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ROLE OF p50 NF-κB IN CANCER RELATED INFLAMMATION

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Milioni di persone hanno visto la caduta della mela, ma Newton è stato colui che ha chiesto "perchè"
(Bernard M. Baruch)

Contents

CHAPTER ONE

1.1 Inflammation and cancer

The idea of a relationship between inflammation and cancer dates back 1863, when Rudolf Virchow observed leukocyte infiltration in neoplastic tissues and hypothesized that the origin of cancer was at sites of chronic inflammation. Yet, it was only during the last decade that numerous studies undoubtedly demonstrated the critical role of inflammation in tumorigenesis, and that some of the underlying molecular mechanisms have been elucidated [1]. Nowadays several lines of evidences [2-4] (Box 1) – based on a range of findings from epidemiological studies of patients to molecular studies of genetically modified mice – have led to a general acceptance that inflammation and cancer are linked.

Box 1 | The evidence that links cancer and inflammation

- Inflammatory diseases increase the risk of developing many types of cancer (including bladder, cervical, gastric, intestinal, oesophageal, ovarian, prostate and thyroid cancer).
- Non-steroidal anti-inflammatory drugs reduce the risk of developing certain cancers (such as colon and breast cancer) and reduce the mortality caused by these cancers.
- Signalling pathways involved in inflammation operate downstream of oncogenic mutations (such as mutations in the genes encoding RAS, MYC and RET).
- Inflammatory cells, chemokines and cytokines are present in the microenvironment of all tumours in experimental animal models and humans from the earliest stages of development.
- The targeting of inflammatory mediators (chemokines and cytokines, such as TNF-α and IL-1β), key transcription factors involved in inflammation (such as NF-κB and STAT3) or inflammatory cells decreases the incidence and spread of cancer.
- Adoptive transfer of inflammatory cells or overexpression of inflammatory cytokines promotes the development of tumours.

In pathological conditions, as after tissue injury, the inflammatory process is the first response of the body designed to "heal" the afflicted tissue. This involves activation and direct migration of leukocytes (neutrophils, monocytes and eosinophils) from the venous system to sites of damage. This cellular migration is controlled by a family of chemotactic cytokines, named chemokines, which possess a relatively high degree of specificity for specific leukocyte populations chemoattraction [5, 6], recruits downstream effector cells and dictates the natural evolution of the inflammatory response.

Hence, inflammation is a fundamental process both for physiological conditions and to protect the body against different exogenous and/or endogenous treats and it is strictly controlled and self-limiting: the disregulation of this mechanism can become an health-treatening event. In fact, in chronically

inflamed tissues, a subversion of cell death and/or repair programmes might occur, resulting in uncontrolled proliferation of cells that carries DNA mutations and predisposes tissues for cancer development.

Indeed, it is estimated that 20% of all cancers is associated with chronic infection and inflammation [7] and that underlying infections and inflammatory responses are linked to 15-20% of all deaths from cancer worldwide [8].

In addiction, a "smouldering" inflammation is present also in tumors not causally related to an obvious inflammatory process. Recent evidence have indeed demonstrated that different classes of oncogenes (e.g. RET [9], RAS and MYC) and tumor-suppressor genes (e.g.VHL, TGFβ and PTEN) regulate the expression of inflammation-related programs [10, 11].

As well as clinical correlations, also molecular evidences show that infiltrating leukocyte can be involved in carcinogenesis and/or tumor invasion and metastasis, [12-15] indicating inflammation as the "Seventh hallmark of cancer"(Fig.1)[16-18].

In some types of cancer, inflammatory conditions are present before a malignant change occurs (extrinsic pathway). Conversely, in other types of cancer, an oncogenic change induces an inflammatory microenvironment that promotes the development of tumours (intrinsic pathway) (Fig. 2).

The extrinsic pathway starts with unresolved and prolonged inflammatory conditions that produce activation of transcription factors and DNA mutations with consequent alteration of the physiological cellular processes like proliferation, survival and apoptosis.

On the other side, in the intrinsic pathway an early genetic event is necessary and sufficient for the development of tumor and directly promotes the build-up of an inflammatory microenvironment.

The activation of transcription factors, mainly nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α), in tumour cells are the points in which the two

pathways converge and, as a consequence, link DNA mutations with the production of inflammatory mediators. These, in turn, recruit and activate various leukocytes, most notably cells of the myelomonocytic lineage, and activate the same key transcription factors in inflammatory cells, stromal cells and tumor cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated in a sort of positive feedback loop. This uncontrolled and non self-limiting cancer-related inflammation has many tumour-promoting effects including induction of genomic instability, alteration in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation and resistance to apoptosis of initiated cells, induction of tumour angiogenesis and tissue remodelling with consequent promotion of tumour cells invasion and metastasis [19].

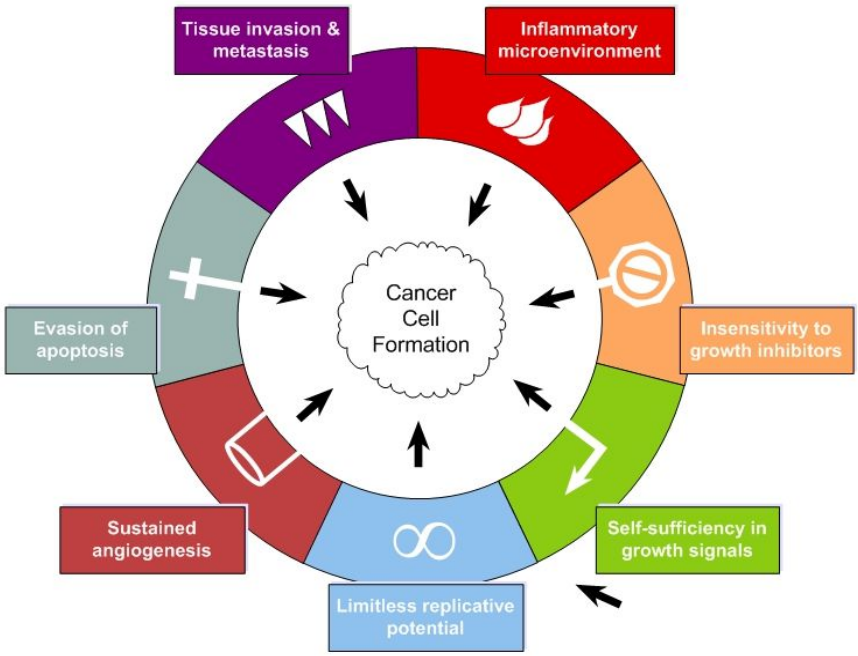

Figure 1. Inflammation as the seventh hallmark of cancer
In 2000, Hanahan and Weinberg proposed a model to define the six properties that a tumour acquires. These are unlimited replicative potential, ability to develop blood vessels (angiogenesis), evasion of programmed cell death (apoptosis), self-sufficiency in growth signals, insensitivity to inhibitors of growth, and tissue invasion and metastasis. Next studies indicate that this model should be revised to include cancer-related inflammation as an additional hallmark. Adapted from [17] and [18].

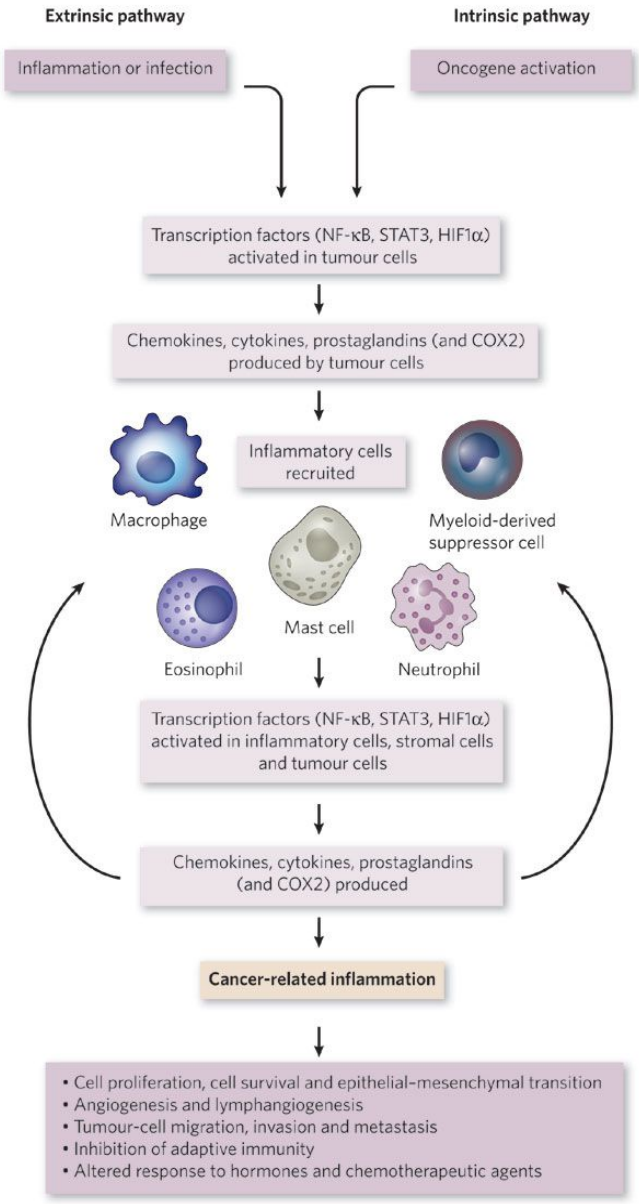

Figure 2. Pathways that connect inflammation and cancer.

Cancer and inflammation are connected by two pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is activated by genetic events that cause neoplasia. These events include the activation of various types of oncogene by mutation, chromosomal rearrangement or amplification, and the inactivation of tumour-suppressor genes. Cells that are transformed in this manner produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumours for which there is no underlying inflammatory condition (for example, breast tumours). By contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites (for example, the colon, prostate and pancreas). The two pathways converge, resulting in the activation of transcription factors, mainly nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α), in tumour cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines, as well as the production of cyclooxygenase 2 (COX2) (which, in turn, results in the production of prostaglandins). These factors recruit and activate various leukocytes, most notably cells of the myelomonocytic lineage. The cytokines activate the same key transcription factors in inflammatory cells, stromal cells and tumour cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated. Smouldering cancer-related inflammation has many tumour-promoting effects. From [19].

However, despite these evidences, genetic studies of mouse models have demonstrated that the inflammatory response supported by innate immune cells is crucial for the activation of an adaptive immune response capable of eliminating nascent tumors [20].

It is generally accepted that immune cells continuously recognize and destroy nascent tumor cells but, due to the genetic instability that characterized

neoplastic cells, the arising of new variants able to evade the immune surveillance results in tumor establishment and progression (immunoediting process; Fig.3) [21]. Only when tumor cells reach to escape the immune cells surveillance, the cancer-related inflammation develops. This is mostly dependent on cytokines and chemokines produced by tumor cells that subvert the anti-tumor activity of inflammatory cells toward a tumor-promoting condition. In this regard, several studies have emphasized that the "smouldering" inflammation associated with tumors is mainly oriented to tune the adaptive immune response.

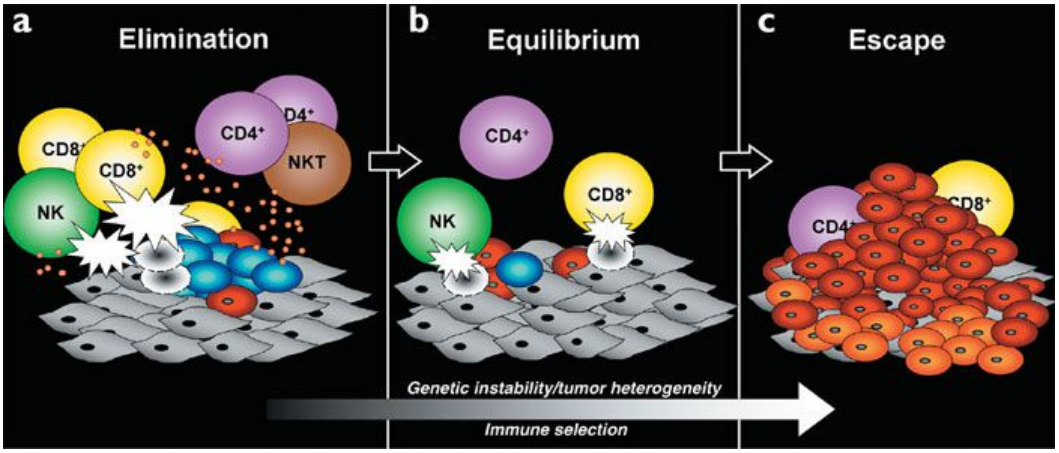

Figure 3. Immunoediting process
Cancer immunoediting encompasses three processes: (a) Elimination corresponds to immunosurveillance. (b) Equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. (c) Escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host. In a) and b), developing tumor cells (blue), tumor cell variants (red) and underlying stroma and nontransformed cells (gray) are shown; in c, additional tumor variants (orange) that have formed as a result of the equilibrium process are shown. Different lymphocyte populations are as marked. The small orange circles represent cytokines and the white flashes represent cytotoxic activity of lymphocytes against tumor cells. From [21].

Myeloid cells in cancer

Myeloid cells are the most abundant haematopoietic cells in the human body and have diverse functions. All myeloid cells arise from multipotent haematopoietic stem cells (HSCs) that develop into mature myeloid cells through sequential steps of differentiation. The three groups of terminally differentiated myeloid cells — macrophages, dendritic cells (DCs) and

granulocytes — are essential for the normal functions of the innate and adaptive immune systems [22].

In addition to their physiopatological roles, infiltrating myeloid cells are an abundant component of solid tumours. Thanks to molecular interactions with tumour and stroma, cells of the myeloid lineage recruited at tumour site change their transcriptional program toward a pro-tumoural phenotype that supports tumour growth inducing immunosuppression, angiogenesis and tissue remodeling. It is well recognized that tumor-derived factors (TDFs), such as cytokines, chemokines and inflammatory messengers like prostaglandins, act in paracrine or systemic fashion to 'reprogram' non-cancerous host cells to promote tumour progression.

Myelopoiesis is a tightly regulated process of cellular development occurring in the bone marrow. Consequently, chronic exposure of the bone marrow microenvironment to non-physiologic levels of ordinarily tightly regulated myelopoietic-like growth factors corrupts the normal process of myeloid cell development and differentiation. This phenomenon drives the increase of circulating myeloid cells in tumour-bearing hosts, originally termed "emergency myelopoiesis", and it is associated with a partial blockade of myeloid cell differentiation and a consequent accumulation of highly immunosuppressive, immature myeloid cells (iMCs) [17, 22]. Indeed, many TDFs are myelopoietic factors making the myeloid compartment a major target of this 'tumour reconditioning' [23]. For example, cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF; also known as KIT ligand), vascular endothelial growth factor (VEGF) and IL-3 are described to promote myelopoiesis and to contribute, in part, to a blockade of myeloid cell maturation. Myeloid deficiencies can occur at developmental and/or functional levels in essentially all myeloid lineages. To distinguish "normal" myeloid cells from their dysfunctional counterparts, the latter

populations have been variously renamed myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs), tumour-associated neutrophils (TANs), immature DCs or tolerogenic DCs (Fig.4).

