancers induce "emergency" hematopoiesis and expansion of tumor-associated myeloid cells (TAMCs), including myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). These are heterogeneous cell populations that suppress antitumor adaptive immune responses, while promoting angiogenesis and tissue remodelling, as well as resistance to chemo-immunotherapy. Hence, the identification of mechanisms driving the tumor-promoting reprogramming of cancer-associated myeloid cells is a crucial step for the development of new effective therapies. Recently, we identified RORC1/RORγ as master inducer of emergency myelopoiesis during cancer disease. RORγ is a member of the nuclear receptor family of intracellular transcription factors that is co-activated by cholesterol-related molecules (i.e. desmosterol, oxysterols). Of note, hypercholesterolemia predisposes to cancer and induces expansion of $Ly6C^{hi}$ immature monocytes. Based on this, we hypothesized a direct correlation between cholesterol metabolism and RORγ-driven TAMCs expansion and differentiation. Interestingly, tumor bearing-mice and colorectal cancer patients displayed increased blood levels of LDL-cholesterol. Moreover, diet-induced hypercholesterolemia in tumor-bearing mice increased the metastatic rate, RORγ expression in myeloid cells, expansion of immunosuppressive monocytic-MDSCs and tumor infiltration by the $F4/80^{high}$ TAMs subset. Expansion of these myeloid subsets produced systemic immunosuppression, resulting in impaired effector memory T cells frequency in favour of quiescent naïve T lymphocytes. Our results indicate that cholesterol-related molecules guide the differentiation of suppressor myeloid cells in cancer bearers, enhancing RORγ transcriptional activity, and indicate cholesterol levels as potential markers of tumor progression. The interplay between cholesterol, activation of myeloid-specific RORγ and cancer-related myelopoiesis highlights a new immunometabolic pathway susceptible of new therapeutic developments.

Introduction

The expansion of relatively immature myeloid-derived suppressor cells (MDSCs) (emergency myelopoiesis), as well as the skewing of tumor-associated macrophages (TAMs) toward an M2-like polarization are key immune alterations of tumor bearers.[1–3] Hallmarks of MDSCs are their ability to suppress T- and NK-cells cytotoxicity and to modulate TAM functions, by conferring them anti-inflammatory/suppressive properties.[4] Further, MDSCs support tumor progression by promoting tumor cell survival, angiogenesis and metastasis.[5] Their clinical relevance has been reported in various cancers, in which frequency of circulating MDSCs correlates with cancer stage, and negatively affects cancer therapy.[6]

MDSCs are heterogeneous in terms of morphology, differentiation, distribution and functions. They are mainly subdivided into two subsets: monocytic (M-MDSCs) and granulocytic (PMN-MDSCs) identified by surface markers as $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ and $CD11b^{+}Ly6C^{lo}Ly6G^{+}$, respectively.[3,7] Recently, our lab identified the isoform 1 of retinoic acid-related orphan receptor gamma (RORC1/RORγ) as one of the lead orchestrator of cancer-driven MDSCs expansion. Indeed, MDSCs from cancer patients and tumor-bearing mice had a myeloid-specific up-regulation of RORγ. By side, *Rorc*-deficient mice showed a reduced tumor progression and an impaired expansion of MDSCs.[8]

RORγ is a nuclear receptor which, by definition, acts as a ligand-activated transcription factor.[9] The recently identified natural ligands for RORγ are cholesterol precursors[10,11] and derivatives (i.e. oxysterols)[12,13], which enhance the RORγ intrinsic activity.[14,15] Oxysterols are a wide range of biologically active end-products of cholesterol[12,16], produced enzymatically[12] or non-enzymatically after reaction with oxidative stress species (e.g. ROS)[17,18] aberrantly expressed in several cancers.[19]

Obesity is an established risk factor for the onset of many cancer types and a poor prognostic indicator in patients with established disease.[20–22] However, the multifactorial nature of obesity has made it difficult to characterize cause and effect relationships in cancer pathophysiology. A prevalent and clinically relevant factor linking cancer disease and obesity is inflammation.

Indeed, obesity is associated with a systemic chronic inflammation, resulting in altered immune cell composition in different tissues.[23–26] Obesity is generally characterized by elevated cholesterol levels and to changes in oxysterols production and metabolism.[27–29] Epidemiologic data regarding cholesterol and cancer onset are controversial and it is unclear whether total, LDL or HDL cholesterols impart risk.[30–32] Studies investigating the correlation between patients taking lowering cholesterol drugs and risk of cancer onset are conflicting.[33–35]

In addition, oxysterols are among the main components of oxidized Low Density Lipoproteins (oxLDLs),[18] which raise in dyslipidemic and hypercholesterolemic conditions, and are directly involved in the atherogenic process.[36] It has been also shown that the lectin-type oxidized LDL receptor-1 (LOX-1), one of the major scavenger receptors for oxLDLs highly expressed by macrophages and endothelial cells,[36] is mainly associated with the immunosuppressive subset of PMN-MDSCs in cancer patients.[37] Of note, whereas in liver RORγ regulates lipid metabolism by inducing the transcription of enzymes involved in oxysterols production (e.g. CYP27A1, CYP7A1),[38] dyslipidemias as well hypercholesterolemia induce an expansion of immature Ly6C^{hi} monocytes[39] and represent a risk factor for cancers.[40] The cholesterol metabolite 27-hydroxycholesterol has been described to induce expansion of polymorphonuclear cells facilitating breast cancer metastatic process.[41]

Moreover, additional studies have described that the cholesterol/oxysterols efflux transporters ABCA1 and ABCG1 are strictly associated with the expansion and the immunosuppressive properties of both MDSCs and

TAMs.[42–45] In agreement, macrophages, which are the main scavenger cells of LDL cholesterol in hypercholesterolemic conditions, have been described to be influenced in their inflammatory activity by a wide range of oxysterols (i.e. 27- and 25-hydroxycholesterol).[46,47]

Together, these evidences suggest that cholesterol metabolism and its interaction with RORγ nuclear receptor could mediate changes in myeloid cell differentiation during cancer development. Here, this thesis describes that a hypercholesterogenic Western-like diet influences the monocytic lineage in cancer-bearing mice, in a RORγ-dependent manner, promoting the metastatic process.

Results

Cancer progression alters circulating cholesterol levels

Previous observations in both cancer patients and murine models highlighted a positive correlation between hypercholesterolemia and carcinogenesis, with the major concept that high LDL cholesterol levels correlate with a poor clinical outcome in many cancer types, in contrast to the protective role of high HDL cholesterol.[48–53] In contrast, other studies suggest that no association exists between cholesterol and cancer progression[34], or even that lowering cholesterol levels with statins exacerbates disease progression.[35,54]

To clarify the significance of cholesterol levels in cancer bearers, we collected blood serum samples from wild-type mice bearing the transplantable MN/MCA1 fibrosarcoma and measured the levels of total, HDL and LDL cholesterols, in comparison with tumor-free wild-type mice. After 24-26 days of tumor growth, when mice are in advanced stages of disease, with tumor volume (MN/MCA1 fibrosarcoma) of about 2.00 cm^3 (Supplementary Fig.1A), we detected enhanced levels (mg/dl) of total and LDL cholesterols as compared to tumor-free mice, whereas the HDL levels decreased (Fig.1A). To assess the entity of this imbalance, we calculated the

ratios of total and LDL cholesterol levels upon HDL levels. Both total/HDL and LDL/HDL ratios increased in tumor-bearing mice (2-fold and 3-fold, respectively), as compared to tumor-free mice (Supplementary Fig.1B). Next, we repeated the same analysis in mice bearing the transplantable melanoma model B16/F10 (Supplementary Fig.1C) and detected a similar imbalance in serum cholesterol levels, in comparison with to tumor-free mice (Fig.1B), as observed in fibrosarcoma-bearing mice, although to a lesser extent (total/HDL ratio=1.5; LDL/HDL ratio=2) (Supplementary Fig.1D).

We next monitored the changes of cholesterol levels during disease progression: before tumor cells transplantation, 12-14 days after tumor cells inoculation (early disease stage) and after 24-26 days (late disease stage)[8]. Alteration of serum cholesterol levels was much evident 24-26 days after tumor implantation (Fig.1C), suggesting that alterations of lipid metabolism occur drastically in late stages of disease progression.

To validate this observation in cancer patients, we analysed the serum levels of total, HDL and LDL cholesterols in: healthy donors as controls (HC; n=10), colorectal cancer patients with non-metastatic disease (NM; n=10), colorectal cancer patients with a metastatic advanced disease (MTS; n=10). Noteworthy, colorectal cancer (CRC) patients showed a significant increase of total and LDL cholesterols in advanced disease, that was paralleled by decreased HDL cholesterol levels (Fig.1D). Tot/HDL and LDL/HDL ratios increased by 1.5-fold (Fig.1E).