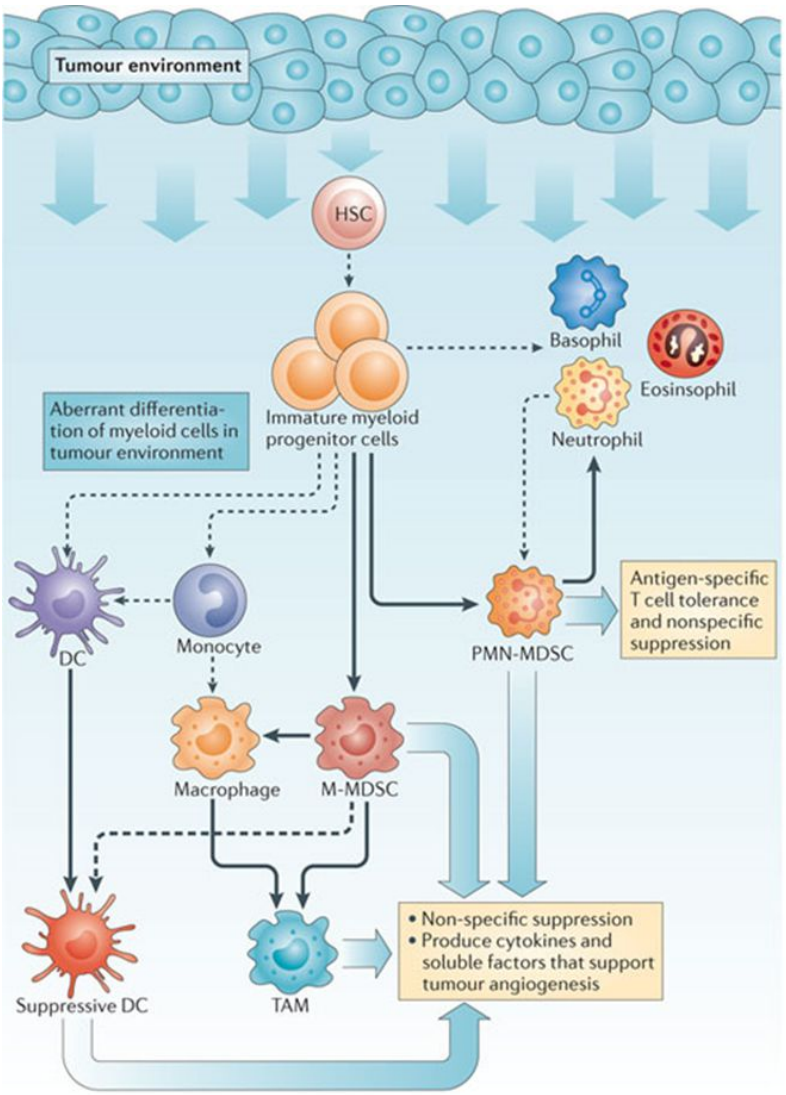

Figure 4. Myeloid cells in cancer. Factors produced in the tumour microenvironment by tumour cells and stromal cells promote the aberrant differentiation of myeloid lineage cells. The dotted lines show the normal pathways of myeloid cell differentiation from immature myeloid precursor cells to dendritic cells, macrophages and granulocytes. The solid bold lines indicate the aberrant pathways of myeloid cell differentiation that occur in cancer, in which the tumour environment can promote the development of various immunosuppressive populations, including monocytic MDSCs, polymorphonuclear MDSCs, suppressive DCs and TAMs and TANs. From [22].

These classifications are largely based on assays (*in vitro* and/or *in vivo*) that measure how these cells affect immune activation or tumour growth and on surface marker expression. Among these, for example, we have recently identified subsets of MDSCs and TAMs based on the expression of retinoic-

acid-related orphan receptor (RORC1/RORγ) in human and mouse tumor bearers. In these disfunctyonal myeloid cells, RORC1 orchestrates myelopoiesis by suppressing negative (Socs3 and Bcl3) and promoting positive (C/EBPβ) regulators of the key transcriptional mediators of myeloid progenitor commitment and differentiation to the monocytic/macrophage lineage (IRF8 and PU.1). RORC1 supported tumor-promoting innate immunity by protecting MDSCs from apoptosis, mediating TAM differentiation and M2 polarization, and limiting tumor infiltration by mature neutrophils [24].

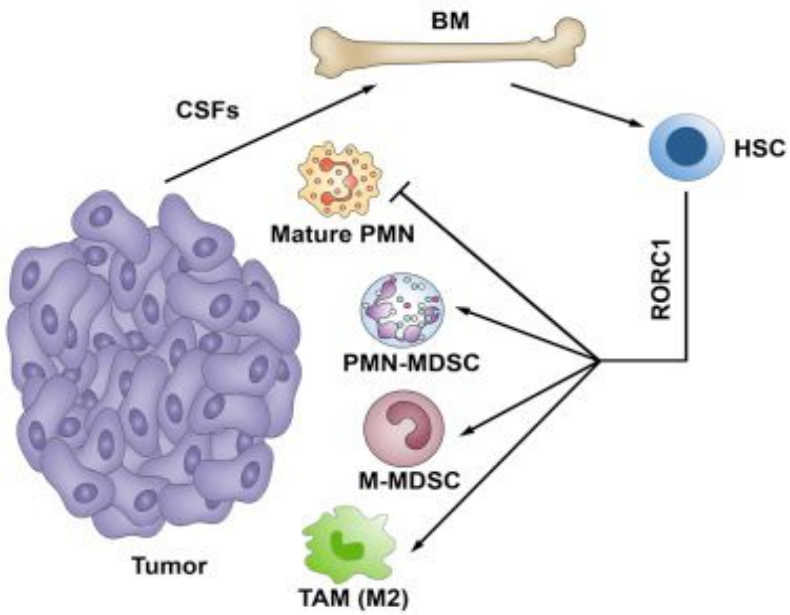

Figure 5. Role of RORC1 myelopoiesis associated with cancer. From [24]

1.2 The Transcription Factor NF-kB

The NF-κB transcription factor family is considered the central mediator of the inflammatory process and a key participant in innate and adaptive immune responses; moreover during the last years it has been proved to play a crucial role in cancer development [25].

NF-κB is an inducible transcription factor that regulates immediate and long-lived cellular responses to environmental changes. NF-κB is evolutionarily conserved and plays a critical role in many biological systems, above all the immune system, where it acts as the major orchestrator of the transcriptional responses to many different stimuli. The engament of several immune receptors such as B and T cell receptor (BCR, TCR), Toll Like Receptors (TLRs), Tumor Necrosis Factor Receptor (TNFR) or CD40 triggers NF-kB activation which in turn results in the expression of cytokines, growth factors and effector enzymes [26-28]. NF-κB also regulates the expression of genes outside the immune system, playing a crucial role even in embryo, mammary gland, skin, bone and nervous system development and physiology [29-35]. At present, more than 150 genes under control of NF-κB have been identified, as a demonstration of its vast spectrum of biological functions [36]. Indeed, it is very well known that NF-κB disregulaton is linked to various pathological situations.

Misregulation of NF-κB activity, such as constitutive activation, could be associated with pathological conditions such as rheumatoid arthritis, asthma, intestinal bowel diseases (IBDs), multiple sclerosis and cancer [37-41].

Given this great variety of biological roles, a better understanding of NF-κB pathways could provide the basis for the development of therapeutic strategies with a relevant impact on human diseases.

NF-κB family

NF-κB family is composed of five members: RELA (p65), RELB, cREL, NF-κB1 (p105-p50) and NF-κB2 (p100-p52). All these proteins possess a conserved 300-amino acid REL homology domain (RHD) that is located toward the N-terminus of the proteins and is responsible for dimerization, binding to inhibitors of nuclear factor κB (IκBs) and binding to DNA. Instead, the carboxy-terminal non-homologous transactivation domain (TAD), which strongly activates the transcription of targeted genes, is present only in cREL, RELB and RELA. p105 and p100 after proteolytic degradation generate p50 and p52 [28] which lack the transactivation domain. Therefore if they form homodimers they still bind the DNA consensus sites, but they don't activate transcription [16] (Fig.4).

Each member of NF-κB family except for RELB can form homodimers as well as heterodimers with one another but the main activated form of NF-κB is the heterodimer composed by p65 and p50 or p52.

Mice lacking cREL, NF-κB1 (p105-p50) or NF-κB2 (p100-p52) subunits display a normal development except some defects in lymphocytes activation, whereas p65 or RELB knockout mice are embryonic lethal by liver degeneration and died postnatally from multiorgan inflammation, respectively. Mice lacking more than one subunit, for instance $p50^{-/-}p52^{-/-}$ or $p50^{-/-}RelB^{-/-}$ display more severe phenotypes demonstrating redundancy between NF-κB members.

NF-κB regulation: IκB proteins

Inhibitor of nuclear factor κB (IκB) family comprises four members: IκBα, IκBβ, IκBε and BCL-3 (Fig.6). These proteins share ankyrin repeats which mediates protein-protein interactions [16].

IκBα, IκBβ and IκBε exhert their regulatory function binding NF-κB proteins and masking their Nuclear Localization Sequence (NLS). So, the complexes IκBs-NF-κB cannot translocate into the nucleus and are retained in the

cytoplasm in inactive forms. Unlike the other members of IκB family, BCL-3 binds specifically to p50 and p52 homodimers and induce the transcription of NF-κB regulated genes [16, 42]. In addition, IκB proteins act only masking p65 NLS, but p50 NLS remains accessible [43-46].

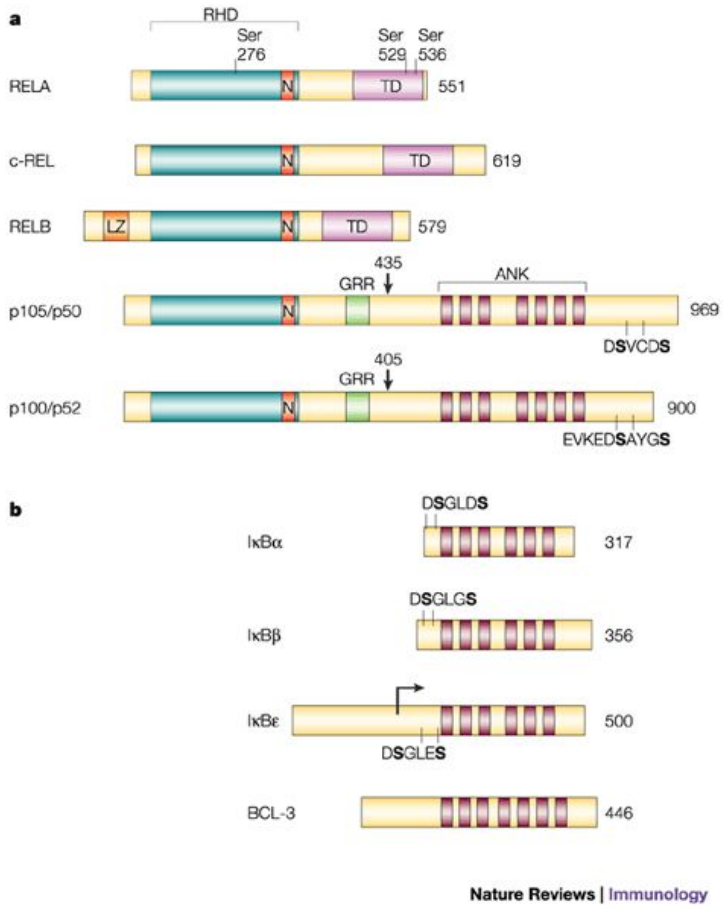

Figure 6: Mammalian NF-κB- and IκB-family members.

A. NF-κB family comprises five members: RELA (p65), cREL, RELB, p105/p50 (NF-κB1) and p100/p52 (NF-κB2). LZ: leucine-zipper motif. **B**. IκBs family contains IκBα, IκBβ, IκBε and BCL-3, and it is identified by the presence of many ankyrin (ANK) repeats. The amino-acid sequences of the sites of induced phosphorylation of IκBα, IκBβ, IκBε for their degradation are shown (D**S**GLD**S**, D**S**GLG**S** and D**S**GLE**S**, respectively). Proteolytic processing of p105 and p100 at residues 435 and 405 (as indicated by arrows), respectively, generates the p50 and p52 NF-κB proteins. The glycine-rich region (GRR) and the carboxy-terminal sites of inducible phosphorylation (in the D**S**VCD**S** and EVKED**S**AYG**S** sequences for p105 and p100, respectively) are required for processing. Phosphorylation of RELA at Ser276, Ser529 and Ser536 is important for its transactivation activity. The size of each human protein is shown on the right (number of amino acids). From [47].

NF-κB Activation

NF-κB activation is mainly dependent on IκBs degradation thus leaving NF-κB complexes free to translocate into the nucleus, bind promoter and enhancer regions containing κB sites with the consensus sequence GGGRNNYYCC (N=any base, R=purine, Y=pyrimidine) and activate gene transcription. In addition to IκBs, also post-translational modification, like acetylation or phosphorylation, can modulate NF-κB activation. For example, IL-1/TNFα-induced phosphorylation of p65 Ser276 after IκBα degradation is essential for the efficient binding of p65 to the transcription co-activator CREB-binding protein (CBP) [4, 48]. Similarly, phosphorylation of p65 Ser529 enhances its transcriptional activity [4, 49] and the loss of p65 phosphorylation influences both its DNA binding and transactivation activities.

Triggering of many different receptors can induce NF-κB activation that is initiated upon phosphorylation of IκBs by IκB Kinases (IKK). IKK is a complex made by kinase subunits IKKα and IKKβ and the regulatory subunit IKKγ or NEMO (NF-κB Essencial Modifier) [50, 51].

Upon phosphorylation by IKKs, IκB proteins are recognized and ubiquitinated by ubiquitin ligases [52, 53] leading to NF-κB activation.

NF-κB could be activated through two different pathways: classical and alternative (Fig.7).

Classical pathway

The common or classical NF-κB signalling pathway is particularly active in innate immunity [54, 55] and is activated predominantly by the subunit IKKβ in a NEMO dependent manner. The released NF-κB dimers, that in this pathway are predominantly p65-p50 heterodimers, go to the nucleus and activate gene transcription [51]. Many different pattern recognition receptors (PRRs) has evolved to recognize microbial invaders and are able to activate NF-κB classical pathway; among these, there are TLRs, members of the CATERPILLAR/NOD

family of cytoplasmic receptors, scavenger receptors and the complement system.

TLRs are evolutionarily conserved PRRs that recognize molecules characteristic of various classes of microbes [56]. The function of TLRs as arbitrators of self/non-self discrimination highlights their central role in innate immunity as well as in the initiation of the adaptive immune response.

Signalling through TLRs leads to activation of canonical IKKs complexes, degradation of IκBs and activation of RELA and cREL containing NF-κB dimers. TLR signalling to NF-κB is divided into two pathways: those that are MyD88 (myeloid differentiation primary response gene 88)-dependent and those that are MyD88-independent.

The beginning of an inflammatory response is strictly dependent from NF-κB classical pathway. Signals coming from the environment lead to the recruitment and activation of effector cells, initially neutrophils and later macrophages and other leukocytes, resulting in the tissue changes characteristic of inflammation – *rubor, calor, dolor* and *tumor* (redness, heat, pain and swelling, respectively).

Alternative pathway

The alternative pathway of NF-κB activation (Fig.6) is particularly active in cells of the adaptive immunity, such as B and T lymphocytes. This pathway is independent of IKKβ and NEMO [57, 58], but it is dependent of IKKα homodimers, which selectively phosphorylate p100 associated with RELB. Therefore, the consequence is the release of active RELB-p52 heterodimers [59, 60].

Activation of NF-κB downstream B cell receptor (BCR) and T cell receptor (TCR) is a critical step for mounting adaptive immune responses allowing antigen specific maturation and proliferation of lymphocytes into effector cells [61].

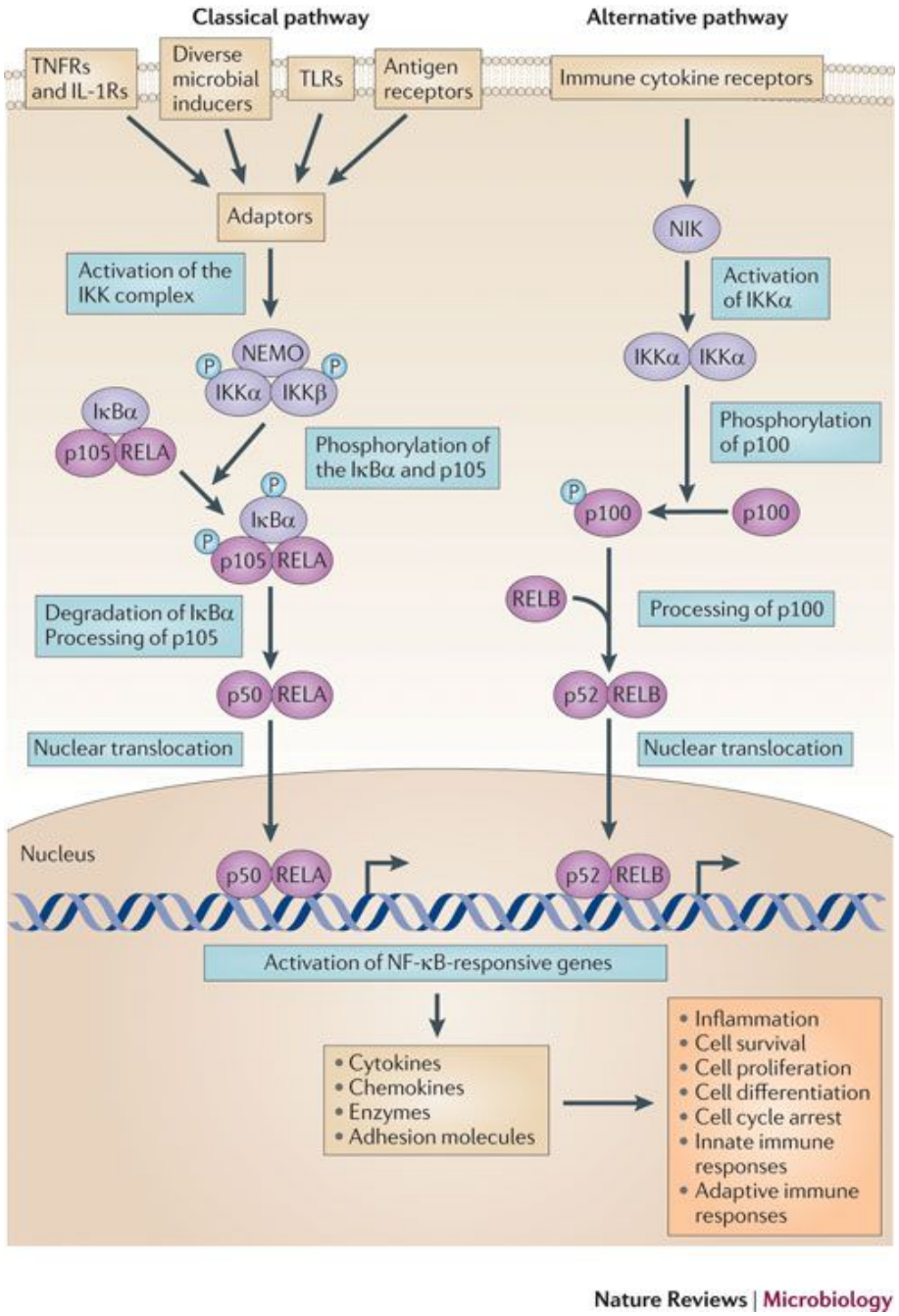

Figure 7: Classical and alternative NF-κB pathway.
Protein levels and activity of signalling molecules can be regulated through post-translational modifications such as phosphorylation, ubiquitylation and acetylation. The activation of nuclear factor-κB (NF-κB) ultimately results in the transcription of genes that encode pro-inflammatory factors and factors that influence cell proliferation. IκBα, NF-κB inhibitor-α (also known as NF-κBIα); IKK, IκB kinase; IL-1R, interleukin-1 receptor; NEMO, NF-κB essential modulator (also known as IKKγ); NIK, NF-κB-inducing kinase (also known as MAP3K14); TLR, Toll-like receptor; TNFR, TNF receptor. From [62].

NF-κB and cancer

NF-κB exerts a great variety of biological roles; this means that disregulations of NF-κB pathways can have broad deleterious consequences.

For its function in activating the transcription of genes important for cell proliferation (e.g. cyclin D1, c-Myc) survival (cIAPs, A1/BFL1, BCL-2, c-FLIP) adhesion, and angiogenesis (e.g. IL-8, VEGF) [4], NF-κB is considered a potential molecular bridge between inflammation and cancer [4].

In fact, as a master regulator of inflammation, NF-κB triggers the transcription of several proinflammatory mediators such as IL-1β, TNFα, IL-6 and IL-8. These factors are able to induce higher NF-κB activation, thus providing a positive feedback loop at the site of inflammation which creates an environment in which DNA damage, cell proliferation, transformation and survival and consequently cancer initiation, growth and progression are facilitated [36]. In addition, NF-κB is involved not only in tumor development at early stages, but also in the migration, invasion and metastasis of malignant cells. The invasive capacity of malignant cells can increase in the presence of inflammatory cytokines such as TNFα, IL-1β and IL6 [2]. In particular TNFα is a potent stimulator of epithelial-mesenchimal transition by breast cancer cells [63] for its ability of activate NF-κB signaling. NF-κB was also found to promote metastatization in a genetic mouse model of prostate cancer, in which inactivation of IKKα was found to reduce metastatic spread [64].

In many cancers, NF-κB is constitutively active, even if the exact mechanism that sustain this activation is not fully understood and several mechanisms have been proposed (Fig.8), such as IL-1β and TNFα production, shorter IκBα half life or IκBα mutations [65-69].

For these reasons, NF-κB represents an ideal therapeutic target for the development of new anti-tumor strategies. Proteasome inhibitors as well as IKK inhibitors block NF-κB activation. Similarly, inhibitors of histone acetylation

can inhibit NF-κB as well as stimulation of histone deacetylase like HDAC3[70] [70].

Another mechanism to block NF-κB activation is the transfer of mutated genes that encode for NF-κB inhibitory proteins, most commonly IκB genes. IκB are mutated at the site of phosphorylation or ubiquitination, therefore they cannot be degraded.

In many studies, the super repressor of NF-κB was delivered into intestinal epithelial cells through adenoviral vectors and inhibition of NF-κB was very successful. These studies are very important because they provide a possible in vivo approach for the treatment of intestinal malignances [71].

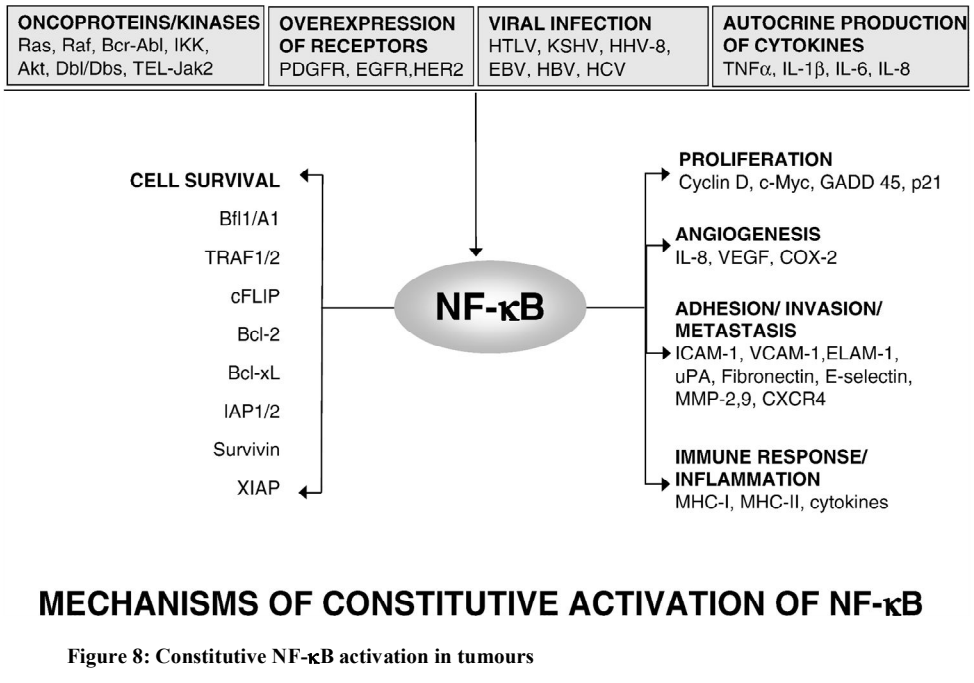

Figure 8: Constitutive NF-κB activation in tumours

p50/NF-κB1

The role of p50 and its precursor p105 in cell physiology and function is very complex. Although originally considered a repressor of transcription, p50 could also be a transcriptional activator: the balance between pro- and anti-inflammatory activity of p50 depends on cell type and environmental conditions.

The NF-κB1 gene encodes two functional proteins: p50 and p105. It is thought that a third protein, IκBγ, could be generated by alternative splicing (Fig.9)[72].

p105 is the precursor of p50 which is the active form of the protein and could form dimers with itself and with other NF-κB subunits.

Since p50 homodimers mainly act as repressors of transcription, and given the importance of NF-κB during inflammation, it is likely that they act also as repressors of inflammation. Indeed, it has been demonstrated that in LPS-tolerant macrophages increased expression of the p50 subunit of NF-κB directly results in the downregulation of LPS-induced TNFα production, whereas in $p50^{-/-}$ macrophages long-term pre-treatment with LPS was unable to induce tolerance. In line, our group has demonstrated that TAMs express a tolerant pro-tumoral phenotype that is controlled by massive nuclear accumulation of p50 NF-κB [73].

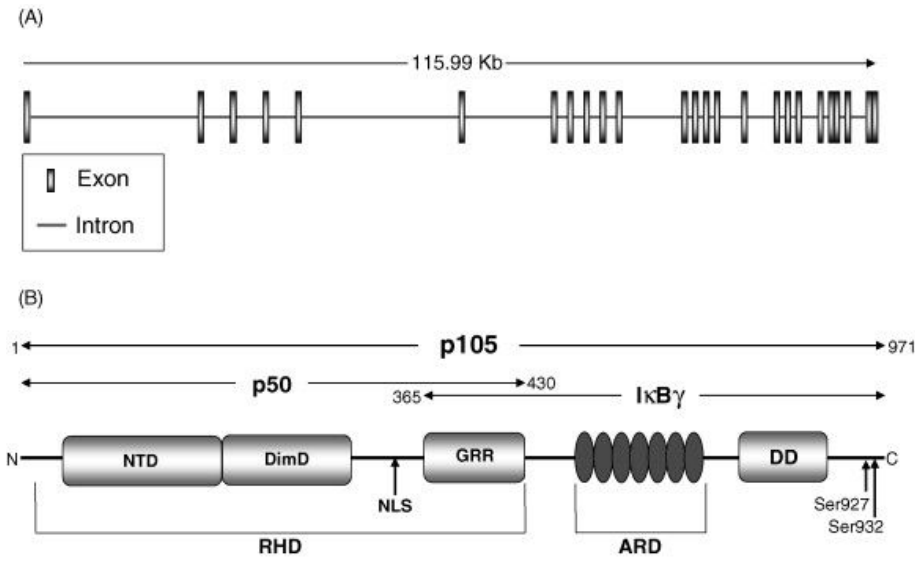

Figure 9: NF-κB1 gene and protein structure.
A. *NF-κB1* gene is 115.6 kb long and encodes a 3452 bp-long transcript containing 25 exons. **B.** Protein structure of p105, p50 and IκBγ. p105 is a 971 amino acids peptide containing a Rel homology domain (RHD), seven ankyrin repeats in the ankyrin repeat domain (ARD) and the death domain (DD). The RHD includes the N-terminal domain (NTD), dimerisation domain (DimD), nuclear localisation sequence (NLS) and glycine-rich region (GRR). p105 is phosphorylated at serine residues 927 and 932, a signal for poly-ubiquitination and subsequent degradation by the 26S proteasome releasing the active subunit p50. p50 spans amino acids 1–430 which encompass the RHD, in this form the

NLS is exposed and promotes p50 nuclear translocation. IκBγ spans amino acids 365–969 and contains the GRR, ARD and DD. From [74].

Even if p50 homodimers lack the transactivation domain, they may activate gene expression by recruiting trascritional co-activators. In particular, Cao et al. demonstrate that in LPS (or other TLRs agonists) stimulated macrophages, p50 homodimers form complexes with the transcriptional co-activator CREB binding protein. This complex binds to the IL-10 promoter and stimulates transcription of this anti-inflammatory gene [75]. In fact, $p50^{-/-}$ mice display reduced IL-10 production and increased TNFα and IL-12 production [76]. Accordingly, we have shown that LPS stimulated $p50^{-/-}$ TAMs recover an IL-12^{high}TNFα^{high}IL-10^{low} phenotype and that this correlates, *in vivo,* with tumor growth inhibition [73]. 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 of key regulator of M2-driven inflammatory responses [77]. Accordingly, p50-deficient mice show exacerbated M1-driven inflammation and defective capacity to mount allergy and helminth-driven M2-polarized inflammatory reactions [77]. Hence p50 NF-κB regulates the orientation of macrophage polarization, playing a crucial role in the control of M1- vs M2-driven inflammation.

For all these reasons, p50 could represent a good therapeutic target for human diseases; in one way, p50 could be inhibited to enhance M1 pro-inflammatory response in situation when it is strongly required (e.g. tumours), whereas in the other way, its activity could be augmented to promote exhintion of exacerbates inflammatory conditions (e.g. IBDs, rheumatoid arthritis, multiple sclerosis).

1.3 Macrophages

A century ago Metchnikoff received the Nobel Prize for the discovery of macrophages and innate immunity. After their first discovery, macrophages acquired many different functions both immunological and non-immunological; they have roles in almost every aspect of an organism's biology from developmet and homeostasis to repair through immunity. Resident macrophages regulate tissue homeostasis by acting as sentinels and responding to changes in physiology as well as challenges from outside. Unfortunately, in many cases, these homeostatic and reparative functions can be subverted by continuous insults, resulting in diseases such as chronic inflammation, autoimmune diseases, obesity and cancer (Fig.10).

Macrophages play an indispensable role in the immune system with decisive functions in both innate and acquired immunity. In innate immunity, resident macrophages provide immediate defence against foreign pathogens and coordinate leukocyte infiltration [78]. Macrophages contribute to the balance between antigen availability and clearance through phagocytosis and subsequent degradation of senescent or apoptotic cells, microbes and possibly neoplastic cells. Their role is essential for triggering, instructing and terminating the adaptive immune response. Macrophages collaborate with T and B cells, through both cell-to-cell interactions and fluid-phase mediated mechanisms, based on the release of cytokines, chemokines, enzymes and reactive radicals.

Macrophage origins and differentiation

In mammalian, macrophages are found in all tissues and display great anatomical and functional diversity. The density of macrophages changes in many tissues during development [79].

Macrophages are differentiated cells of mononuclear phagocytic lineage [80] that are characterized by a specific phenotype and by the expression of particular markers, none of which are entirely restricted to the lineage [81]. In mice, macrophages express CD11b, F4/80, CD68, colony-stimulating factor-1 receptor (CSF-1R; CD115) and do not express Gr1. In humans they are described as $CD68^{+}CD33^{+}CD14^{+}$ cells.