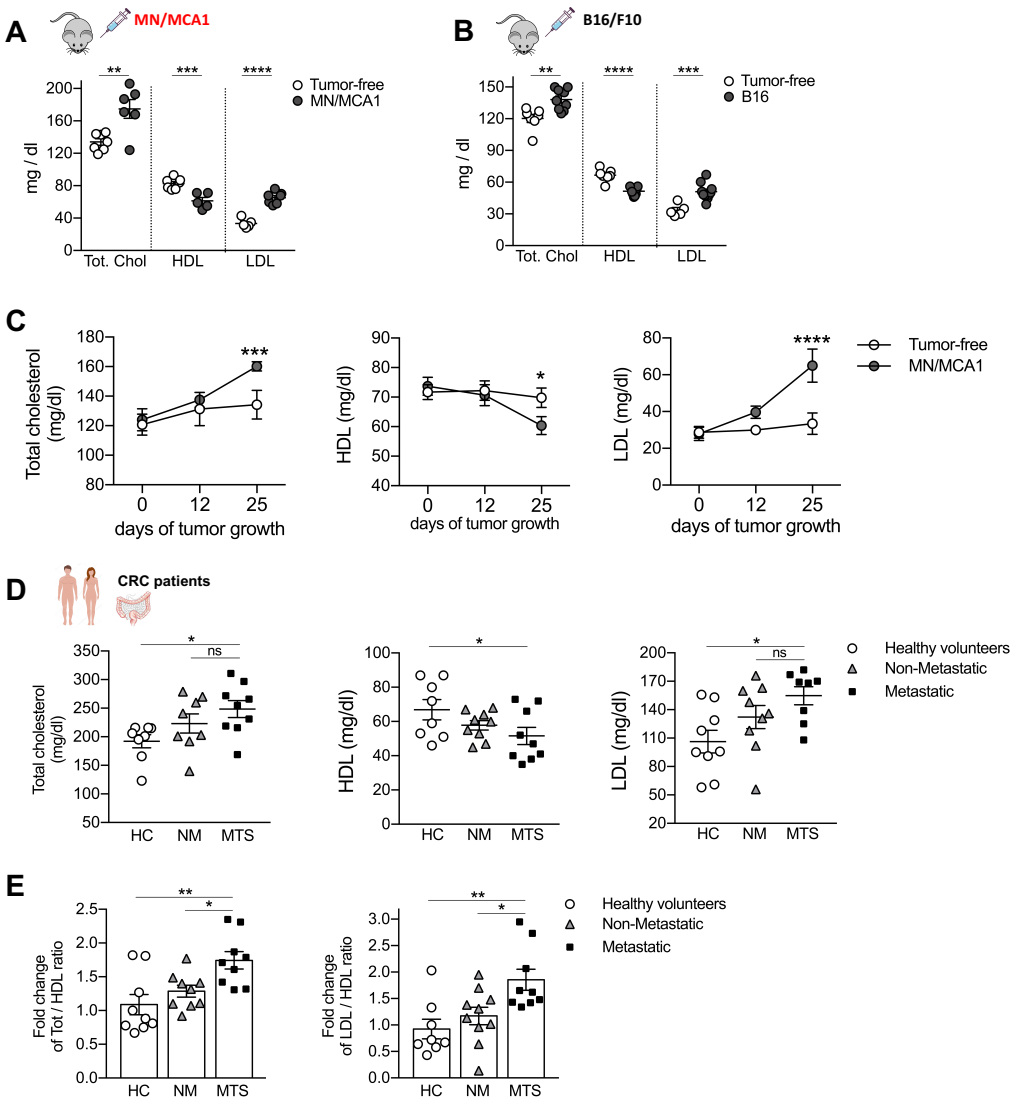

Figure 1. ***Cancer progression induces increased serum levels of total and LDL cholesterol and decreased HDL cholesterol.*** Serum levels of total, HDL and LDL cholesterols (mg/dl) in wild-type mice bearing the MN/MCA1 fibrosarcoma (A) or B16/F10 melanoma models (B) in late stage disease (26 days and 19 days of tumor growth, respectively) in comparison with tumor-free control mice. C) Serum levels of total, HDL and LDL cholesterol levels before MN/MCA1 cells transplantation, after 12 days (early disease) and 25 days (advanced disease) of tumor growth, in comparison with tumor-free control mice. D) Serum cholesterol levels of total (left panel), HDL (central panel) and LDL (right panel) cholesterols in colorectal cancer patients (CRC) in non-metastatic (NM) and metastatic disease stages (MTS), compared to healthy donors (HC). E) Values of total and LDL cholesterols in mg/dl from (D) were respectively normalized to HDL levels. Then, the ratios obtained were graphed as fold change relative to healthy donor ratios. Graphs from mice experiments are representative of 4 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

Hypercholesterogenic Western-like diet exacerbates cancer metastasis in a RORγ-dependent manner

There is compelling evidence that hypercholesterolemia, and more generally obesity, enhances tumor progression.[30,55] The nuclear receptor RORγ is activated by binding with cholesterol derivatives (i.e. oxysterols)[12,14], which are dramatically increased in obesity conditions[29]. To determine whether the anti-tumoral effect of RORγ-deficiency[8] is correlated to the hypercholesterolemia-associated cancer promotion, and to imbalance of serum cholesterol levels, we fed *Rorc*$^{-/-}$ mice and wild-type control mice with a hypercholesterogenic Western-like diet (WD), containing 0,2% w/w cholesterol and 20% w/w fat, or with a standard chow diet (CD) (containing 0,006% w/w cholesterol and 5% w/w fat). After 6 weeks of pre-conditioning with the Western diet, we transplanted obese mice with MN/MCA1 fibrosarcoma cells, keeping mice in the same dietary regimen (Fig.2A).

After 6 weeks of WD-diet pre-conditioning, we observed increased levels of total and LDL cholesterols, as well as reduced HDL, in both wild-type and *Rorc*$^{-/-}$ tumor free mice (Fig.2B). Surprisingly, after MN/MCA1 cells transplantation, we detected an exacerbated increase of total and LDL cholesterols in WD fed mice (Fig.2B, Supplementary Fig.2), as compared to chow diet-fed mice (Fig.1A,C and Fig.2B). Of note, no differences in cholesterol levels were observed between wild-type and *Rorc*$^{-/-}$ mice (Fig.2B). Moreover, while confirming tumor growth reduction in RORγ-deficient conditions (Fig.2C, D), WD-diet increased the metastasis number and bulk only in RORγ-proficient (wt) mice (Fig.2E-G). These data suggested that the pro-metastatic effects of Western diet are dependent on RORγ.

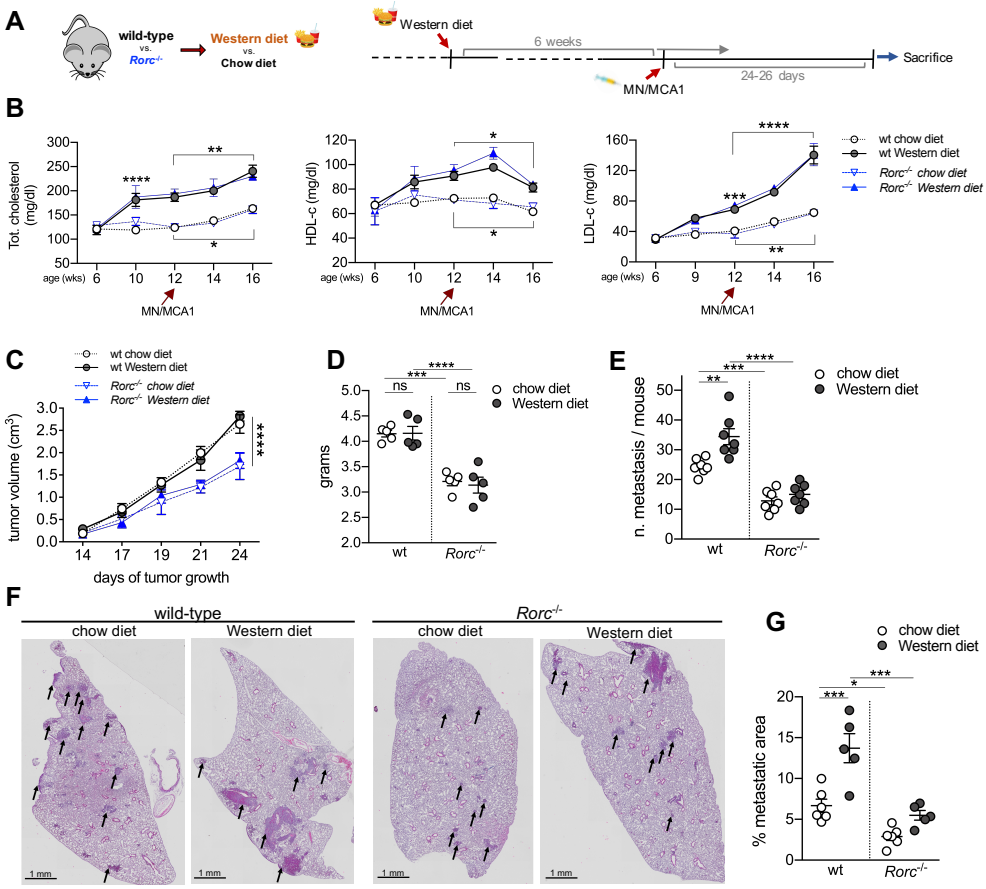

Figure 2. ***Hypercholesterogenic Western-diet exacerbates cancer metastasis in a RORγ-dependent manner.*** A) Scheme showing the experimental design: wild-type and *Rorc*$^{-/-}$ mice were pre-conditioned with hypercholesterogenic Wester-like diet (WD) or with a standard chow diet starting at 6 weeks of age. After 6 weeks of Western diet, mice were transplanted with the MN/MCA1 fibrosarcoma. Mice were kept in their respective dietary regimens until 24 days after tumor cells implantation. B) Variations of total (left panel), HDL (central panel) and LDL (right panel) cholesterol levels during the experimental period. C) Tumor volume growth curve obtained by measurements three times per week, starting from day 14 after tumor cells injection. D) Weight of tumors explanted after the sacrifice of mice at day 24 of growth. E) Number of lung metastatic lesions in tumor-bearing mice. F) Representative lung sections of 4 experimental groups underling the presence of metastatic lesions (arrows) and the differential area of lesions. G) Percentage of lung metastatic lesions. Each dot value represents the mean area of 3 serial longitudinal sections from a single lung. Graphs are representative of at least 3 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

RORγ mediates the Western diet-dependent expansion of monocytic MDSCs and pro-tumoral macrophages.

Myeloid-derived cells are key regulators of the metastatic process[56], not only in the primary tumor, but also on the distal metastatic site, where they facilitate the establishment and subsequent growth of metastatic lesions[57,58]. Since hypercholesterolemia is a condition driving the expansion of the Ly6C^{high} monocytes, precursors of both monocytic myeloid-derived suppressor cells (M-MDSCs) and tumor-associated macrophages (TAMs) [39,59], we speculated that the RORγ-dependent metastatic phenotype observed in Western diet-fed mice may involve cells of myeloid lineage. In order to test this hypothesis, we analysed by FACS the MDSC and TAM subsets in our experimental groups. In blood, as well as in the spleen, we detected an increased number of Cd11b^{+}Ly6C^{hi} M-MDSCs in wild-type mice under WD feeding as compared to chow diet-fed control mice, which was dampened in *Rorc*$^{-/-}$ mice (Fig.3A-B, left panels). In contrast, the Cd11b^{+}Ly6G^{+} PMN-MDSC subset was not affected by WD (Fig.3A-B, central panels). Interestingly, Western diet feeding induced an increase in the expression of RORγ in M-MDSC from wild-type mice, which resulted in higher levels as compared to the expression in PMN-MDSCs (Fig.3A-B, right panels; Supplementary Fig.3A).

Intratumoral analysis of infiltrating myeloid cells confirmed the WD-dependent increase of monocytic MDSCs in wild-type mice, which was impaired in RORγ deficient mice (Fig.3C, left panel), along with a higher number of Ly6C^{hi} RORγ^{+} cells expressing increased RORγ protein (Fig.3D). Regarding the intratumoral PMN-MDSC subset, we confirmed the increased frequency of Ly6G^{+} cells in *Rorc*$^{-/-}$ mice, which was not affected by different diet regimens (Fig.3C, right panel, and Ref. 8). Interestingly, intratumoral *Rorc*-deficient PMN-MDSCs displayed a more mature (CD16/CD32^{high}) inflammatory phenotype[8] (data not shown).