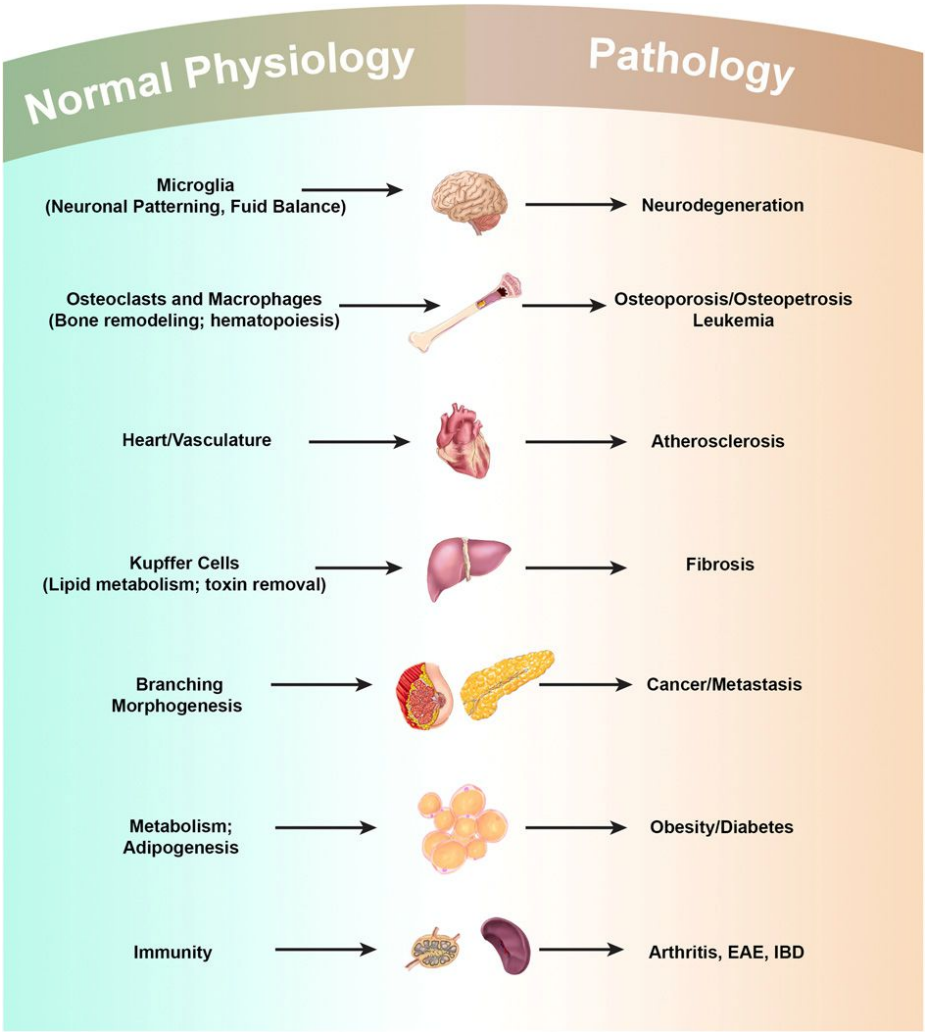

Figure 10: Macrophages in Development, Homeostasis and Disease
Macrophages play many developmental roles shaping the architecture of tissues ranging from the brain to bone to the mammary gland. Once development is over macrophages modulate homeostasis and normal physiology through regulating diverse activities including metabolism and neural connectivity and by detecting damage. These trophic and regulatory roles however, are often subverted by continuous insult and macrophages contribute to many diseases that are often associated with aging. EAE: Experimental Autoimmune Encephalomyelitis; IBD: Inflammatory Bowel Disease. From [82].

The term “macrophage” comes from the Greek, emphasising the ability of these cells to phagocyte. Lots of definition were given to these cells during time; nowadays macrophages are classified basing on ontogeny and phagocytosis by the term *mononuclear phagocytic system (MPS)* [83]. It includes bone-marrow derived precursor cells, monocytes in peripheral blood and mature macrophages in tissues. In the MPS schema, adult macrophages are end cells of the mononuclear phagocytic lineage and tissue macrophages derive from circulating monocytes that originate in BM.

More recently the classification of the MPS has been refined because several studies pointed out that macrophages have several origins during ontogeny and each of these different lineages persist into adulthood displaying great diversity [84]. In addition, some dendritic cell subsets can differentiate from monocytes and macrophages [85].

The origin of mononuclear phagocytic cells in ebryo is complex: in mice, the first population of macrophages is observed at embryonic day (E)7.5 and is of maternal origin [86]. Embryonic macrophages derive from the primitive ectoderm of the yolk sac and do not go through a monocytic stage but differentiate directly from mesenchymal progenitor cells [87]. By E10.5 to E11 liver represents the main site of hematopoiesis followed then by a second wave of progenitor cells from the aorta-gonads-mesonephros region of embryo [88, 89]. After birth, the bony structures are formed and, from that moment, the bone marrow become the source of circulating monocytes (resident $Ly6C^-$ and inflammatory $Ly6C^+$ in mice) [90]; at this stage, the MPS is established [83].

Nowadays, this first model of the MPS formation has been expanded. In fact, in a few tissue, such kidney and lung, macrophages were shown to have a chimeric origin (Hematopoietic Stem Cells [HSC]- and yolk sac-derived) as well as Langerans cells (LC) of the skin, which have a mixed origin from yolk sac and fetal liver [91, 92]. So it is possible to identify at least three lineages of

macrophages in mouse during different stages of development and persisting in the adult (Fig.11).

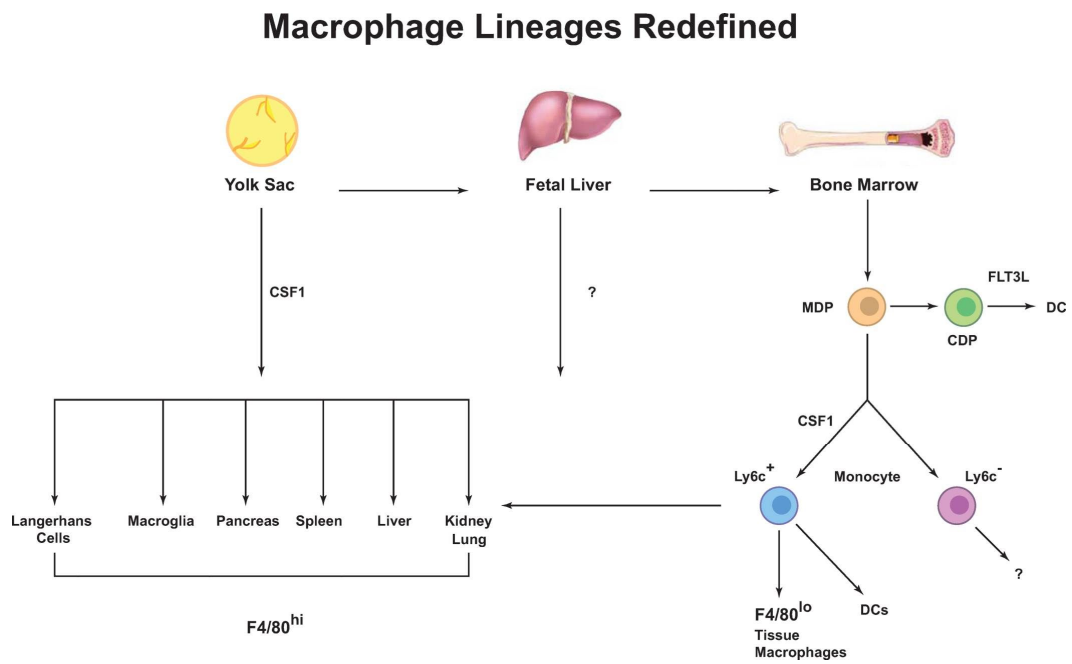

Figure 11. Macrophage Lineages Re-Defined in Mice
The mononuclear phagocytic system in adults derives from at least three sources. The first is the yolk sac (YS) that results in progenitors that populate all tissues and their progeny persist throughout life as F4/80 bright resident macrophages. These lineages are largely regulated by CSF1R. The second from the fetal liver is less well defined but seems to contribute to adult LCs perhaps through a progenitor derived from the YS. The third lineage derives from the bone marrow (BM) to give circulating monocytes and their progeny F4/80^{low} macrophages and DCs. In this case the Ly6c^{+} monocytes give rise to classical DCs under the regulation of FLT3 and these are continuously replenished. Other macrophages that are F4/80^{low} also emanate from Ly6c^{+} monocytes and in some cases such as kidney and lung, co-exist with those derived from YS to give chimeric organs. The exact role of the patrolling Ly6c^{+} macrophages remains unclear, as is the contribution of fetal liver to adult tissue macrophages. From [82].

Yolk sac macrophages first appear at E9.0 in both mouse and rat, and develop without passing through a monocytic intermediate stage [93]. They are the primary source of microglia, the resident macrophages of the central nervous system [91], and also give rise to a minor fraction of Langherans cells (LCs) [92]. The major fraction of adult LCs derives from fetal monocytes generated in the fetal liver from E12.5 and recruited into fetal skin around E14.5 [92]. Fetal monocytes also contribute to populations of adult macrophages in lung alveoli [94, 95] and in the heart [96]. Using fate-mapping to distinguish cells arising from primitive versus definitive hematopoiesis initially suggested that adult

macrophage populations in lung, dermis, and spleen arise predominantly from definitive hematopoiesis with negligible contribution from yolk sac-derived macrophages [91]. However, a new approach exploiting the differential dependence of macrophages on the transcription factor c-Myb has since indicated that c-Myb-independent yolk sac-derived macrophages may be the sole origin of macrophages in the lung, liver, and pancreas, as well as of microglia and LCs[97]. In this scenario it seems that the expression of c-Myb between early and late stage is different and many reports indicate that primitive hematopoiesis in yolk sac can occur in the absence of c-Myb [98] because the earliest yolk sac progenitors, which give rise to microglia, do not express c-Myb [99], whereas progenitors from definitive hematopoiesis both express and depend upon c-Myb [100-102]. Because fetal monocytes are absent in c-Myb-deficient embryos [97, 103] and c-Myb expression is upregulated during fetal monopoiesis, it is likely that the change in the progenitors fate between the yolk sac and the fetal liver is orchestrated by c-Myb. Microglia represents an exception because it have a unique origin, arising from yolk sac macrophages that maintain themselves by proliferating in situ throughout adulthood, and not from fetal monocytes [91, 99].

In addition, recent studies have shown that even in absence of hematopoietic stem cells, yolk sac progenitors were capable of giving rise to the major tissue resident population of macrophages in skin, spleen, pancreas, liver, brain and lung [97].

Hence, the idea that macrophages derive from circulating monocytes has been questioned. In fact, complete loss of $CD16^+$ monocytes in humans appears to be of little consequence [104] and in this scenario the function of monocytes needs to be re-defined. It is possible that patrolling monocytes ($Ly6C^-$) act to maintain vessel integrity and to detect pathogens while inflammatory monocytes ($Ly6C^+$) are recruited only to site of infection or injury or to tissues that have continuous cyclical recruitment of macrophages such as the uterus.

The development of macrophages from monocytes is regulated by several growth factors. Early studies indicate that, in mice and rats at least, the most important of these was the colony stimulating factor 1 receptor (CSF1R) which not only drove the differentiation of macrophages from progenitors, but also controlled their proliferation and viability in vitro. More recent studies, basing on the ablation of Csf1r, demonstated a severe depletion of macrophages in many, but not all, tissues [105]. These new data opened a different scenario and following experiments confirmed that CSF1R is expressed both on yolk sac macrophages and fetal monocytes, but only the development of the former is dependent on CSF1R [91, 92]. In fact, for example, Hoeffel and Ginoux discovered that the major fraction of adult LCs is derived from fetal monocytes that are generated in the fetal liver independently of CSF-1R expression [91, 92].

Macrophages in development

Macrophages present important roles in tissue development as demonstrated by the cluster of abnormalities that characterize *Csf1* null mice, which lack many macrophages populations. This mice survive to adulthood because of extra-medullary hematopoiesis in the spleen and liver [80].

As professional phagocytes, macrophages perform critical functions in the remodelling of tissues, both during development and in the adult animal. For istance, during erythropoiesis, maturing erythroblasts are surrounded by macrophages, which ingest the extruded erythrocyte nuclei. This function of macrophages is of critical importance because in its absence, erythropoiesis is blocked and lethality ensues [106]. Remodeling deficiencies in the absence of macrophages have been noted in many other tissues, suggesting a general requirement for macrophages in tissue remodeling and morphogenesis [80, 107].

Macrophages also regulate angiogenesis through a variety of mechanisms, among which there is the synthesis and release of Wnt7b that delivers a cell death signal to the vascular endothelial cells (VEC) and either its absence or the absence of macrophages drives vascular over-growth [108]. This is not restricted to the vascular arm of the circulatory system since macrophages also play roles in lymphangiogenesis during development [109].

Also brain development is influenced by a specific population of macrophages called microglia, whose presence is dependent on CSF1R signalling [110, 111] that promotes neuron viability [80] , modulates neuronal activity [112], prunes synapse during development [113] as well as expresses a range of neuronal growth and survival factors including NGF [80].

Macrophage phenotypes

Peculiarity of macrophages is their plasticity, that means the ability to finely modulate their programs in response to different microenvironmental conditions [114].

The diversity of macrophages functions has led to various classification attempts. “Classical activated” M1 macrophages are characterized by high levels of inducible nitric oxide syntase (iNOS) and the production of reactive oxygen intermeadiateds (ROI). They are potent effector cells and strongly antigen presenters involved in T helper 1 (Th1)-cell-mediated immune resolution of infection [115]. Signals that led to M1 polarization (LPS, IFNγ) trigger the activation of NFκB- and STAT1- pathways with subsequent transcription of NFκB- and STAT1- dependent proinflammatory cytokines (e.g. IL-12 and IL-23). On the contrary the “alternative activated” M2 macrophages, which respond to Th2-type cytokines, such as IL-4 and IL-13, are involved in fibrosis, scavenge debris, tissue remodelling and repair, angiogenesis and humoral immunity [116] and are able to tune inflammatory response. They show strong activation of

arginase pathway with generation of ornithine and polyamines [12] (Fig.12). M2-polarizing signals generally inhibit the expression of M1 cytokines and chemokines. These inhibitory effects principally relay on STAT-3 dependent mechanisms [117] and the direct inhibition of NF-κB [47].

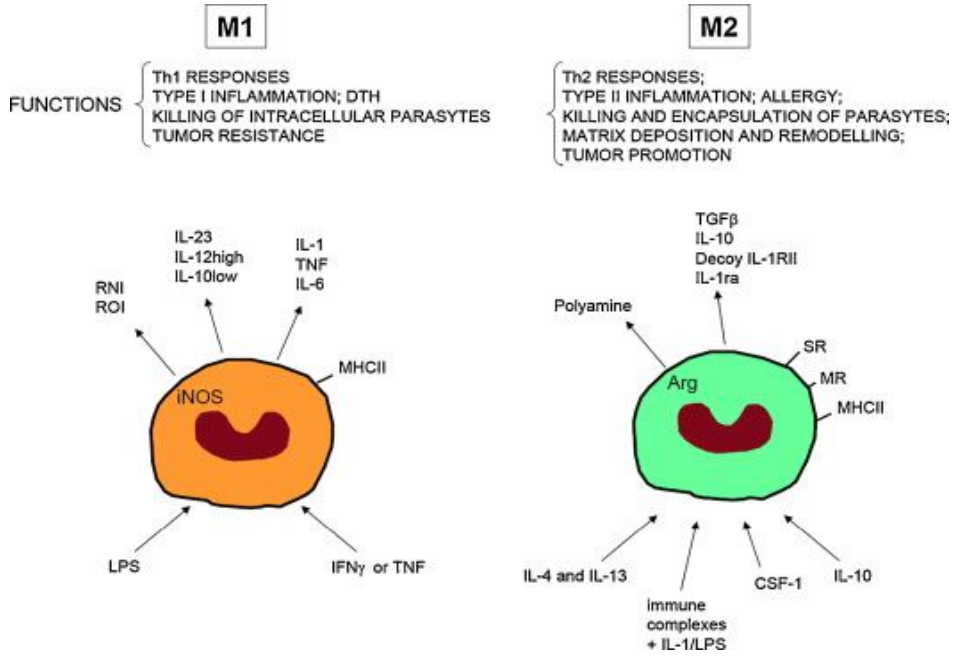

Figure 12: M1 and M2 macrophages.
In the presence of interferon (IFN)–γ, lipopolysaccharide and other microbial products, monocytes differentiate into M1 macrophages. In the presence of macrophage colony-stimulating factor (CSF-1), interleukin (IL)-4, IL-13, IL-10 and immunocomplexes in association with either IL-1R or TLR-ligands, monocytes differentiate into M2 macrophages. M1 and M2 subsets differ in term of phenotype and functions. M1 cells have microbial activity, immuno-stimulatory functions and tumor cytotoxicity. M2 cells have high scavenging ability, promote tissue repair and angiogenesis and favour tumor progression.

Signals from the microenvironment are able to drive macrophages towards M1 or M2 polarization; moreover macrophages are exposed to a multiplicity of opposite signals in vivo with different temporal pattern. Nevertheless there is considerable plasticity between distinct types: M1 and M2 polarization states are often referred to as the extremes of a continuum (Fig.13) [116].

When tissues are damaged following infection or injury, inflammatory monocytes (Ly6C+ in mice) are recruited from the circulation and differentiate into macrophages as they migrate into the affected tissues [90]. These recruited

macrophages exhibit a M1 pro-inflammatory phenotype in the early stages of a wound healing response.

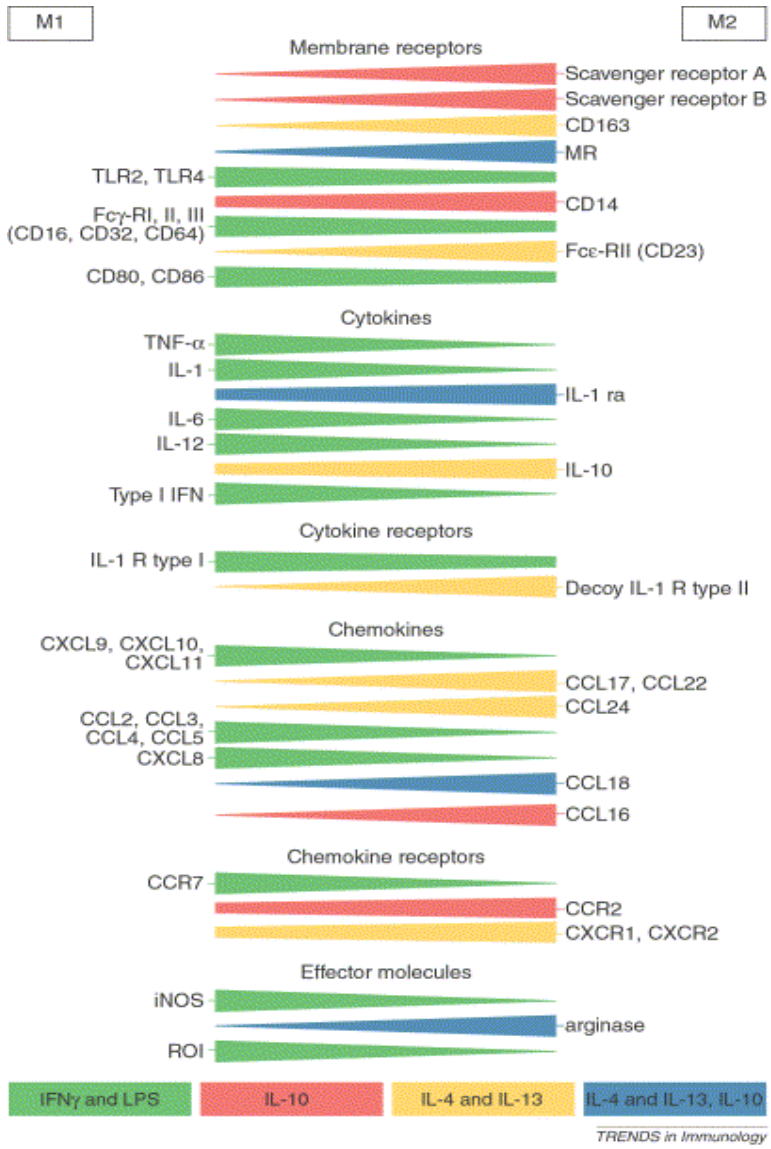

Figure 13: M1 and M2 macrophages are the extremes of a continuum.

M1 and M2 macrophages, the extremes of a continuum. Essential properties of polarized macrophage populations are shown. For M1 cells, molecules induced by IFN-γ and LPS are shown in green. For M2 cells, molecules induced by IL-4 and IL-13 are shown in yellow, those induced by IL-10 in red and those induced both by IL-4 and IL-13, and IL-10, in blue. Macrophages exposed to the classic activation signals, IFN-γ and LPS, express opsonic receptors (e.g. FcγRIII/CD16), whereas type II macrophages are characterized by abundant levels of non-opsonic receptors (e.g. the MR). M1 cells also have a higher ratio of reduced-to-oxidized glutathione, with opposite effects of IFN-γ and IL-4 on the reductive status. Components of the IL-1 system are differentially regulated in polarized macrophage populations. IL-4, IL-13 and glucocorticoid hormones induce expression of the IL-1 type II decoy receptor, whereas IFN-γ and LPS inhibit it. IFN-γ and LPS upregulate the signaling type I receptor, and IL-1R accessory protein (IL-1RacP). IL-4 and IL-13 induce IL-1ra production and inhibit IL-1. Therefore, pro- and anti-inflammatory components of the IL-1 system are coordinately regulated by signals that polarize macrophages in a type I or type II direction. IL-10 upregulates the CC chemokine receptors CCR1, CCR2 and CCR5. By contrast, CXCR2 and CXCR4 are partially downregulated under the same conditions. An increase in CCR2 expression is also observed in monocytes exposed to dexamethasone. IL-4 and IL-13 do not modify the expression of CC chemokine receptors but induce functional CXCL8 (IL-8) receptors in human monocytes. By contrast, monocytes exposed to LPS or IFN-γ downregulated CCR1, CCR2 and CCR530. and 73.. Similar to what was reported for DCs, exposure of monocytes to classical proinflammatory signals induces the expression of functional CCR7 and the effect is inhibited by IL-10. Abbreviations: DC, dendritic cell; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MR, mannose receptor; ra, receptor agonist; ROI, reactive oxygen intermediates; TLR, toll-like receptor; TNF, tumor necrosis factor. From [116].

Although these inflammatory macrophages are initially beneficial because they facilitate the clearance of invading organisms, they also trigger substantial collateral tissue damage because of the toxic activity of reactive oxygen and nitrogen species [118]. Indeed, if the inflammatory macrophage response is not quickly controlled, it can become pathogenic and contribute to disease progression, as seen in many chronic inflammatory and autoimmune diseases [119, 120].

To counteract the tissue damaging potential of the inflammatory macrophage response, macrophages undergo apoptosis or switch into a M2 anti-inflammatory or suppressive phenotype that dampens the pro-inflammatory response, while facilitating wound healing [121].

So, the pathophysiological adaptations to regulate over-exuberant inflammation serve as an important mechanism for host protection and one of the classic examples of such a protective mechanism is endotoxin tolerance [122-126].

Tolerance is a state of hyporesponsiveness acquired after prolonged exposure of macrophages to inflammatory agents including bacterial products such as LPS. Tolerant macrophages enter into a transient unresponsive state and are unable to respond to further challenges with endotoxin. This phenomenon has been observed both *in vitro* and *in vivo* in animal models as well as in humans [123, 124, 126-130]. Tolerance is caused by a profound "gene expression reprogramming" in macrophages [122, 126, 129] which develop poor inflammatory capacity coupled with upregulation of anti-inflammatory cytokines. Overall this characteristics contribute to protection against septic shock and increased phagocytosis allow efficient bacterial clearance (Fig.14).

Interestingly, many of the characteristic of endotoxin-tolerant monocytes/macrophages resemble that of the immunosuppressive M2 macrophages [77].

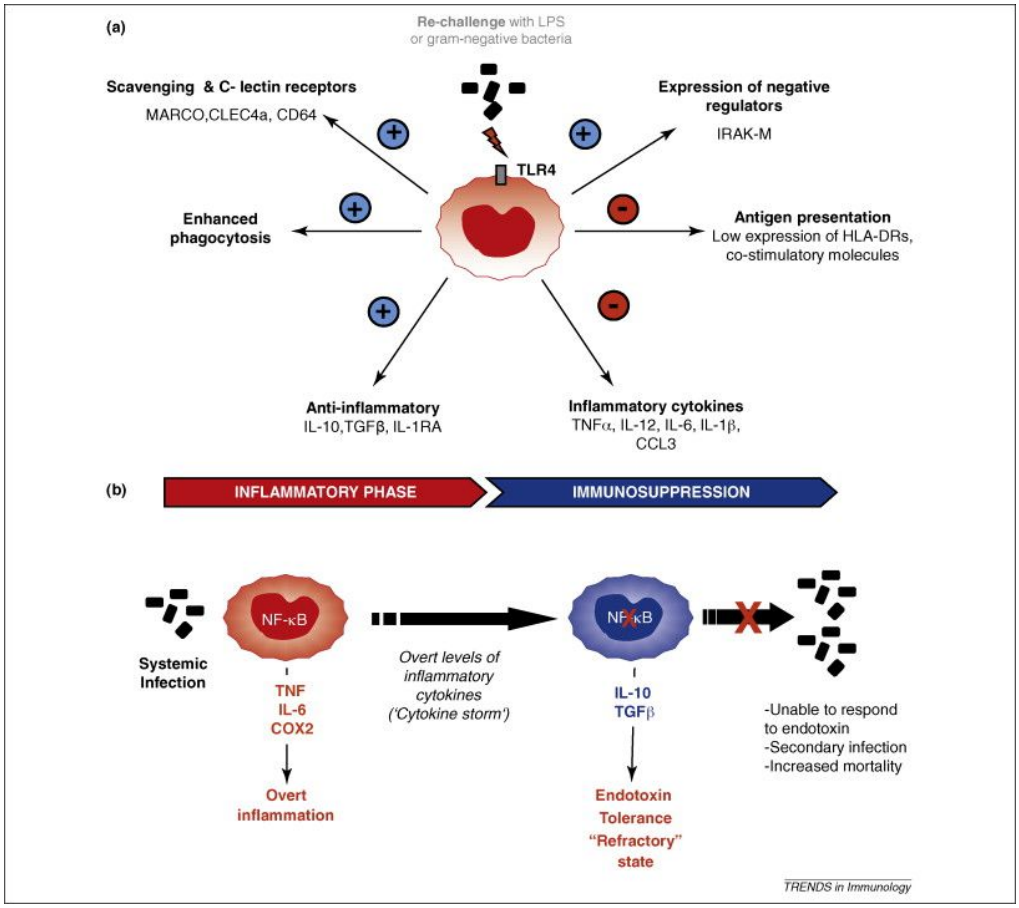

Figure 14: phenotype of endotoxin tolerance monocytes/macrophages.
A. Upon endotoxin re-challenge with gram-negative bacteria or LPS, monocytes/macrophages show a drastic downregulation of inflammatory cytokines (e.g. TNFα, IL-6, IL-1β, IL-12) but an upregulation of anti-inflammatory cytokines like IL-10, TGFβ and IL-1RA as compared to non-tolerized cells challenged with the same stimuli. The tolerant monocytes/macrophages also show an impaired antigen presenting capacity correlated with decreased expression of HLA-DR and some co-stimulatory molecules. In contrast, these cells show upregulated expression of a number of scavenging/C-type lectin receptors like MARCO, CD64, CLEC4a 10 and 11 is linked to enhanced phagocytic capacity. Upregulation of negative regulators of TLR4 signaling like IRAK-M is also a characteristic of these cells. (+) denotes upregulation, while (-) denotes downregulation during ET. **B.** Schematic representation of the biphasic nature of sepsis wherein an initial overt inflammatory phase leads to a later phase of immunosuppression or "immunocompromise". In parallel with these events, monocytes/macrophages also "switch" from an inflammatory phenotype to an endotoxin tolerant or refractory state. Monocytes respond to the systemic infection by triggering an inflammatory reaction characterized by overt levels of inflammatory cytokines and chemokines (e.g. TNFα and IL-6). However, as sepsis progresses, these monocytes become refractory to further endotoxin challenge whereby they fail to upregulate inflammatory cytokines. Instead, they start producing anti-inflammatory cytokines (TGFβ, IL-10) which promote immunosuppression. Under these conditions, there is a high risk of developing secondary infections, which may lead to mortality. From [131].

We previously demonstrated that, like M2 skewed macrophages, also tolerant macrophages display accumulation of p50 NF-κB subunit in the nucleus, and subsequent defective NF-κB activation (Fig.15) [132]. The importance of p50 for the acquisition of a tolerant phenotype is also demonstrated by the fact that

lack of p50 in murine macrophages prevents the development of tolerance. Macrophages lacking p50 do not dowregulate pro-inflammatory cytokines and are not able to upregulate anti-inflammatory factors even after prolonged exposure to LPS [77].

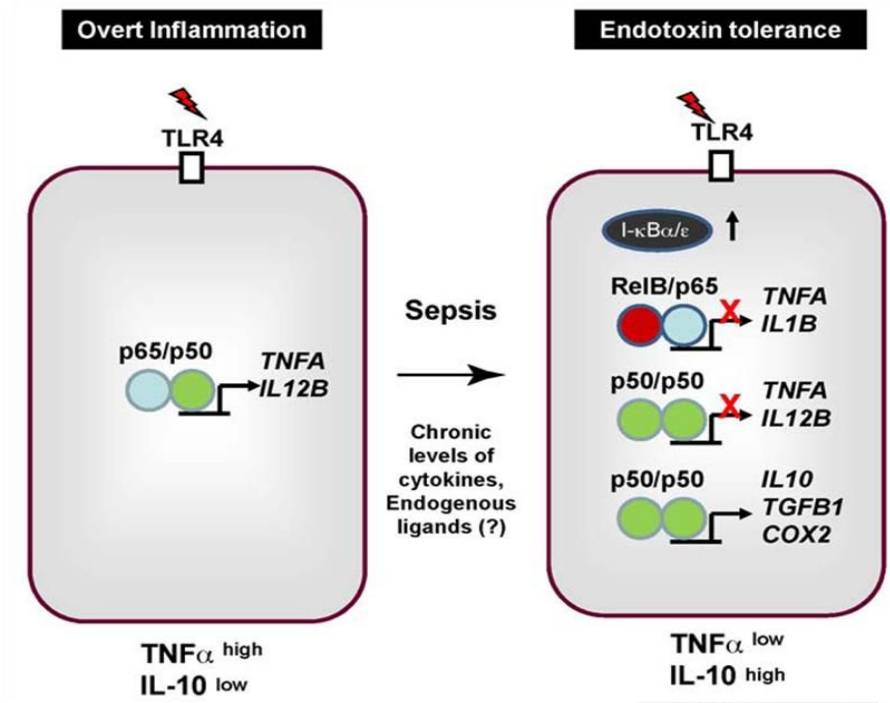

Figure 15: plasticity of NF-kB function in inflammation and endotoxin tolerance.
This figure represents how different combinations of NF-kB hetero- and homodimers can switch-on or -off the same genes under inflammatory or endotoxin tolerant conditions. During overt inflammation (as seen in the first phase of sepsis), the p65/p50 NF-kB heterodimer is responsible for the transcription of inflammatory genes like TNFA, IL12 etc. During endotoxin tolerance, there is an overexpression of p50/p50 NF-kB homodimers, which lack a transcription activating domain. This causes p50/p50 NF-kB homodimers to occupy the promoters of inflammatory genes and thereby prevent p65/p50 NF-kB heterodimer binding and gene transcription. Conversely, p50/p50 NF-kB homodimer triggers the transcription of genes like IL10, TGFB1 and COX2 [133]. RelB/p65 NF-kB heterodimers present in the endotoxin tolerant cells also prevent the transcription of inflammatory genes. Finally, the accumulation of IkBa and IkBe in endotoxin tolerant cells also prevents NF-kB activation. From [131]

Macrophages in cancer – Tumor Associated Macrophages

The major population of leukocyte infiltrating tumors is represented by macrophages.