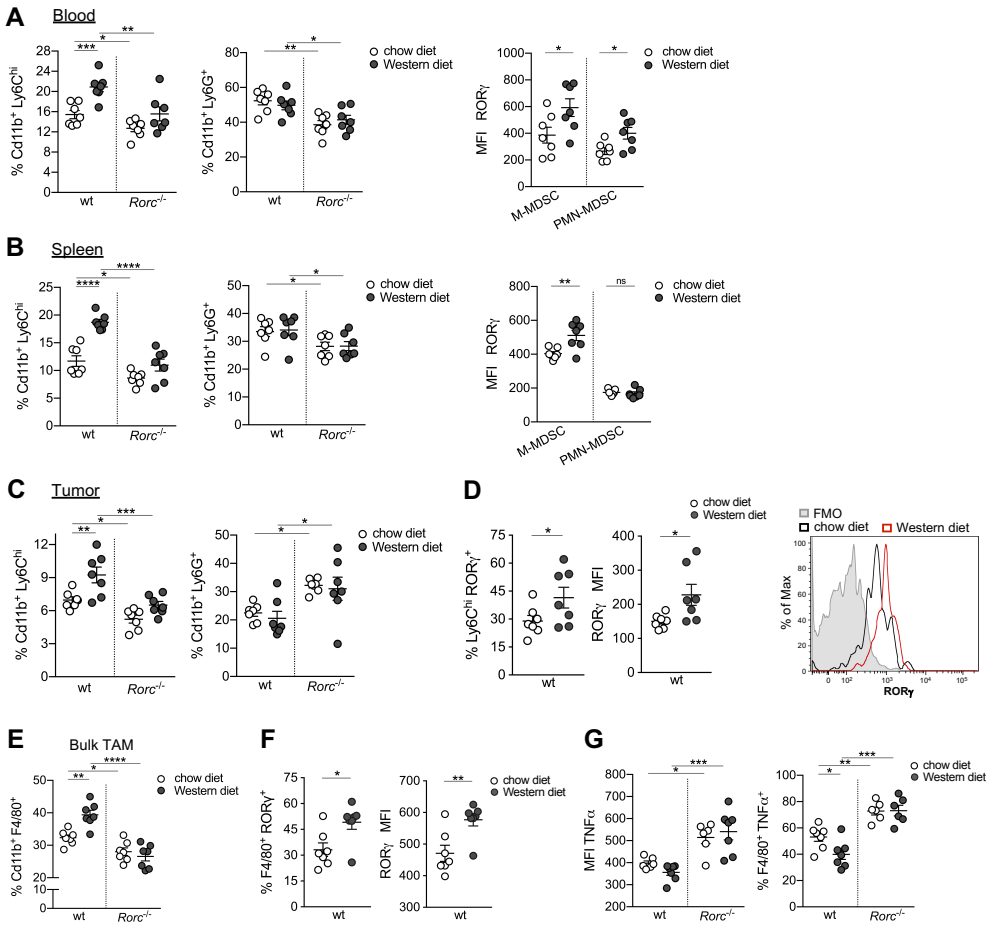

Figure 3. ***RORγ drives the Western diet-dependent increase of monocytic-MDSCs and TAMs.*** Flow cytometry analysis of $CD45^+CD11b^+Ly6C^{hi}$ (M-MDSC) and $CD45^+CD11b^+Ly6G^+$ (PMN-MDSC) cells and mean fluorescent intensity (MFI) of RORγ in blood (A) and spleen (B) of MN/MCA1-bearing mice at day 24 of growth. C) M-MDSC and PMN-MDSC percentages were evaluated in tumor tissues explanted at day 24. D) Percentage of $ROR\gamma^+$ cells (left panel) and RORγ MFI (central panel) in intra-tumoral $Ly6C^{hi}$ M-MDSCs from wild-type mice; right panel represents the differential RORγ expression between chow and Western diet conditions. E) FACS analysis of total $CD45^+CD11b^+F4/80^+$ TAMs and (F) RORγ MFI in TAMs. G) TAMs expression of TNFα. Graphs are representative of at least 3 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

TAMs are composed by heterogeneous macrophage populations.[60–62] Interestingly, FACS analysis of bulk TAMs, obtained from wild-type mice under Western diet feeding, showed significant increased number of both total $CD11b^+Ly6C^{low}F4/80^+$ cells (Fig.3E) and $ROR\gamma^+$ TAMs (Fig.3F), as

compared to chow diet condition. In analogy with M-MDSCs, this heightened presence of TAMs was absent in *Rorc*$^{-/-}$ mice fed with Western diet (Fig. 3E).

Intriguingly, TNFα^{+} TAMs significantly increased in *Rorc*$^{-/-}$ mice, independently from the dietary regimens (Fig.3G, right). These data suggested that the pro-metastatic effects of hypercholesterogenic diet could be due to RORγ-mediated increase of immunosuppressive myeloid cells.

To assess the direct effect of the Western diet on the immune cells infiltrate into metastatic sites, we analysed metastatic lung lesions by flow cytometry, combined with an unsupervised visual implementation of t-distributed stochastic neighbour embedding (tSNE [viSNE]) analysis (Fig.4A). The generated tSNE plot highlighted a marked heterogeneity in myeloid cells among the different four experimental groups (Fig.4A). Notably, major changes were observed on the Ly6C^{hi} M-MDSC subset. M-MDSCs resulted increased in metastatic lungs from wild-type mice fed with the Western diet, as compared to chow diet control mice, and this increase was dampened in *Rorc*$^{-/-}$ WD-fed mice (Fig.4A-B). Moreover, we confirmed an increased expression of RORγ by M-MDSCs (Fig.4C). Further, lung M-MDSCs from tumor-bearing *Rorc*$^{-/-}$ mice displayed a reduced expression of iNOS and increased TNFα levels in both dietary conditions (Fig.4D).

Analysis of lung PMN-MDSCs showed a significant increase in the RORγ-deficient groups, independent of the dietary regimens (Fig.4A, green cluster and Supplementary Fig.3B). No differences were observed also in the number of dendritic cells infiltrating metastatic lungs (Suppl. Fig.3C).

Recent work has highlighted the contribution of tissue-resident macrophages, including pulmonary alveolar macrophages (AM) (Cd11b^{low}CD11c^{+}F480^{dim}) (Fig.4E), in the promotion of cancer development and metastasis formation[63,64]. Interestingly, Western diet feeding increased the number of RORγ^{+} AM (Fig.4F) only in tumor-bearing wild-type mice.

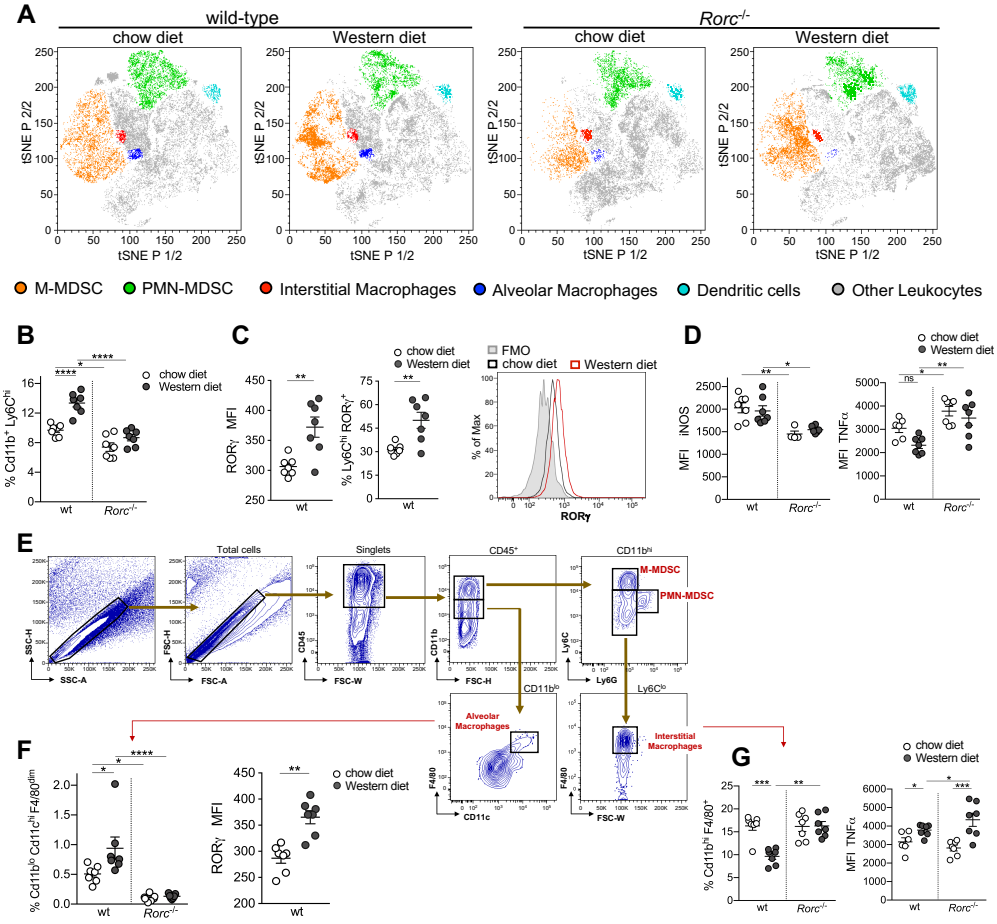

Figure 4. ***Western diet alters myeloid cells landscape in metastatic lungs in a RORγ-dependent manner.*** A) Representative tSNE plot of CD45^{+} cells in metastatic lungs using the markers Cd11b, Ly6C, Ly6G, Cd11c and F480. Cell populations defined by gating strategy (E) were projected onto t-SNE space and assigned with specific colors. B) Percentage of Ly6C^{hi} M-MDSCs in metastatic lungs. (C, left) RORγ MFI and percentage of RORγ^{+}Ly6C^{hi} cells (central panel) in wild-type M-MDSCs; right panel represents the differential RORγ expression between chow and Western diet conditions. D) FACS analysis of iNOS (left panel) and TNFα (right panel) in lung M-MDSCs. E) Representative gating strategy of Cd11b^{lo}CD11c^{hi}F4/80^{+} alveolar macrophages (AMs). F) Percentage of alveolar macrophages in metastatic lungs (left panel) and relative RORγ MFI (right panel). G) Percentage of interstitial (non-alveolar) CD11b^{hi}F4/80^{+} macrophages (left panel) and relative TNFα expression (right panel). Graphs are representative of at least 3 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

Moreover, we observed a reduced number of CD11b^{hi}F4/80^{+} interstitial macrophages (IM) in WD-fed wild-type mice (Fig.4G, left panel and Supplementary Fig.3D), expressing higher levels of TNFα (Fig.4G, right panel). In *Rorc*-deficient conditions, WD did not affect the number of IM,

which, however, expressed higher levels of TNFα (Fig.4G, right panel). This evidence suggests that the frequency of TNFα$^+$ IMs is decreased by hypercholesterogenic diet in a RORγ-dependent manner, hence favouring a pro-metastatic lung microenvironment.