In many cancers, these Tumor Associated Macrophages (TAMs) express an

M2-like phenotype which supports immune escape, tumor growth and malignancy exerting crucial tumor-promoting functions (e.g. induction of tumor cell proliferation and angiogenesis, incessant matrix turnover) [134, 135] and suppress the adaptive immune response [136, 137]. This activities ultimately have an important impact on disease progression [12]. Clinical studies have demonstrated a correlation between high frequency of TAM and the poor prognosis for many different human tumors including lymphoma, cervix, bladder, breast and lung cancers [138]. One of the most important characteristic of TAMs include their ability to directly affect tumor growth through promotion of tumor angiogenesis as well as the survival and metastasis of tumor cells [3, 116, 139-141].

TAMs are recruited to the tumor by a tumor-derived chemotactic factor, originally identified as CCL2, also known as MCP-1. Actually, other chemokines and molecules active on TAMs were detected in neoplastic tissues such as M-CSF, VEGF and angiopoiein-2 [139]. These factors have been showed to promote macrophages recruitment as well as macrophages survival and proliferation and their expression correlates with tumor growth.

Cytokines network at tumor site has a central role in TAMs recruiting and differentiation. Immunosuppressive cytokine IL-10 and transforming growth factor β (TGF β) as well as PGE2 produced by both cancer cells (ovary) and TAMs [116] contribute to a general suppression of anti-tumor activities.

Also in terms of cytotoxicity and expression of inflammatory cytokines, TAMs resemble the M2 macrophages: both are poor producers of nitic oxide (NO) [142, 143] and of ROIs; both are poor antigen presenting cells and not only they are unable to trigger Th1 polarized immune responses, but also they induce T regulatory cells (Treg) [116] and suppress T cell activation and proliferation [116, 144]. Moreover TAMs are unable to produce IL-12, even upon stimulation with IFNγ and LPS [145]. TAMs express high levels of both

scavenger receptor-A (SR-A) [146] and the mannose receptor (MR) together with other M2 markers like Arginase I, YM1, FIZZ1, MGL2.

Angiogenesis is an M2-associated function which represents a key event in tumor growth and progression. Lin and colleagues [147] demonstrated a slower rate of progression to malignancy and fewer pulmonary metastases in CSF-1 null mutant mice (that lack macrophage population) bearing spontaneous mammary carcinoma, than in CSF-1 wild type mice. These data are consistent with clinical findings that high number of TAMs often correlate with increase tumor vascularization. In several human cancer, TAMs accumulation has been associated with angiogenesis and with the production of angiogenic factors such as VEGF and platelet-derived endothelial cell growth factor [8]. Additionally, TAMs participate to the proangiogenic process by producing the angiogenic factor thymidine phosphorylase (TP), which promotes endothelial cell migration *in vitro* and whose level of expression are associated with tumor neovascularisation [148]. These pro-angiogenic TAMs are known as Tie-2 expressing monocytes (TEMs) because they are characterized by the expression of the angiopoietin receptor TIE2 [149, 150] and were found to constitute a small subpopulation of the total tumour infiltrating $CD11b^+$ myeloid cells that could be distinguished from the majority of TAMs by their surface marker profile ($Tie2^+Sca\text{-}1^+CD11b^+$), their preferential localization to areas of angiogenesis, and their marked pro-angiogenic activity [13, 14]. The selective elimination of these Tie2-expressing monocytes dramatically impaired angiogenesis in mouse tumours and induced substantial tumour regression.

TAMs also express molecules which affect dissolution of connective tissues. These include enzymes which regulate the digestion of the extracellular matrix, such as MMPs, plasmin, urokinase-type plasminogen activator (uPA) and the uPA receptor (Fig.16).

TAMs can also be potent immunosuppressors of the cytotoxic activity of $CD8^+$ T cells in progressing tumors: a high stromal TAMs infiltration inversely

correlates with $CD8^+$ T cell number [151]. This immunosuppression is mediated, at least in part, by nitrosylation of T cell receptors *via* ARG1, iNOS and peroxynitrite, inducing T cell apoptosis [22].

For these reasons TAMs have been described as "obligate partners for tumor-cell migration, invasion and metastasis" [141].

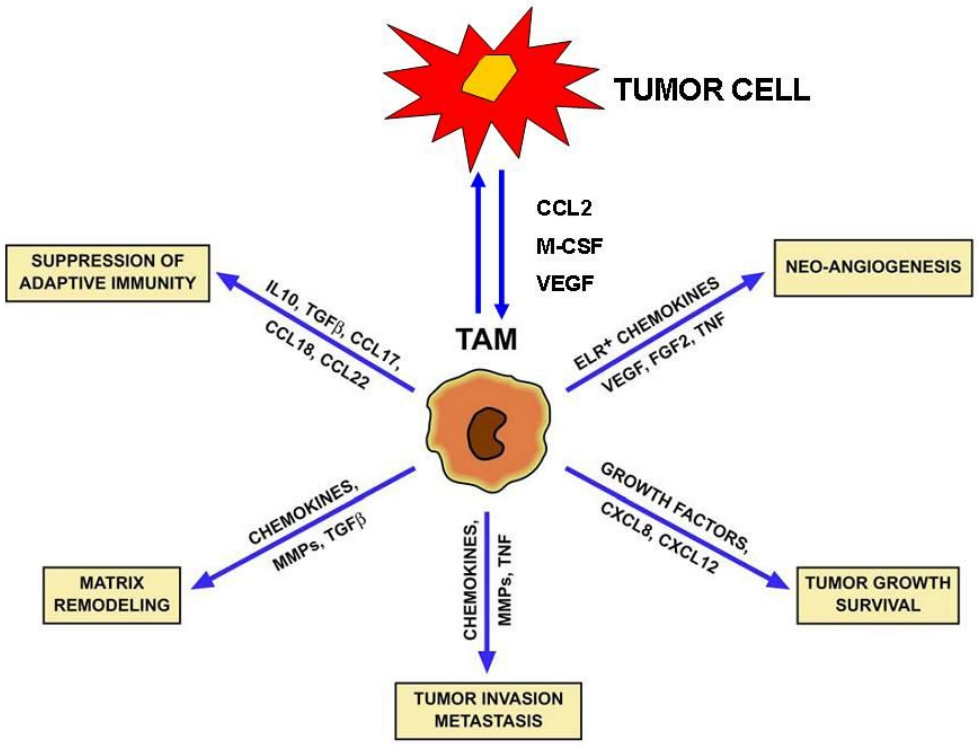

Figure 16: TAMs produce several factors that favour tumor growth and spreading.

NF-kB in TAMs

Activation of the transcriptional factor NF-κB is a necessary event promoting transcription of several proinflammatory genes. TAMs display a defective NF-κB activation in response to M1 polarizing signals LPS and TNFα [145]. The defect in NF-κB was shown to be associated to the over expression of nuclear p50 NF-κB homodimers which inhibit the transcription of proinflammatory genes [73]. The defective NF-κB activity was seen in TAMs isolated from

advanced stages tumors and it is in apparent contrast with TAMs NF-κB dependent pro-tumorigenic functions observed in murine models of inflammation-associated liver and colorectal cancer [152, 153]. This discrepancy might reflect a dynamic change in the tumor microenvironment during the transition from early-neoplastic events to advanced tumor stages, which would result in progressive modulation of the NF-κB activity expressed by infiltrating inflammatory cells and progressive conversion of the TAMs from an M1 to an M2 macrophage phenotype (Fig.17).

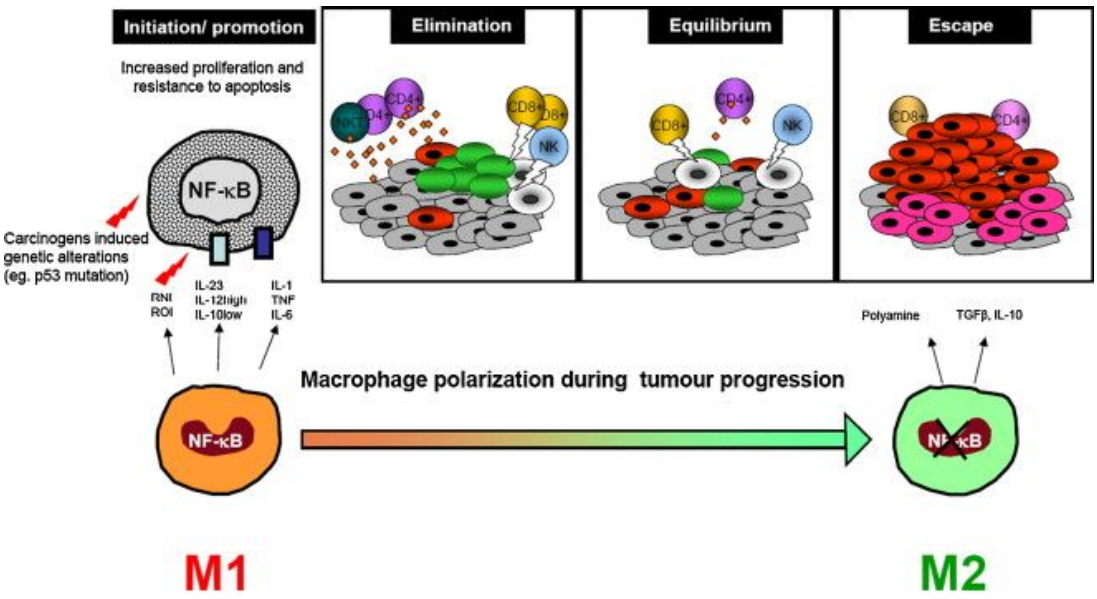

Figure 17: tumor immunoediting and progression: macrophage polarization.
During tumor progression a gradual switching of macrophage polarization, M1 versus M2, is paralleled by the gradual inhibition of NF-kB activity. These events concur to establish permissive conditions for tumor growth and spread. From [21].

Although these experimental and clinical results, some evidence does not fit into this general pattern. For example, in certain tumors or subset of tumors, the presence of inflammatory cells is associated with better prognosis (for example, eosinophilis in colon tumors and TAMs in a subset of breast tumors and pancreatic tumors). In fact, appropriately activated macrophages can kill tumor cells although in most cases their tumor-promoting properties prevail [144]. The

importance of this balance is evident in psoriasis where a marked chronic inflammatory response is not associated with an increased risk of developing skin cancer [154]. This because psoriasis is a M1/Th1-mediated disease and consequently the inflammatory microenvironment that develops has antitumor features. For these reason not only the ablation, but also the repolarization of TAMs from a M2 to a M1 status is considered an interesting therapeutic strategy.

TAMs as therapeutic targets

It is now clear that myeloid cells infiltrating in the tumor represent an important player that can initiate and support tumor development. In addition to these pro-tumoral activities, TAMs can also modulate the efficacy of various form of anticancer therapy. Based on this, both the recruitment and activation of TAMs are are considered putative targets for therapeutic intervention.

The major strategy so far is based upon genetic experiments targeting genes specifically involved in pro-tumoral macrophages phenotype like CSF-1. In this case the approach is based on anti CSF-1 receptor neutralizing antibodies or small molecule inhibitors interfering with this pathway. For example, TAMs depletion by anti-CSF1 antibodies enhanced the efficacy of combination chemotherapy (cyclophosphamide, methotrexate, and 5-fluoro-uracil) in chemoresistant, human breast cancer xenografts grown in immunodeficient mice [151]. Similarly, TAMs depletion improved the efficacy of paclitaxel in mouse models of mammary tumors [155]. Small molecule inhibitors to CSF1R have also been shown to deplete some populations of TAMs and to dramatically enhance responses to chemotherapy. This effect is at least in part consequent to the removal of macrophage-mediated immunosuppression during the tumor recovery period [151, 156].

Furthermore, low-dose irradiation of tumors activates macrophages to orchestrate T cell immunotherapy [157] while the therapeutic efficacy against tumors of Trabectedin in mouse model derives from its ability to directly kill mononuclear phagocytes, including monocytes and macrophages [158].

The role of macrophages in modulating the antitumor efficacy of chemotherapy is very complex and poor understood also because it can be based on both direct and indirect effects. In this regard, innate immune cells like macrophages, are known to activate local antigen presenting cells and increase the immunogenicity of the tumour by inducing the release of danger signals from the tumours cells [159]. These signals can stimulate innate immune responses by operating as adjuvants. This event has been defined as Immunogenic Cell Death (ICD). ICD is an immunogenic type of death that is characterized by a well-known series of events that include: the pre-apoptotic exposure of calreticulin (CRT) and other endoplasmic reticulum proteins at the cell surface (heat-shock proteins, HSPs), the increasing extracellular release of adenosine triphosphate (ATP) during the blebbing phase of apoptosis, and the post-apoptotic release of the chromatin-binding protein high-mobility group B1 (HMGB1) [159]. These molecules act together to promote presentation of tumour antigens [160-162]. Once on the cell surface, CRT serves as an ‘‘eat-me’’ signal, stimulating the engulfment of dying tumour cells and their apoptotic debris by macrophages and immature dendritic cells [160, 163, 164]. Similarly, HSP90 has been demonstrated to be a crucial mediator of immunogenicity [163]. ATP molecules released by dying cells constitute a potent chemotactic signal for myeloid cells including monocytes/macrophages [164] and DC precursors [165]. Cancer cells respond to ICD inducers by secreting ATP, lysosomal exocytosis, and plasma membrane blebbing [164, 166]. Only a few chemotherapeutics are known to induce ICD and Doxorubicin is among them.

The contribution of TAMs to the modulation of tumor responses to chemotherapy can vary markedly among different cytotoxic agents and tumor models. For example, the antitumor activity of the taxane docetaxel involves the depletion of immunosuppressive (M2-like) TAMs and the concomitant activation or expansion of antitumoral (M1-like) monocytes. Indeed, *in vivo* T cell assays showed that docetaxel-treated monocytes/MDSCs are able to enhance tumor-specific cytotoxic T cell responses [167].

TAMs may also release "chemoprotective" factors. Shree and colleagues showed how lysosomal enzymes, cathepsins B and S, secreted by TAMs protected cancer cells from paclitaxel-induced cell death [168]. In addition, also the efficacy of Doxorubicin and Etoposide was seen to be reduced by these TAMs-secreted cathepsins. Furthemore, a recent study demonstrates how the release of these cathepsins from lysosomes by TAMs is induced after 5-fluoro-uracil and Gemcitabine treatment [169]. The chemoprotection produced by cathepsins is correlated to an increase in the production of IL-1β by TAMs, which, in turn, stimulates the secretion of IL-17 by $CD4^+$ T cells, blunting the anticancer effects of chemotherapy.

TAMs may support tumor chemoprotection also by providing survival signals to tumor initiating/cancer stem cells (CSCs). For example, TAMs were found to protect lung and colon CSCs from Cisplatin by releasing milk fat globule-epidermal growth factor 8 protein (MFG-E8) which, in turn, activates STAT3 pathway [170]. In addition, TAMs depletion was demonstrated to improve T cell responses and the efficacy of chemotherapy in pancreatic cancer model, in part by decreasing the tumor-initiating capacity and STAT3 activation of CSCs [156].

Many studies demonstrate the contribution of TAMs to the cytotoxicity of therapeutic monoclonal antibodies (moAbs) [171]. In fact, TAMs express surface receptors for the Fc fragment of antibodies and enable them to engange in Ab-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP).

Trastuzumab, a moAb against the human epidermal growth factor receptor-2 (HER2), not only interrupts HER2 signaling in breast cancer cells, thereby slowing their proliferation rate, but also induces Fcγ receptor (FcγR)-mediated activation of macrophage cytotoxicity [172] and priming of antigen-specific $CD8^+$ T cell responses [173]. Macrophages also enhance lymphoma elimination in mice in response to rituximab, a moAb against CD20, primarly through FcγR-dependent ADCP [174]. Furthemore, high number of TAMs correlates with better prognosis in rituximab-treated patients [175].

Tumor irradiation is widely used to treat many cancers types. Early studies correlated high TAM number in mouse with poor tumor responses to irradiation [176]. Recent data suggest that DNA damage induced by irradiation promotes the transcription of *Csf1 via* the v-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1) kinase, which, in turn, recruits CSF1R-expressing myeloid cells (including TAMs) that enhance post-irradiation tumor regrowth. Indeed, a CSF1R inhibitor improved tumor response to radiotherapy in a prostate cancer model [177]. Antibody-mediated depletion of $Cd11b^+$ myeloid cells in human head and neck tumors grown in immunodeficient mice also reduced tumor regrowth after therapy [178]. In a model of orthotopic human glioblastoma, local irradiation dramatically enhanced tumor infiltration by $CD11b^+$ myeloid cells [179]. It has been proposed that TAMs activity in post-irradiated tumors is similar to that of M2-like macrophages driving tissue repair after injury [180]. TAMs drive reparative mechanisms in tumours after not only radiotherapy, but also treatment with vascular-targeting agents.

Docetaxel have a strong antitumor activity dued to the depletion of immunosuppressive (M2-like) TAMs and the concomitant activation or expansion of antitumoral (M1-like) monocytes in 4T1-Neu mammary tumour implants [167]. Moreover, Trabectedin, a DNA-damaging agent approved for soft tissue sarcomas, inhibited the growth of mouse fibrosarcomas primarily by depleting mononuclear phagocytes, including monocytes and TAMs [158].

Intriguingly, increasing data suggest that the efficacy of some forms of immunotherapy may also depend on effective reprogramming of TAMs toward an M1-like phenotype. For example, macrophage-mediated killing of bladder cancer cells relies on both direct effector-target cell contacts and the release of soluble cytotoxic factors, such as TNF-α, IFN-γ, and NO, from the macrophages [181]. An agonistic antibody to the TNF receptor superfamily member, CD40, was recently reported to bind to circulating monocytes, trigger their recruitment into mouse pancreatic tumors, and activate their tumoricidal functions. These CD40-activated, cytotoxic (M1-skewed) TAMs were also found to enhance the efficacy of gemcitabine in a small cohort of patients with surgically incurable pancreatic cancer [182]. Finally, macrophages and DCs express programmed cell death ligand-1 (PDL1, also known as B7-H1), a major negative regulatory ligand that suppresses T cell activation through its receptor-programmed cell death protein 1.

1.4 Colorectal cancer

Colorectal cancer (CRC) is one of the most common tumor in industrialized countries and it represents the best example of pathological association between chronic inflammation and cancer development. In fact, only about 20% of CRC cases can be genertically attributed to familiar history [183], while a very important numbers of CRC are linked to a condition of chronic inflammation (Colitis Associated Cancer [CAC]) of different inflammatory bowel diseases (IBDs) like Chron's disease (CD) or Ulcerative colitis (UC) [184-188]. Indeed it has been estimated that inflammatory bowel disease (IBD) patients show an almost 3 –fold higher risk to develop CRC than general population [189] and a "smoldering inflammation" is present even in sporadic and familial CRC tumors that are not causally related to an obvious inflammatory process [1]. In mouse models, only single injection of the carcinogen azoxymethane (AOM) give rise to multiple colonic tumors, when coupled to the induction of chronic colitis [190, 191], while it takes multiple injection of carcinogen and longer time for tumors to form when inflammation is absent. For these reasons, mouse models of CAC are considered extremely valuable for our understanding of general mechanisms which connect inflammation and cancer [1, 192, 193].

In fact, like most solid tumours, CRC exhibits a characteristic inflammatory signature including the expression of inflammatory mediators and the infiltration of different leukocytes population. For example, Cyclooxygenase-2 (COX-2) is over expressed in up to 40% of colon adenomas and 85% of colon adenocarcinoma compared to matched control tissues [194, 195] and its tumor promoting role is supported by multiple independent trials based on the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin or sulindac) in Familiar Adenomatous Polyposis (FAP) patients [196, 197]. In IBDs patients, long-term inflammation is thought to sustain cancer initiation by the continuous

release of reactive oxygen species (ROS), which are thought to ultimately cause DNA damage and the dysplastic degeneration of the repairing epithelium [198].

Several studies indicates NF-κB as link between genetic-dependent CRC and CAC. In fact in CRC tumorigenesis a series of genetic alterations happen and, among them, abnormal activation of Wnt/β-catenin pathway is present in over 90% of CRCs. The most common mutations that influence Wnt/β-catenin pathway happen in adenomatous polyposis coli (APC) tumor suppressor gene, GSK3β, a kinase which controls APC and β-catenin stability and β-catenin itself [199-203].

Wnt/β-catenin signaling pathway is instrumental for the renewal of intestinal epithelium and is a critical regulator of normal and malignant cell proliferation. In mouse models of CAC activatory β-catenin mutations can be found [152], but mutations in APC or other activatory mutations happen rather late during the disease progression and follow earlier mutation in p53 and K-Ras, quite differently from what is observed during CRC development [187, 204, 205]. To explain how, despite its pivotal role in proliferation and tumor development, genetic alteration in Wnt/ β-catenin arm of signaling are observed late during CAC development, several line of evidence suggest that different inflammatory pathways can enhance β-catenin signaling in the absence of mutations. Mice deficient for IL-10 spontaneously develop colitis and, later on, colorectal tumors which tipically do not display APC mutation but exhibit elevated nuclear activity of β-catenin [206, 207]. In addition, several inflammatory pathways, including NF-κB, PI-3K and Akt pathways [208-210] can drive β-catenin nuclear accumulation even without any mutations in APC [211]. So, the initial step, which requires APC mutation to initiate CRC tumorigenesis, in some cases may be bypassed by inflammatory signals during CAC development.

As described above, inflammation can temporarily bypass the mutation requirement for tumor initiation, but continuous inflammatory injuries are able to cause mutations. Indeed, IBD and colitis induces robust genotoxic response

[212]. In genetic search of early oncogenic events in CAC, mutations in p53 gene have been identified, not only in dysplasia or carcinoma areas, but also in inflamed intestinal mucosa [213]. Further, the inflammatory infiltrate, mainly represented by T lymphocytes and macrophages, may exert divergent effects on CRC outcome. Similar to other solid cancers, high number of intra-tumoral macrophages correlates with CRC tumor malignant index [214] but high density of tumor associated macrophages (TAM) at the invasive front seems to be beneficial for patient outcome [215]. These controversial results may find an explanation in the plasticity that characterizes macrophages, underlining their ability to express either pro- or anti-tumoral programs [116]. The importance of polarized inflammation on CRC development was strengthen by the observation that different tumor infiltrating T cell subsets have opposite prognostic value [216]. Indeed, studying a large cohort of CRC patients, it has been observed that infiltrating $CD4^+$ T-helper1 (Th1) cells and $CD8^+$ cytotoxic T cells constitute a positive prognostic sign in CRC outcome [216, 217], whereas high expression of a cluster of genes associated with the T-helper interleukin (IL)-17-producing (Th17) cells is associated with a poor prognosis [216]. In line, IL-23 and IL-23R expression markedly increased from adenoma to CRC as compared to adjacent normal tissue suggesting that IL-23/IL23R pathway drives malignant progression [218]. Recently, the link between IL-23 and Th17 in CRC development was undoubtedly proved. Using a genetic model of CRC it has been shown that tumor associated myeloid cells are the major producers of IL-23 which in turn stimulates IL-17-mediated tumor growth and progression [219]. Accordingly different pre-clinical studies have demonstrated the pro-tumorigenic role of Th17-dependent inflammation even in colitis associated cancer (CAC). Indeed, using the AOM/DSS model, IL-17A-/- or IL-21-/- mice showed decreased inflammation and tumor development [220, 221]. These observations seem to be relevant also in human, indeed the IL23/Th17 pathway is recognized as one of the most important etiological factors in IBD [222] and

higher IL-21 was observed in the mucosa of both UC colitis and CAC patients [221].

On the other side, both T regulatory (Treg) cells and immunosuppressive cytokines (TGFβ and IL-10) are abundantly present in the healthy gut while their decreased number or activity is associated with chronic intestinal inflammation [223, 224]. It is well established that Tregs accumulation in tumor bearing promotes cancer growth by inhibiting antitumor immunity [225]. Accordingly, Treg accumulation in various human carcinomas is generally associated with a poor prognosis [226]. Strikingly several clinical studies demonstrated that high Treg-cell infiltration is associated with a favorable prognosis of CRC patients [216, 227]. This immune paradox, may find an explanation in the Treg mediated attenuation of Th17 pro-tumorigenic rather than Th1 anti-tumorigenic inflammation, but additional studies are required to address this issue.

Although both IL-10 and TGFβ play a crucial role in the maintenance of intestinal immune homeostasis, their involvement in CRC development is controversial.

Mouse studies indicate that ablation of IL-10 drives both colitis and CAC development in the presence of certain enteric bacteria [207, 228]. Accordingly, in genetic model of CRC, inhibition of IL-10 production and Treg generation result in increased inflammation and tumorigenesis [229]. In contrast human studies failed to find a correlation between IL-10 and CRC risk [230] or prognosis [216].

CAC mouse studies in which TGFβ signaling was specifically modulated in $CD4^+$ Tcells by using different approaches showed divergent results [231] [232]. In human, sporadic CRC showed altered TGFβ signaling [233] but TGFβ levels were not predictive of CRC patient outcome [216]. TGFβ plays multiple roles in cancer related inflammation including regulation of epithelial cell differentiation and growth arrest, Th17-mediated immunity and Treg-mediated tolerance.

Inflammation and tolerance are two critical mechanisms in gut fisiology and the presence of STAT3-inducing cytokines, such as IL-6 or IL-23, tip the balance between them [234, 235]. Accordingly, several evidences indicate that IL-6 plays a crucial role in both intestinal inflammation and CRC development. Indeed blockade of IL-6 signaling ameliorated colitis in both mouse models and in a clinical trial of patients with Crohn's disease [236]. Serum levels of IL-6 are linked to a higher risk of developing colorectal adenomas in IBD patients [237] and significantly correlate with tumor staging and poorer survival rate of CRC patients [238]. Different mouse studies contributed to understand the molecular basis of IL-6 tumor promoting activities. Besides IL-6-induced-Th17 differentiation, this cytokine links colonic inflammation with carcinogenesis through direct effects on IECs. Indeed, IL-6 promotes neoplastic transformation by regulating epigenetic tumor suppressor gene silencing [239] and stimulates the survival and proliferation of premalignant IECs, by inducing the oncogenic transcription factor STAT-3 [240]. Lamina propria myeloid cells are the major producers of IL-6 in an NF-κB-dependent manner.

As a master regulator of inflammation, balanced NF-κB activity in intestinal epithelial cells versus innate immune cells is crucial to maintain tissue integrity and gut immune homeostasis [241]. Indeed impaired NF-κB activation in intestinal epithelial cells results in chronic inflammation [242-245] but also increased NF-κB activity, as observed in the inflamed intestinal mucosa (lamina propria and epithelial cells) of IBD patients [246, 247], has a central pathogenic role in chronic intestinal inflammation [248]. In addition, as a crucial orchestrator of cancer related inflammation, NF-κB pathway drives CAC development (Fig.18) [4]. Cell specific inactivation of the IκB kinase/NF-κB pathway demonstrated that NF-κB activation in intestinal epithelial or in myeloid cells promotes tumor incidence and growth, respectively [152]. In fact, despite increased intestinal inflammation, these mice developed much fewer CAC tumors, presumably because inflammation can stimulate tumorigenesis

only as long as transformed cells retain their abililty to survive and proliferate amid adverse conditions. Because of the NF-κB inactivation, more epithelial cells underwent apoptosis and less transformed cells survived, in full agreement with the role of NF-κB as transcriptional regulator of anti-apoptotic gene expression program [1, 4].

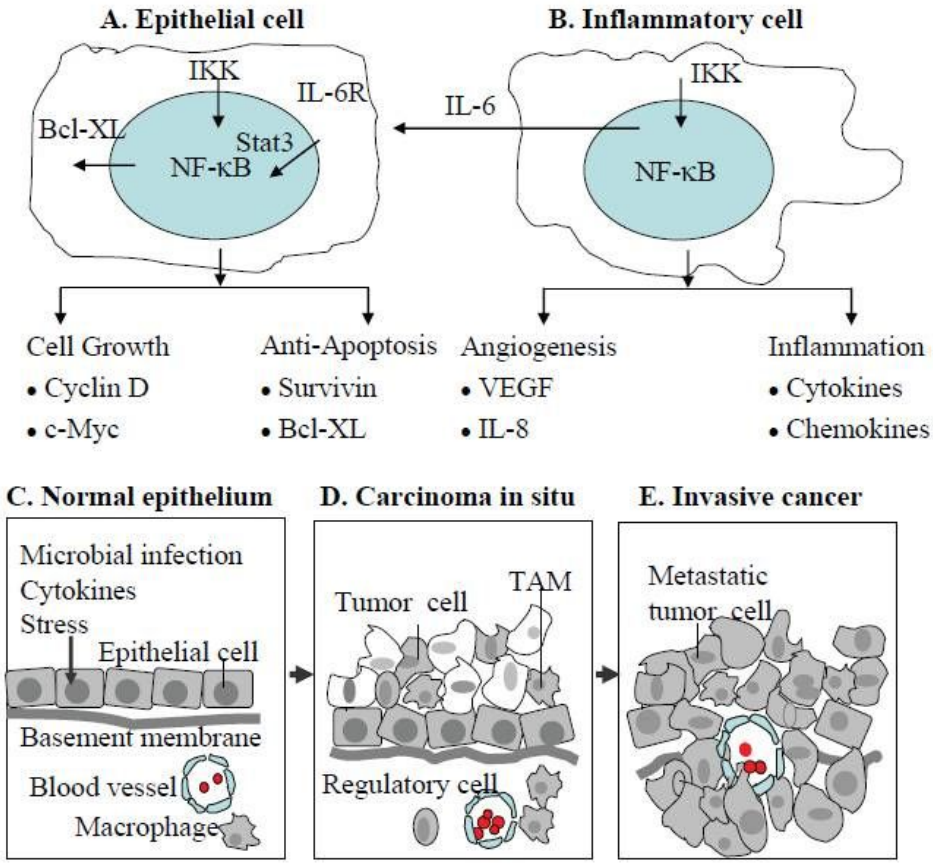

Figure 18. Inflammation and progression of CRC

NF-κB promotes the development of CRC by acting in two different cells. (A) NF-κB activation in intestine epithelial cells (IEC) results in proliferation of IEC by upregulating survival gene such as CyclinD and Bcl-XL. (B) Activation of NF-κB in inflammatory cells also contributes to CRC development by inducing expression of angiogenic factors, chemokines and epithelial cell growth factors, such as IL-6. (C) Normal epithelium. Chronic inflammation in the IEC resulted from microbial infection, cytokines, and stress, can lead to gene mutation of IEC. (D) Carcinoma in situ. Activation of NF-κB induced production of chemokines and cytokines, which attract tumor associated macrophages (TAM) and regulatory T cells. The chemkines receptor can be induced on initialted cells, and be necessary for tumor cell proliferation and invasion. (E) Invasive cancer. The chemokines and cytokines mediated signaling promotes expression of genes associated with invasion and metastasis. From [249].