Influence of RORγ/Western diet on T cells

To assess how RORγ and Western diet-conditioned myeloid cells influence adaptive immunity, we analysed by FACS the percentage of various T lymphocyte subsets. We identified an increase in primary tumor of CD3$^+$ (Fig.5A) and CD4$^+$ (Fig.5B) T cells in *Rorc*$^{-/-}$ mice in CD, (Supplementary Fig.4A,B). Western diet regimen and RORγ deficiency further increased their accumulation in the lungs. Surprisingly, an increased expression of the immune checkpoint PD-1, a marker of T cell exhaustion and inactivation[65,66], was observed in CD4$^+$ T cells from *Rorc*$^{-/-}$ and was dampened by Western diet (Fig.5C, left panel). In addition, CD4$^+$ T cells from *Rorc*$^{-/-}$ under WD feeding showed increased production of IFNγ (Fig.5C, right panel and Supplementary Fig.4C). To further characterize CD4$^+$ cells, we analysed naïve and effector memory T cell populations, defined by differential expression of CD44 and CD62L[67]. RORγ deficiency dramatically reduced the number of CD44$^+$CD62L$^-$ naïve T cells (T_n) in favour of CD44$^-$CD62L$^+$ effector memory T cells (T_{em}) (Fig.5D). Further, both wild-type and *Rorc*$^{-/-}$ WD-fed mice showed a statistically significant increase in the number of naïve T cells, as compared to the chow diet-fed mice (Fig.5D, right panel).
Analysis of CD8$^+$ T cells in metastatic lungs did not show differences in terms of number and IFNγ expression (Fig.5E, F), despite an increase in PD-1 expression was detected in wild-type mice (Fig.5G). Similarly, with CD4$^+$ T cells, we observed strong expansion of effector memory CD8$^+$ T cells in *Rorc* deficiency (Fig.5H). Together, these results indicate that a significant reshaping of the T cell compartment parallels the observed myeloid cell modifications controlled by diet regimen and RORγ.

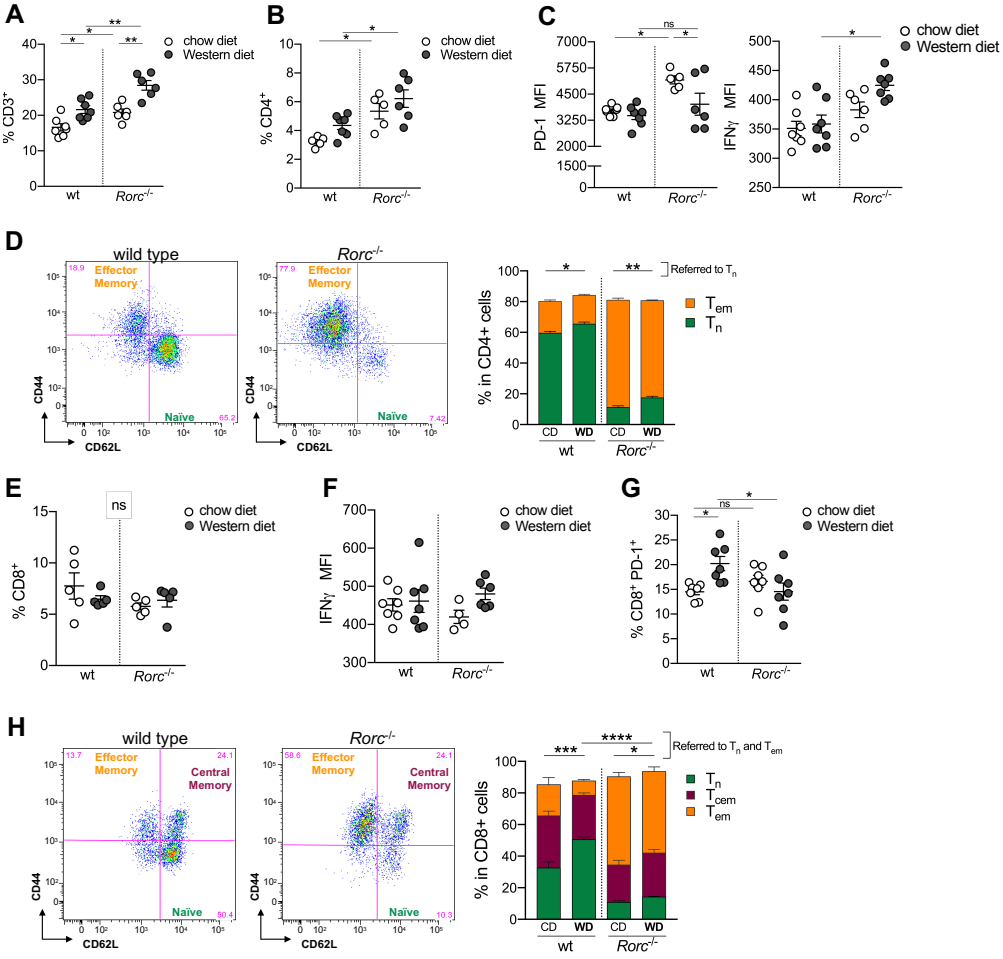

Figure 5. ***T lymphocytes rearrangement accompanies the RORγ/Western diet-dependent myeloid alterations.*** FACS analysis of $CD3^+$ (A) and $CD4^+$ T cells in metastatic lungs from wild-type and *Rorc*$^{-/-}$ mice under Western or chow diet. C) PD-1 expression levels in $CD4^+$ cells (left) and IFNγ (right panel). D) Representative gates of $CD44^-CD62L^+$ naïve $CD4^+$ T cells (T_n) and $CD44^+CD62L^-$ effector memory $CD4^+$ T cells (T_{em}) from metastatic lungs of wild-type and *Rorc*$^{-/-}$ mice, regardless of dietary conditions, which are represented as percentages in the right panel. The statistical values reported represent differences in T naïve subset (green sub-bar). E) Analysis by FACS of $CD8^+$ T cells in metastatic lungs, F) their IFNγ expression and G) percentage for PD-1 positivity. H) Representative gates of $CD44^-CD62L^+$ naïve $CD8^+$ T cells (T_n), $CD44^+CD62L^+$ central memory $CD8^+$ T cells (T_{cem}), and $CD44^+CD62L^-$ effector memory $CD8^+$ T cells (T_{em}) from metastatic lungs of wild-type and *Rorc*$^{-/-}$ mice, regardless of dietary conditions, and represented as percentages in the right panel. The statistical values reported represent differences in both T naïve and T effector memory subsets (green and orange sub-bars, respectively). No statistical differences were observed in T_{cem} subset among groups. Graphs are representative of at least 3 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

Western diet increases the highly pro-tumoral TAM F4/80^{high} subset in a RORγ-dependent manner.

We and others have recently identified two distinct subsets of TAMs on the basis of the differential expression levels of F4/80 (F4/80^{high} and F4/80^{low})[68–70]. TAM F4/80^{high} are characterized by higher in situ proliferation[70], MHC-II expression[69], M2-like polarization (higher production of *Arg1*, *Il10*, *Tgfb*, and lower *Il1b*, *Il6*, *Il12*), and inhibition of T cell activity[69]. Hence, we decided to monitor these two subsets of F4/80^{high} and F4/80^{low} in our experimental conditions.

As shown (Fig.6A-B), Western diet induced a selective increase of F4/80^{high} TAMs in wild-type mice, which was prevented in *Rorc*-deficiency conditions. Lack of RORγ reduced the F4/80^{high} TAM population also in chow diet feeding (Fig.6A, and 6B-left panel). Moreover, in *Rorc*$^{-/-}$ mice a reduction of TAM F4/80^{low} was observed in both dietary regimens (Fig.6A, and 6B-right panel). F4/80^{high} TAMs displayed higher RORγ positivity (Fig.6C and Supplementary Fig.5A) than F4/80^{low} cells, that was enhanced by Western diet (Fig.6C, Supplementary Fig.5A). In addition, F4/80^{high} TAMs expressed higher RORγ in response to WD feeding (Supplementary Fig.5C).

F4/80^{high} cells, despite any change in the iNOS expression (Supplementary Fig.5C), in wild-type mice under Western diet feeding, produced lower TNFα, which is restored in lack of RORγ (Supplementary Fig.5C).

Interestingly, Western diet enhanced PD-L1 expression by both F4/80^{high} and F4/80^{low} TAMs from wild-type mice (Fig. 6D, Supplementary Fig.5D), indicating a potential reinforcement of their suppressive activity.

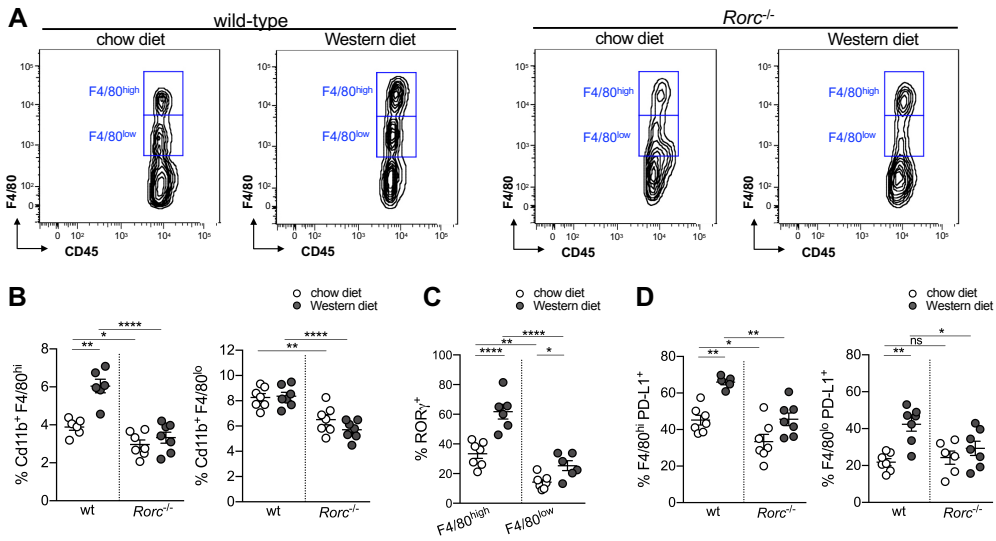

Figure 6. ***Western diet promotes expansion of F4/80^{high} TAMs in a RORγ-dependent manner.*** A) Representative gating strategy of F4/80^{high} and F4/80^{low} TAMs in wild-type and *Rorc*$^{-/-}$ mice bearing the MN/CA1 fibrosarcoma, during different dietary regimens. B) Quantification of F4/80^{high} and F4/80^{low} subset percentages in CD45^{+} cells. C) Percentages of RORγ^{+} F4/80^{high} and F4/80^{low} TAMs. D) FACS analysis of PD-L1^{+} F4/80^{high} and F4/80^{low} TAMs. Graphs are representative of at least 3 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

TAM F4/80^{high} and F4/80^{low} subsets possess different expression profiles of cholesterol metabolism-related genes

To better define the possible differential roles of F4/80^{high} and F4/80^{low} TAMs in cholesterol metabolism we performed genome wide analysis through the Agilent DNA microarray platform, comparing global gene profiling of F4/80^{high} TAMs, F4/80^{low} TAMs and control peritoneal macrophages (PEC). The results showed that F4/80^{high} and F4/80^{low} TAMs are profoundly distinct in terms of global gene expression profile (not shown, under publication). Focus on genes involved in the cholesterol metabolism regulation, synthesis, uptake, efflux and lipoprotein packaging, and downstream managing and enzymatic modifications, revealed strong differences among the different populations (Fig.7A). In particular, the expression profiles of cholesterol-related genes from F4/80^{high} subset showed distinctive signatures in each cholesterol 'sub-pathway', as compared to TAM F4/80^{low} (Fig.7A).

By qPCR we validated the expression of two additional cholesterol-related genes not included in the analysis: *Ldlr,* which encodes for the ubiquitously expressed LDL receptor, the main mediator of the uptake of exogenous cholesterol and unmodified LDL, directly involved in pro-inflammatory response of macrophages[71]; *Cyp27a1* gene encoding an enzyme converting cholesterol into 27-hycroxycholesterol, known as a RORγ activating ligand[12,15] and directly involved in several activities exerted by tumor-associated myeloid cells[41,47,72]. While *Ldlr* did not show differential expression (Fig.7B, right), *Cyp27a1* was significantly more expressed by F4/80^{high} TAMs, as compared to F4/80^{low} TAMs (Fig.7B, left panel).

Next, we monitored the protein expression of genes differentially expressed by F4/80^{high} and F4/80^{low} TAMs (Fig.7A). In particular, the macrophage scavenger receptor 1 (*Msr1*/MSR1), also known as CD204 or Scavenger receptor A-1 (SR-A1), that acts by binding and internalizing a number of ligands that predominantly include modified LDL particles[73]. Of note, CD204 is considered a marker of M2-polarized macrophages[74,75] and its expression in TAMs correlated with poor prognosis in patients[75,76].

As compared to PEC, MSR1 gene expression was up-regulated in F4/80^{high} cells and inhibited in F4/80^{low} TAMs (Fig.7A, red arrow). This trend was confirmed at protein level (FACS) (Fig.7C). Of note, lack of RORγ reduced the induced expression of MSR1 by WD feeding (Fig.7C).

The *Ch25h* gene encodes the enzyme cholesterol 25-hydroxylase (CH25H), which converts cholesterol in oxysterol 25-hydroxycholesterol (25-OHC), identified as RORγ ligand[13]. In the context of immune response CH25H and its product showed contradictory pro- and anti-inflammatory functions[77]. In macrophages CH25H is highly induced by short term stimulation with LPS, and strongly down-regulated in tolerogenic conditions.[46] We identified a higher expression of *Ch25h* mRNA in F4/80^{high} cells in comparison with F4/80^{low} cells and PEC control (Fig.7A, blue arrow). This differential expression was confirmed by FACS (Fig.7D). Notably, both F4/80^{high} and

F4/80^{low} cells from wild-type CD-fed mice showed higher CH25H expression in comparison with Western diet regimen (Fig.7D).

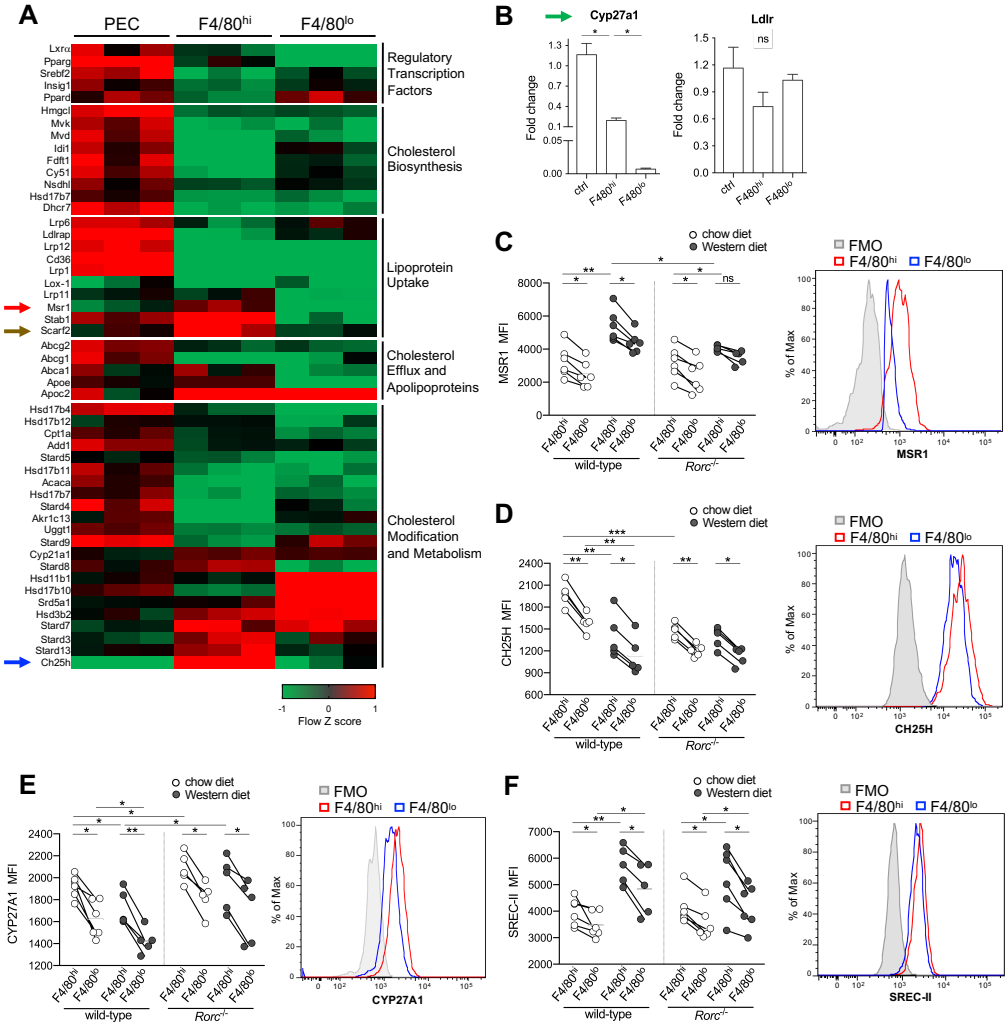

Figure 7. ***TAM F4/80^{high} and F4/80^{lo} subsets possess different expression profiles of cholesterol metabolism-related genes.*** A) Heatmap showing the differential expression of genes related to 5 sub-pathways of the cholesterol metabolism, among PEC, F4/80^{high} and F4/80^{low} TAMs. Coloured arrows indicate genes investigated for protein expression in our experimental groups (Fig.2A). B) RT-qPCR gene expression of *Ldlr* and *Cyp27a1* in PEC, F4/80^{high} and F4/80^{low} TAMs. Data are represented as mean +/- SEM of three biological replicates. FACS analysis of MSR1 (C), CH25H (D), CYP27A1 (E) and SREC-II (F) in F4/80^{high} and F4/80^{low} TAMs from wild-type and *Rorc*$^{-/-}$ mice, fed with Western or chow diet. Right panels in C), D), E) and F) are representative of the differential protein expression between the F4/80^{high} and F4/80^{low} subsets. Graphs are representative of 2 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

CYP27A1 is a key enzyme in cholesterol metabolism which, produces 27-hydroxycholesterol (27-OHC), a RORγ ligand[12] and key intermediate in bile acid production and in steroid hormone synthesis.[78] 27-OHC has direct activity on immune cells in cancer: *Cyp27a1* is highly expressed in TAMs and its product 27-OHC increases the number of inflammatory monocytes in breast cancer models.[41,72] Moreover, 27-OHC drives the M2 polarization of human monocyte-derived macrophages.[47] We observed higher expression of *Cyp27a1* mRNA in F4/80^{high} TAMs (10-fold), as compared to in F4/80^{low} TAMs (Fig.7B, green arrow), which was corroborated by its higher protein levels (Fig.7E) and decreased by Western diet in wild-type mice. F4/80^{high} from *Rorc*$^{-/-}$ mice in CD feeding showed higher levels of CYP27A1 as compared to wild-type control (Fig.7E).

We also found a marked differential expression of *Scarf2* gene. *Scarf2* (scavenger receptor class F-2) encodes for SREC-II protein whose function and activity is poorly investigated. SREC-II is known to functionally interact with its isoform SREC-I[79], which acts as a receptor for modified LDL (primarily acetylated) and is emerging for its role in innate immunity and regulation of cell signalling through Toll-Like Receptors (TLR)[80]. We revealed up-regulation of Scarf2 mRNA in F4/80^{high} TAMs, as compared to both F4/80^{low} cells and PEC (Fig.7A, brown arrow), which was confirmed by FACS (Fig.7F). Noteworthy, SREC-II was induced by Western diet feeding (Fig.7F).

In summary, these data indicate that different TAM populations express distinct signatures of genes involved in cholesterol metabolism and that this class of genes is significantly modulated by diet regimens, in a RORγ-dependent manner. The impact of this metabolic-immune network in cancer is under further investigation by our group.

Conclusions

Increasing evidences are underlining obesity as a strong risk factor for the onset of several cancers.[20–22] This association reflects the established link between inflammation and cancer, since obesity generates chronic inflammatory conditions.[21,81] Here, we investigated the influence of Western-like diet-induced hypercholesterolemia on cancer development and, in particular, its impact on RORγ-driven differentiation of tumor-associated myeloid cells.

We demonstrate in both preclinical and clinical settings that cancer progression itself induces alterations in serum cholesterol, indicating cancer as a cause (not a consequence[27,30]) of dyslipidemias. We describe that alterations of total and LDL cholesterols, as opposed to reduced HDL levels, is a hallmark of advanced cancer stages, which coincides with the expansion of myeloid suppressor cells.

Exacerbation of these events by hypercholesterogenic Western-like diet amplifies the RORγ-driven, cancer-related myelopoietic alterations[8]. In agreement with previous reports[25,41], by using a murine fibrosarcoma model, we observed that obesity-induced hypercholesterolemia alters the myelopoietic output, enhancing metastasis formation. This process is RORγ-dependent, as *Rorc* deficiency impairs cancer-driven myeloid cell expansion and dampens the metastatic burden. Indeed, tumor progression towards advanced stages increased the frequency of $Ly6C^{hi}$ $ROR\gamma^{+}$ monocytic-MDSCs in blood, spleen, as well as in the metastatic lung. These events were further magnified by Western diet and paralleled by elevation of infiltrating $ROR\gamma^{+}$ $CD11b^{+}F4/80^{+}$ TAMs. Consistently, RORγ deficiency significantly prevented these events and resulted in enhanced production of TNFα by TAMs.

Previous observations[25,41] also showed that the Western diet promotes changes in the neutrophilic subsets that accumulate in metastatic lungs. In

our tumor model (MN/MCA1) the cell types primarily affected by Western diet stemmed, in a RORγ-dependent manner, from the monocytic/ macrophage lineage and were identified as the RORγ^{+} M-MDSCs population.

Interestingly, in diet-induced hypercholesterolemia, we observed a RORγ-dependent increase of RORγ^{+} alveolar macrophages, which were described to directly impact the formation of the pre-metastatic niche in the lungs[64]. Of note, this event correlated negatively with the number of interstitial (non-alveolar) macrophages. It needs to be established whether the balance between alveolar and non-alveolar macrophages may determine metastasis formation. This evidence highlights the key role of RORγ activity in metastatic lung, since its ablation resulted in higher production of TNFα by lung macrophages and reduces metastasis formation.

T cell functions were affected along with these events. Indeed, absence of RORγ augmented the number of CD4^{+} T cells, which in Western diet conditions showed pronounced activation, characterized by low PD-1 and high IFNγ expression levels.

In parallel, CD8^{+} T cells displayed higher levels of PD-1, which were reduced by RORγ deficiency. Of interest, while lack of RORγ restrained the CD4 and CD8 naïve T cell compartment and expanded the pool of anticancer effector memory T cells[82,83], Western diet elicited an opposite effect.

Western diet-induced RORγ activity strongly influenced the functions of distinct TAM subsets. In particular, WD reduced TNFα production, while increasing frequency, RORγ and PD-L1 expression by pro-tumoral F4/80^{high} TAMs[69,70]. Minor effects were elicited on F4/80^{low} TAMs. This evidence was corroborated by the expression profile of cholesterol metabolism-related genes in the two TAMs subsets. F4/80^{high} TAMs highly expressed different scavenger receptors with the capacity to uptake modified LDL cholesterol

(i.e. Msr1, Stab-1, Scarf2), as well as two major enzymes promoting oxysterols production (CYP27A1 and CH25H) and the cholesterol efflux transporter ABCA1, which has been directly correlated with the pro-tumor activity of TAMs[42,44,45]. Additional genes controlling inflammatory pathways (MSR1, CYP27A1, CH25H, SREC-II), were also influenced to different extents by Western diet and by RORγ expression.

Overall, our work demonstrates that hypercholesterolemia induces profound changes in the myeloid cells expansion, as well as in their inflammatory and metabolic traits, which are controlled by RORγ. We conclude that the network hypercholesterolemia–RORγ activity promotes myeloid alterations and consequent disarmament of adaptive immunity in target organs, which together favour metastasis formation through organization of pre-metastatic loci[5,84–86].

Material and Methods

Ethics statement. The study was designed in compliance with: Italian Governing Law (Legislative Decree 116 of Jan. 27, 1992); EU directives and guidelines (EEC Council Directive 86/609, OJ L 358, 12/12/1986); Legislative Decree September 19, 1994, n. 626 (89/391/CEE, 89/654/CEE, 89/655/CEE, 89/656/CEE, 90/269/CEE, 90/270/CEE, 90/394/CEE, 90/679/CEE); the NIH Guide for the Care and Use of Laboratory Animals (1996 edition); Authorization n. 11/2006-A issued January 23, 2006 by Ministry of Health. The study was approved by the scientific board of Humanitas Clinical and Research Center. Mice have been monitored daily and euthanized when displaying excessive discomfort. Septic patients were enrolled in the study after signing Cancer Research Center Humanitas IRB-approved consent.

Animals. C57BL/6 mice were purchased from Charles River (Calco, Italy). Homozygous RORC mutant mice (B6.129P2(Cg)-Rorctm1Litt/J) (Sun et al., 2000) were donated by Prof. Dr. Dan Littman (New York University). Starting from 6 weeks of age mice were fed with a high cholesterol/high fat Western-like diet (ssniff ® EF D12079 mod. with 21% butterfat & cholesterol -1% corn oil) or with standard chow diet for further weeks in an IACUC-approved animal facility.

Patients. Peripheral blood was collected from healthy donors (n=10), from non-metastatic colorectal cancer patient (n=10; ≤Stage 2) and metastatic patients (n=10; ≥Stage 3). All the subjects were sex and aged mixed.

MN/MCA1 tumor model. After 6 weeks of hypercholesterogenic diet, 12 weeks old mice were injected intramuscularly in the left leg with 10^5 cells of murine fibrosarcoma (MN/MCA1). Tumor growth was monitored three times a week, and it was measured with a caliper when become palpable, starting around from day 14.

B16 tumor model. 8 weeks old wild-type mice were injected subcutaneously with $5*10^5$ cells of melanoma (B16/F10) in 200 ul of saline solution into the right flank. Tumor growth was monitored 3 times a week with a caliper, starting from day 10.

Blood cholesterol measurement. Blood samples were taken by patients and healthy donor and collected in serum tube gel separating serum from the cells. They were let to clot for 20 minutes at RT and then centrifuged for 15 min at 2000 RPM, RT. Mouse blood samples were collected via puncture of facial vein in Eppendorf tube and kept on ice for 20 min. They were centrifuged for 20 min at 13000 RPM, 4°C. Serum samples were measured for the content of total, LDL and HDL cholesterol by the Clinical Analysis Laboratory of Humanitas Hospital. Test assay kit used were purchased from Abbott (Cholesterol 7D62; Direct LDL 1E31-20; Ultra HDL 3K33-21).

Lung Histology. Histology was performed on three longitudinal serial sections (150 μm apart, 8 μm in thickness) from each left lung, formalin-fixed, paraffin-embedded, stained with hematoxylin and eosin (H&E), and scanned by VS120 DotSlide BX61 virtual slide microscope (Olympus Optical). The total number and area of lung lesions were obtained by manually tracing the perimeter of lesions using the Image Pro-Premiere software (Media Cybernetics).

Flow cytometry. Splenocytes were collected from spleen after disaggregation and filtration through Falcon strainers (70 μm). Primary tumors and right metastatic lungs were cut into small pieces, disaggregated with 0.5 mg/ml collagenase IV and 150 U/ml DNase I in RPMI-1640 for 30 min at 37 °C and filtered through strainers. Cells (10^6) were re-suspended in HBSS (Hank's balanced salt solution, Lonza) supplemented with 0.5% BSA (Sigma) and the staining was performed at 4°C for 20 minutes with specific antibodies. The following antibodies were used: CD45-FITC, -PerCP (Clone 30-F11); CD11b-PerCp- Cy5.5, -APC, -PE-Cy7, -FITC (Clone M1/70); Ly6G-PE, -APC, -FITC, BV570 (Clone 1A8); Ly6C-PE, -FITC,-APC-Cy7, -PE-Cy7, -APC (Clone HK1.4); F4/80-PE, -PE-Cy7, -APC (Clone BM8); CD11c-PE, -FITC, -APC (Clone N418); RORγ-APC, -PE (Clone AFKJS); CD204 (MSR1)-APC-Cy7, -PE-Cy7 (Clone RE148); CD8-APC, (Clone 53-6.7); CD3-PerCP, -PE, -BV650 (Clone 145-2C11); CD4-APC, PE-Cy7 (Clone GK1.5); IFNγ-PE, -FITC (Clone XMG1.2); CD44-FITC (Clone IM7); CD62L-APC, -BV570 (Clone MEL-14); TNFα-APC, -PE-Cy7 (Clone MP6-XT22); PDL1-PE, (Clone 10F.9G2); from BD Bioscience, eBioscience, BioLegend or Miltenyi Biotec. Antibody targeting mouse CH25H-FITC conjugated (bs-6480R-FITC) were purchased from Bioss. Further, we used unconjugated rabbit polyclonal anti-mouse iNOS (ab 15323, Abcam), anti-CYP27A1 (Abcam, Clone EPR7529), anti SREC-II (Invitrogen, SA101027AW) followed by incubation with secondary goat anti-rabbit Alexa Fluor® 488- or 647-conjugated antibody (Life Technologies). Cell viability was determined by LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermo Fisher), negative cells were considered viable. For intracellular staining Foxp3/Transcription Factor Staining Buffer Set (eBioscience) were used according to the manufacturer's instruction. Cells were detected using the BD FACSCanto™ II or BD LSRFortessa™ and analysed with BD FACSDiva and FlowJo (9.3.2) software.

F4/80^{high} and F4/80^{low} TAMs sorting. TAMs were isolated from tumor as previously described and were enriched by positive selection with CD11b microbeads, according to manufacturer's instructions (MACS, Miltenyi). CD11b$^+$ cells were stained with CD45 and F4/80 antibodies, and sorted with cell sorter BD FACS Aria to obtain CD45$^+$F4/80^{high} population and CD45$^+$F4/80^{low} population.

Microarray expression profiling. Thioglycollate-elicited peritoneal exudate cells (PEC) were isolated from healthy as previously described (Porta et al., 2009) and F4/80^{high} and F4/80^{low} TAMs were sorted from three biological replicates. The RNA was purified from PEC and TAMs using the RNeasy Plus Mini Kit, following standard RNA isolation protocol (Qiagen, Italy), and quantified with ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, USA). All the cRNA synthesis/sample-labelling, hybridization, washing, and scanning steps were conducted following the manufacturer's specifications (Agilent Technologies Inc., Italy). After hybridization, the slides were washed and then scanned with the Agilent G2565BA Microarray Scanner. The fluorescence intensities of scanned images were extracted and pre-processed by Agilent Feature Extraction Software (v10.5.1.1). Expression measures were computed using the robust multiarray average.

Real-Time PCR. Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. 1 μg of total RNA was reverse-transcribed by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), amplified using Fast Sybr Green Master Mix (Applied Biosystems), and detected by the 7900HT Fast Real-Time System (Applied Biosystems). The primers used are: *Ldlr (*fw, 5'-agtggccccgaatcattgac-3'; rev, 5'-ctaactaaacaccagacagaggc-3'); *Cyp27a1* (fw, 5'-ggaccggaacgctacaattt-3'; rev, 5'-tggtcttgttcagcacctgg-3'); *Gapdh* (fw, 5'-gcaaagtggagattgttgccat-3'; rev, 5'-ccttgactgtgccgttgaattt-3'). Data were processed using SDS2.2.2 software (Applied Biosystems). Results were normalized to the expression of the housekeeping gene *Gapdh* and then expressed as fold up-regulation with respect to the control cell population.

Statistics. Statistical significance was determined by two-tailed Student's t test, one-way or two-away ANOVA, depending on the experimental conditions and groups. *p value< 0.05; **p value< 0.01; ***p value< 0.001; ****p value<0,0001.

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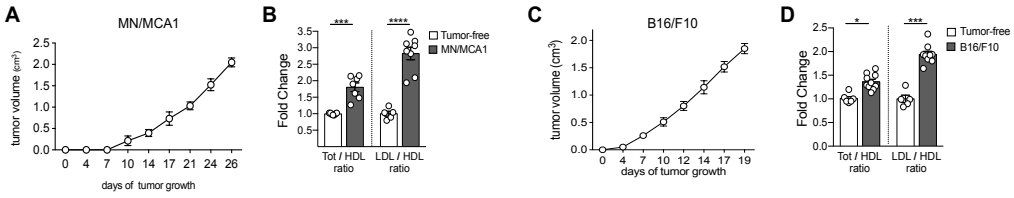
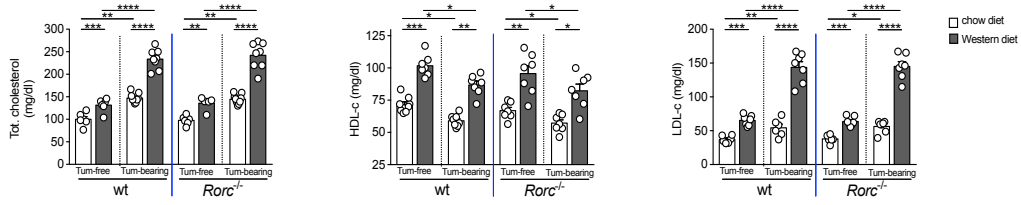
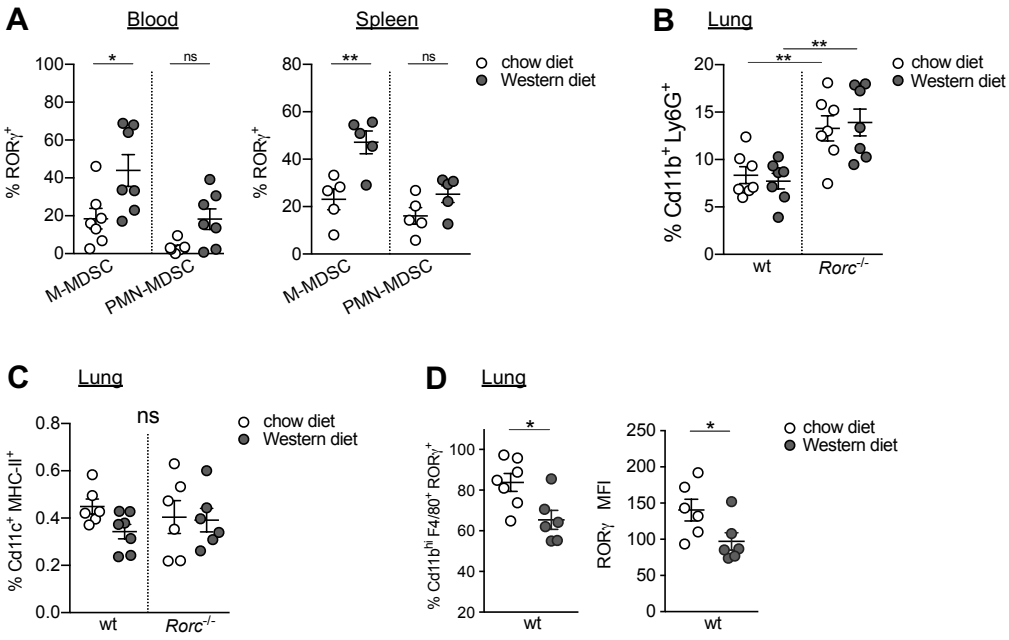
Supplementary figures



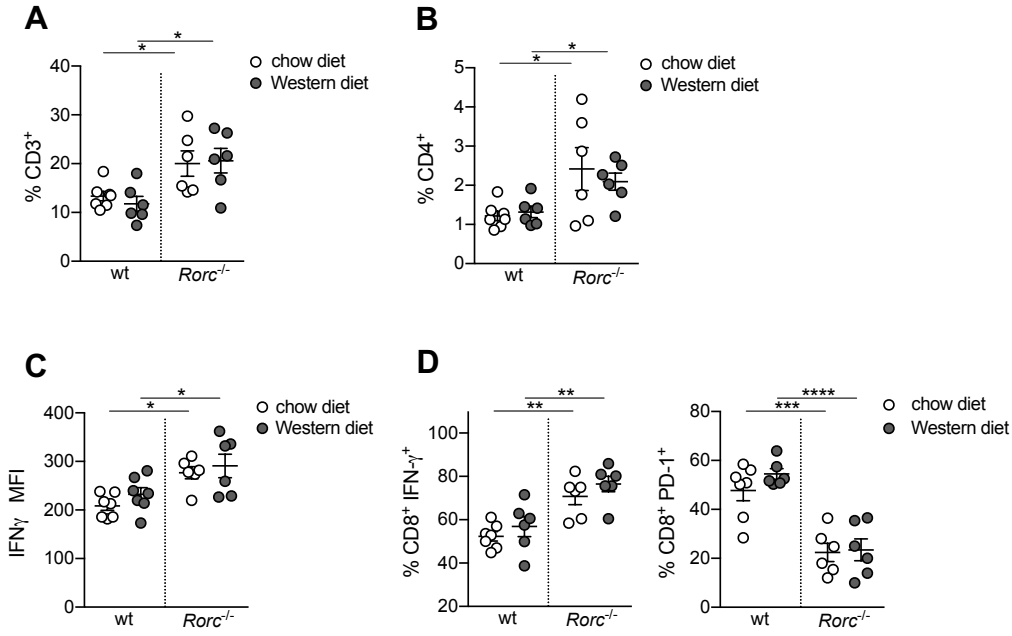
Supplementary Figure 1. Related to Figure 1. A) Representative growth curve of MN/MCA1 fibrosarcoma in wild-type mice. Measures are represented as mean +/- SEM. B) Values of total and LDL cholesterol in mg/dl from tumor-bearing mice (A, and Fig.1A) were respectively normalized to HDL levels. Then, the ratios obtained were graphed as fold change relative to tumor-free ratios. C) Representative growth curve of B16/F10 melanoma in wild-type mice. Measures are represented as mean +/- SEM. D) Total/HDL and LDL/HDL ratios from B16/F10-bearing mice were graphed as fold change relative to tumor-free ratios. Graphs are representative of 4 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.



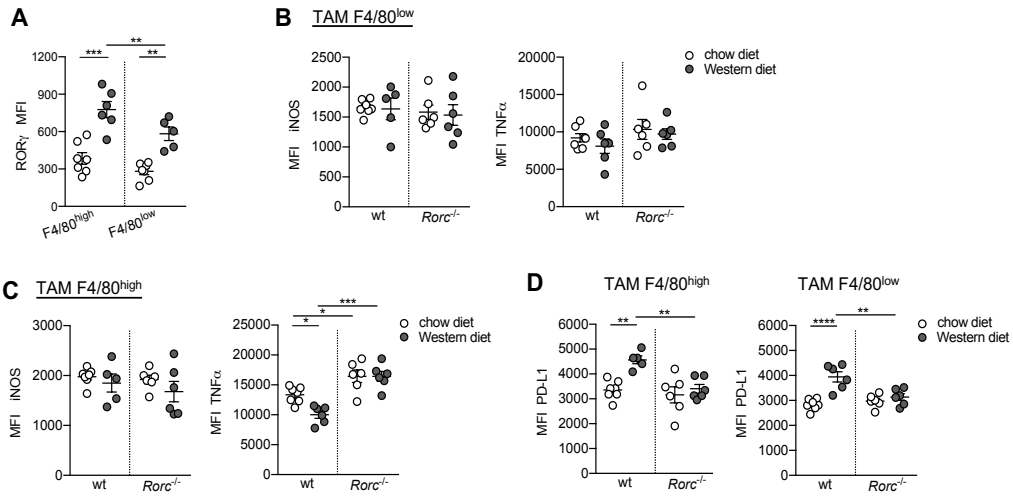
Supplementary Figure 2. Related to Figure 2. Serum levels of total (left panel), HDL (central panel) and LDL (right panel) cholesterol in wild-type and *Rorc*$^{-/-}$ mice under Western or chow diet after 25 days of MN/MCA1 fibrosarcoma growth (tum-bearing), in comparison with respective tumor-free (tum-free) control mice. Graphs are representative of 3 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.



Supplementary Figure 3. Related to Figure 3 and 4. A) FACS analysis of RORγ^{+} cell percentages in M-MDSC (CD11b^{+}Ly6C^{hi}) and PMN-MDSC (CD11b^{+}Ly6G^{+}) subsets in blood (left panel) and spleen (right panel) of wild-type and *Rorc*$^{-/-}$ MN/MCA1 fibrosarcoma-bearing mice after 25 days of tumor growth, and under Western or chow diet feeding. Quantification of (B) PMN-MDSCs and of (C) non-resident CD11b^{+}Cd11c^{+}MHC-II^{+} dendritic cells (DCs) into metastatic lungs. D) FACS analysis of RORγ expression in non-alveolar interstitial macrophages (CD11b^{hi}F4/80^{+}) indicated as RORγ^{+} cells and MFI of RORγ protein. Graphs are representative of at least 2 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.



Supplementary Figure 4. Related to Figure 5. FACS analysis of (A) $CD3^+$ and (B) $CD4^+$ cells into tumor microenvironment of wild-type and *Rorc*$^{-/-}$ MN/MCA1 fibrosarcoma-bearing mice after 25 days of tumor growth, and under Western or chow diet feeding. C) IFNγ expression in tumor-infiltrated $CD4^+$ T cells. D) Quantification of $IFN\gamma^+$ (left panel) and $PD\text{-}1^+$ (right panel) $CD8^+$ T cells in metastatic lungs. Graphs are representative of at least 2 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.



Supplementary Figure 5. Related to Figure 6. A) RORγ expression in TAM F4/80^{high} and F4/80^{low} from wild-type mice bearing the MN/MCA1 fibrosarcoma, under Western or chow diet. FACS analysis of iNOS (left panels) and TNFα (right panels) expression in (B) TAM F4/80^{low} and (C) F4/80^{high} subsets in wild-type and *Rorc*$^{-/-}$ MN/MCA1-bearing mice under Western or chow diet. D) Quantification of PD-L1 expression in F4/80^{high} and F4/80^{low} TAMs. Graphs are representative of at least 2 independent experiments. Data are represented as mean +/- SEM. Statistical analysis: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

Chapter 5

Discussion

The last decade has seen a revolution in cancer treatments, with a definite integration of conventional therapies (e.g. chemotherapy and radiation) with new immunomodulatory drugs, which improve specific immune responses against tumors. In particular, the identification of immunological checkpoints (e.g. CTLA-4, PD-1)[1,2] led to a radical paradigm shift in fundamental immunology, making cancer immunotherapy effective. First generation of immunotherapeutic agents include blocking antibodies of CTLA-4 (ipilimumab) and PD-L1/PD-1 (atezolizumab/nivolumab, pembrolizumab), able to reactivate T cell specific responses against tumor cells.[3] Unfortunately, such immune checkpoint blockade therapy (ICB) today it is effective only in a minority of patients.[4]

Retrospective analyses of patients treated with immunotherapeutic agents have revealed that individual patients may be classified by distinct immune-profiles, which largely predict their clinical responses [5–7].

To clarify the mechanisms that guide the immunological set-up of the individual patient during disease progression, our laboratory is trying to decipher the molecular determinants that tumors use to impose this immunological reprogramming, paying particular attention to the myeloid compartment. Myeloid cells represent the most abundant cell lineage that expand in response to cancer-related inflammation[8]. This event, defined as 'emergency' myelopoiesis, generates distinct subsets of tumor-promoting and suppressive myeloid cells, including tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs)[9]. MDSCs are further subdivided in two subsets: granulocytic and monocytic (PMN-MDSC and M-MDSC, respectively).[10] The high frequency of immunosuppressive myeloid cells into tumor microenvironment has emerged as negative prognostic indicator, in terms of survival and clinical response to therapies.[11,12]

To study the molecular determinants of "emergency myelopoiesis", we hypothesized that tumors release factors that act locally, in the tumor microenvironment, and distally, in the bone marrow, acting on both more mature myeloid cells and on medullary progenitors. We then focused on transcriptional and epigenetic events, as potential determinants of these phenomena, hypothesizing that the correction of such transcriptional and epigenetic aberrations could reprogram the MDSCs and TAMs towards an antitumor modality, improving the outcome of cancer immunotherapies.

In this scenario, our lab has previously identified that p50 NF-κB transcription factor accumulates into the nucleus of tolerogenic macrophages and TAMs, and regulates the transcription of genes mediating immunosuppressive and anti-inflammatory functions.[13,14] Macrophages arise from the same common myeloid progenitor of monocytic MDSCs. Moreover, TAMs and MDSCs have a similar M2-like immunosuppressive gene signature. Therefore, we investigated the potential role of p50 NF-κB in the expansion, recruitment and functional properties of M-MDSCs.

Accumulation of nuclear p50 NF-κB in both mouse and human drive the expansion of M-MDSCs and induced the production of immunosuppressive molecule NO in response to IFNγ. Indeed, the ablation of p50 NF-κB reactivated specific antitumor immunity and reinstated the *in vivo* antitumor activity of IL-12 and IFNγ cytokines. The accumulation of p50 NF-κB in myeloid cells promotes the IFNγ-mediated binding of STAT1 onto specific gene regulatory elements of IFNγ-responsive genes, including *Nos2*. We also observed that lack of p50 results in increased monocytic myeloid cells in the spleen of tumor-bearing mice, with low NO production and suppressive activity.

COX2-stimulated production of PGE2 enhances the expansion of MDSCs and promotes suppression of adaptive immunity[15]. We show that antagonists

of the PGE2 receptors reprogram M-MDSC functions in response to IFNγ towards an anti-tumor inflammatory phenotype. COX inhibitors (e.g. aspirin) were suggested for prevention and treatment of cancer[16]. Nevertheless, due to their severe side effects, targeting specific prostanoid receptors endowed with immunosuppressive properties, such as EP2 [17–19], might provide an alternative selective and safer approach to burst specific immunity in cancer patients.

Overall, our work on p50 NF-κB indicates that its nuclear accumulation corrupts the differentiation and activation of M-MDSCs leading to immunosuppressive pro-tumoral properties. In addition, during cancer disease the chronic inflammatory status and the subsequent constant production of PGE2 mediate the corruption of M-MDSCs at the epigenetic level, establishing a pattern of gene expression which exerts their immunosuppressive phenotype. Thus, this study suggests the use of PGE2 receptor antagonists as potential adjuvants for anticancer immunotherapy.

We have previously shown that the transcription factor RORC1/RORγ is up-regulated in myeloid cell precursors during cancer progression, driving the expansion and the immunosuppressive properties of MDSCs and TAMs.[20]

Of interest, RORγ intrinsic activity is enhanced by cholesterol-related molecules, including its precursors and oxysterols.[21] Further, hypercholesterolemia and cholesterol-derived oxysterols can shape the myeloid cells during cancer and obesity[22–25]. This evidence suggested a possible link between cholesterol metabolism and myeloid cells fate in cancer bearers.

Interestingly, we observed that cancer progression influences lipid metabolism, altering serum cholesterol levels in a way that mimics hypercholesterolemia (elevation of total and LDL, and decreased levels of HDL). In addition, administration of high cholesterol-content Western-like

diet to mice exacerbated metastasis formation. These effects were associated with increased numbers of both RORγ^{+} M-MDSCs and RORγ^{+} TAMs.

Consistently, RORγ ablation reduced the expansion and the immunosuppressive phenotype of both M-MDSCs and TAMs, impairing the pro-metastatic effects of Western diet, but with no influence on the cholesterol levels.

Noteworthy, RORγ, with a participation of Western diet-mediated expansion of RORγ^{+} M-MDSCs and RORγ^{+} TAMs, dramatically dampened the frequency of antitumor effector memory cells (both CD4 and CD8), in favour of quiescent naïve T cells, indicating a possible link between obesity/hypercholesterolemia, immunosuppression and tumor progression.

New evidence indicates that TAMs are characterized by significant heterogeneity. Indeed, TAMs display different expression levels of various macrophage markers, including of F4/80 and others (e.g. MHC-II), that allow distinction among different TAM subsets. The F4/80^{high} TAM subset was established to have a pronounced pro-tumoral activity, as compared to F4/80^{low} TAMs. We observed that F4/80^{high} TAMs are induced by Western diet in a RORγ-dependent manner. Moreover, genomic analysis revealed that this subset expresses a cluster of genes related to cholesterol metabolism, with the capacity to ‘sense’ and metabolize exogenous cholesterol (oxysterol formation and efflux).

Our results underline new intricate pathways of immune-metabolic interactions, conferring to RORγ the status of key player in cancer-driven dysmetabolism (namely hypercholesterolemia) and alterations of myeloid immune functions. Our observation suggests that RORγ inhibitors and/or of cholesterol-lowering drugs (e.g. statins, ezetimibe) may act as potential immunomodulators, alone or in combination with standard immunotherapy, and supports its potential relevance in cancer patients affected by obesity or hypercholesterolemia.

In summary, this study highlights the relevance of the crosstalk between immunity and metabolism and places myeloid cells at the crossroads of this intricate system. The characterization of the metabolic state and the impact on the patient's immunophenotype appears increasingly relevant for the design of personalized therapies, aimed at correcting dysmetabolic conditions and immune dysfunctions that favour disease progression.

Collectively, our data identify p50 NF-κB as key driver of immunosuppressive M-MDSCs in response to tumor-derived PGE2 and potentiate the evidence that RORγ orchestrates the pathological differentiation of suppressor myeloid cells, with a remarkable action in dyslipidemic conditions. Hence, inhibitors of PGE2/p50 NF-κB and of cholesterol/RORγ axis could be useful in the improvement of anticancer therapy.

Contribution to the work

My contribution to the work described and discussed in Chapter 3 (*Tumor-derived prostaglandin E2 promotes p50 NF-κB-dependent differentiation of monocytic MDSCs*) included the planning and the execution of part of the experiments conducted in our laboratory.
In cooperation with the first authors (C.P. and F.M.C.), I contributed to design and perform the key experiments both *in vivo* and *in vitro*. I gave a considerable contribution to the manufacture, preparation and analysis of samples. I collaborated in the interpretation of the results, provided critical feedback and helped shape the research. I discussed the results with the team and contributed to the setting and the revision of final manuscript.

The work described in Chapter 4 (*Interplay between RORC1 and cholesterol metabolism in cancer*) was fully carried out under my responsibility and with the supervision of Prof. Antonio Sica. We both conceived the presented idea and hypothesis.
I studied, designed and planned all the experiments which were performed with the technical support of F.M.C and A.M. I analysed, interpreted and discussed the results with the contribution of the team. I produced critical feedback and consequently shaped the research and the experiments, with critical indication by Prof. Antonio Sica.
I took the lead in setting and writing the manuscript in consultation with the supervisor, who revised the final version.

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Chapter 6

List of publications

1. **Membrane Cholesterol Regulates Macrophage Plasticity in Cancer.**

   Sica A, Bleve A, Garassino MC.

   Cell Metabolism (2019); 29(6):1238-1240.

2. **Insulin-Driven PI3K-AKT Signaling in the Hepatocyte Is Mediated by Redundant PI3Kα and PI3Kβ Activities and Is Promoted by RAS.**

   Molinaro A, Becattini B, Mazzoli A, Bleve A, Radici L, Maxvall I, Sopasakis VR, Molinaro A, Bäckhed F, Solinas G.
   Cell Metabolism (2019); 29(6):1400-1409.e5.

3. **Myeloid-Derived Suppressor Cells: Ductile Targets in Disease.**

   Consonni FM, Porta C, Marino A, Pandolfo C, Mola S, Bleve A, Sica A.

   Frontiers in Immunology (2019); 10:949.

4. **Metabolic influence on the differentiation of suppressive myeloid cells in cancer.**

   Porta C, Marino A, Consonni FM, Bleve A, Mola S, Storto M, Riboldi E, Sica A.
   Carcinogenesis (2018); 39(9):1095-1104.

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