In addition, inhibition of p65 expression in mice impedes IBD development [250] and many current therapies of IBD, such as sulfasalazione, mesalamine and methotrexate are directly or indirectly targeting NF-κB activation [204]. Indeed, in intestinal epithelial cells (IECs) NF-κB enhances the survival of newly emerging pre-malignant cells, whereas in myeloid cells it drives the expression of inflammatory cytokines that induce tumor growth [152]. Genetic ablation of NF-κB in intestinal epithelial cells blocks the expression of several anti-apoptotic genes, including Bcl-xL, Bcl-2 and c-IAP [152, 240, 251]. The observed increased epithelial cell death and tissue injury in the absence of NF-κB in epithelium results in more severe colitis, which tipically would be expected to lead to enanched tumorigenesis. Remarkably, however, a very pronounced decrease in tumor number is observed [152, 240, 251]. The likely explanation is that inflammation through NF-κB enhances the resistance to cell death, and as more mutated pre-neoplastic cells are given a chance to survive, more tumors form.

As described above, we can say that there are three kinds of molecules in IBD and CAC:

1) The first group is composed by molecules whose inactivation leads to decrease intestinal inflammation concomitant with reduction in CAC;
2) In the second group there are molecules whose inactivation results in exacerbation of both IBD and CAC;
3) Third group of molecules regulates IBD in CAC in opposite directions, i.e. inactivation of such molecules aggravates IBD but, despite increased local inflammation, CAC tumorigenity is reduced. Such molecules can be deemed critical for the cross-talk between inflammation and oncogenesis.

Undoubtedly, strategies aimed at blocking NF-κB have a great therapeutic potential but also relevant side effects; hence, the challenge is to design

therapeutic protocols that prevent the pathogenic effects of NF-κB without hindering its beneficial functions. For these reasons strategies to deplete TAMs or block cancer-induced M2-like macrophage programming may have the potential to enhance T cell-mediated antitumor responses and improve the efficacy of immunotherapies [252-254].

Therefore, different approaches have been explored to harness the potency of the immune system to target cancer. These have been essentially focused on enhancing the immunogenicity of the tumour or on the induction and expansion of immune effectors to potentially target and eradicate the tumour. However, till now, efforts to actively stimulate the immune system against tumours in patients have been largely disappointing despite substantial evidence that peripheral immune responses against tumour antigens can be generated. Moreover, immune-modulating activities of chemotherapeutic agents are often very complex to understand, in fact, the same molecules may play opposite roles depending on tumour type, immune contexture, and/or precise therapeutic strategy. For example, gemcitabine and 5-fluorouracil, have been reported to deplete immunosuppressive MDSCs but also to induce the release of cathepsin B from lysosomes and the activation of the NLRP3 inflammasome and caspase-1, which causes IL-1β secretion from MDSCs, resulting in IL-17 production by T cells and promotion of tumour growth [169]. To overcome this limitation, a possible approach could be the combination of specific chemotherapeutic agents with specific immunotherapeutic approaches for cancer treatment: for example, IL-1 receptor antagonist was shown to enhance the antitumor effect of 5-fluorouracil [255].

These complexities underscore the need for an ever more profound comprehension of the dynamic changes in the tumour microenvironment and in systemic immune responses as tumours evolve, progress, and respond to therapy. An improved knowledge of these aspects will facilitate the rational

design of highly efficient, synergistic regimens that combine anticancer agents and immunotherapies.

In addiction, the experimental data discussed above suggest that targeting TAMs either by ablation or repolarization can be beneficial in cancer therapy. This is an attractive approach because this diploid normal cells do not have the enhanced mutation rates of tumor cells and, for this reason, are less prone to develop drug resistance. On the other side the pan-macrophage therapeutic approaches will have systemic toxicities as they target all macrophages.

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CHAPTER TWO

2. Outline of the thesis

Macrophages are phagocytic cells with remarkable functional plasticity that play an essential role in the balance between immunity and immune tolerance.

Because of their unique role in linking the innate and adaptive immunity, macrophage-based immunotherapy is widely considered in clinical trials with cancer patients. However, major problems remain as the tumor microenvironment expresses high levels of immunosuppressive cytokines (eg. IL-10, TGFβ) that drive the anti-tumor phenotype of macrophages towards tolerogenic. immunosuppressive and anti-tumor properties. As a consequence, a major challenge in optimizing macrophage-based immunotherapy is the identification of new mechanisms controlling macrophages polarization, compatible with protective antitumor immune responses.

We have reported that massive nuclear localization of the p50 NF-κB inhibitory homodimers in Tumour Associated Macrophages correlates with impaired inflammatory functions and tumor promotion. More recently, we have reported that similarly to TAM, p50 NF-κB accumulation in monocytes/macrophages is essential for endotoxin tolerance as well as for M2 polarized activation.

Several evidence have demonstrated that physiologic levels of inflammation are necessary for intestinal tissue homeostasis and immune tolerance, but excessive inflammation is deleterious and is at the basis of inflammatory bowel disease (IBD) and inflammation-promoted colorectal cancer (CAC). A smoldering inflammation is present also in tumors not causally related to an obvious inflammatory process such as familial colorectal cancer (CRC). Hence intestinal tumor is considered one of the best model to elucidate cellular and molecular pathways underlying cancer-related inflammation.

As a master regulator of inflammation and a key inducer of cell proliferation, survival, adhesion, differentiation and angiogenesis NF-κB represents a

molecular link between inflammation and cancer. Indeed, disregulated NF-κB activation in both intestinal epithelial cells and myeloid cells results in intestinal inflammation and colon cancer development. Strategies blocking NF-κB activity have a great therapeutic potential but also relevant side effects. Hence, the challenge is to design therapeutic protocols that block the pathogenic effects of NF-κB without hindering its beneficial functions.

Based on this we decided to investigate the role of p50-driven polarized inflammation in CRC development and progression by using two distinct models of colitis-associated (CAC) and genetic- (Apc^{Min} mice) cancer.

Using a chemical model of CAC, we evaluated $p50^{-/-}$ as compared to wt mice in terms of both intestinal inflammation (as scored by weight loss, colon shortening and histology) and tumor development (as scored by tumor incidence, growth and stage). To elucidate the molecular basis linking p50-dependent inflammation with tumor development we analysed the expression of several inflammatory genes in both early inflamed colon tissues and tumors. Similarly, to investigate the role of p50 in a genetic model of intestinal tumor development we crossed ApcMin and $p50^{-/-}$ mice, to obtained double mutant mice (ApcMin-$p50^{-/-}$). We analysed mice survival, tumor multiplicity, size and histopathological stage, in ApcMin p50 sufficient versus p50 deficient mice.

We also evaluated the accumulation of p50 NF-κB in the nuclei of TAMs from CRC patient biopsies and correlated this accumulation with the prognosis.

CHAPTER THREE

The p50 NF-κB subunit promotes intestinal tumor development and progression by shaping gut associated inflammation

Unpublished results

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Abstract

Plasticity is a key feature of macrophages, in both steady state and pathological conditions. In several types of human cancers, tumor associated macrophages (TAM) express an M2-skewed phenotype and are therefore, associated with unfavorable prognosis. However, the impact of TAM infiltration on colorectal cancer (CRC) development and outcome is still controversial. Since we previously demonstrated that in response to lipopolysaccharide, nuclear accumulation of p50 NF-κB drives M2-polarization, here we investigated whether p50 NF-κB-driven inflammation influences CRC development and progression. This study demonstrates, in murine models of both colitis-associated CRC (CAC) and spontaneous intestinal cancers that p50 NF-κB is a crucial regulator of polarized inflammation associated with promotion of tumor development. Whereas progression from colitis to cancer was associated with up-regulation of M2-related genes, ablation of p50 NF-κB exacerbated the colitis score and reduced CRC development. This latter event was associated with reduced number of lamina propria and tumor-associated monocytes/macrophages, together with increased number of NK, NKT, $CD8^+$ T cells and apoptotic cancer cells. Colons from $p50^{-/-}$ tumor bearers expressed enhanced levels of M1/Th1 cytokines, including IL-12 and CXCL10, whose administration in vivo restrained CAC development. Accordingly, analysis of tumor tissues in a cohort of CRC patients indicated that high nuclear p50 in TAM and low expression of M1/Th1 genes correlates with poor prognosis. Hence our study provides first evidence that both p50 nuclear accumulation in TAM and M1/Th1 cytokines may represent both prognostic indicators and promising therapeutic targets in CRC.

Introduction

Colorectal cancer (CRC) represents one of the best example of pathological association between chronic inflammation and cancer development. Indeed, according to extrinsic and intrinsic pathways of cancer related inflammation [1] the risk to develop CRC is almost 3–fold higher in IBD patients than general population [2] and a "smoldering inflammation" is present even in sporadic and familial CRC tumors that are not causally related to an obvious inflammatory process [3, 4]. Hence, independent on their origins, CRCs exhibit a characteristic inflammatory signature, including the presence of inflammatory mediators and immune cells, associated with tumor promotion. For example, Cyclooxygenase-2 (COX-2) is over expressed in up to 40% of colon adenomas and 85% of colon adenocarcinoma compared to matched control tissues [5] and its tumor promoting role has been recognized since as early as 1981 when tumor suppressive effects of NSAIDs were originally reported [6]. Accordingly, several clinical trials confirmed that chronic use of high doses of COX-2 inhibitors exerts chemopreventive effects for colorectal tumor development, unfortunately the severe side effects on cardiovascular system does not recommend their routine use in the general population [7].

As a master regulator of inflammation, the transcription factor NF-κB is a key regulator of gut functions in both physiology and pathology. Indeed, an aberrant activation of NF-κB was frequently found in human CRC [8, 9] where actually correlates with the occurrence of lymph node metastases [10]. Accordingly, in preclinical models of CRC, IKKβ-dependent activation of NF-κB in neoplastic cells promotes their proliferation, survival and Epithelial-Mesenchymal Transition (EMT) [10, 11]. Moreover, in myeloid cells IKKβ-driven NF-κB activation induces the expression of inflammatory molecules like IL-6, which in turn activate further crucial pathways (e.g. STAT3) for proliferation and survival of cancer cells [12, 13]. The mechanisms underlying IKKα pro-tumorigenic functions further strengthen the importance of intestinal

epithelial cells (IEC)-myeloid cells cross-talk along with the complexity of NF-κB regulated pathways. Indeed in IKKα deficient mice, IKKβ-driven NF-κB activation in IEC is essential for both myeloid cells recruitment and M1-skewed activation. In turn, macrophages restrain intestinal tumor development through the release of anti-tumor molecules such as interferon-γ (IFNγ), or directly, as effector cells [14]. Undoubtedly, NF-κB represents a promising target for CRC therapy however, being a central regulator of several physiological immune and non-immune functions, its systemic inhibition will lead to severe adverse effects [15-17]. Even locally, a balanced NF-κB activity in intestinal epithelial cells versus innate immune cells is crucial to maintain tissue integrity and gut immune homeostasis [18]. Indeed either an increase [19-21] or a block [22-25] of the canonical pathway of NF-κB activation lead to chronic intestinal inflammation. Hence, the challenge is to design therapeutic protocols that prevent the pathogenic effects of NF-κB without hindering its beneficial functions.

Inflammatory cells abundantly infiltrate human cancers where depending on their functional activation may exert beneficial or detrimental activities [17, 26] Indeed, several experimental and clinical studies indicate that the high number of cytotoxic T cells, memory T cells and Th1 cells is positively associated with favorable CRC outcome whereas Th17 cells infiltration correlates with disease progression [27-32]. Accordingly, the immunoscore defined by type density and location of T cells has been widely recognized as a crucial predictive factor for CRC patients [33].

Tumor Associated Macrophages (TAM) are the major population of leucocytes infiltrating tumors, despite their potential anti-tumor activities several evidence indicate that tumors generally co-opt macrophages to promote their own development, growth and malignant progression [34, 35]. Indeed, in established cancers TAMs largely express an M2-skewed phenotype, associated with suppression of adaptive immune functions and promotion of angiogenesis and

invasion [26]. Accordingly, high number of TAMs has been associated with poor prognosis in many human cancers [36]. However, due to the well known functional plasticity of macrophages, these cells can also express an M1 phenotype, associated with anti-tumor activities [37]. As a consequence, in some studies (e.g. esophagous, gastric, prostate, lung cancers) TAMs appear to restrain rather than promote cancer progression [38]. The results are particularly conflicting in CRC [39-41]. Despite macrophages are the only innate immune cells associated with the T cell network, in both tumor center and invasive margin their presence tends to correlate with a bad outcome [31]. In contrast other studies suggest that TAMs exerts different biological activities in relation to their localization, in particular those located in the center of tumor mainly express pro-tumoral functions [42], while those situated at the invasive front exert beneficial activities [31, 39, 43]. Overall these controversial results could find an explanation in the high degree of plasticity that characterizes macrophages and that has not yet been investigated. M1 and M2 functional phenotypes are the extremes in a universe of different activation states [44], hence, in response to the complex tissue-derived signals that macrophages receive, cells in different functional states or with a mixed phenotype can coexist in the same tumor [45], consequently , in vivo, one or two markers are not sufficient to discriminate macrophage polarized activation [46]. Accordingly, simultaneous accumulation of $NOS2^{+}$ (M1) and $CD163^{+}$ (M2) macrophage populations as well as cells co-expressing both markers are observed in human CRC tumors [47]. As a reflection of dynamic changes occurring during the transition from early neoplastic events towards advanced tumor stages an M1 towards M2 switch of TAM functions could occur during tumor development [26]. Accordingly, using a pre-clinical model of colitis associated cancer it has been observed that M1 and M2 macrophages populations are dynamically recruited and functionally modulated during disease progression [48]. However, the molecular basis and the clinical

relevance of this dynamic “reprogramming” of macrophage polarization have not been fully elucidated. We have found that nuclear accumulation of p50 NF-κB in macrophages is a key event controlling both tolerance in tumor associated macrophages [49] and alternative (M2) polarized activation [50]. Basis on these finding, we investigated the impact of p50 NF-κB-driven inflammation in two different murine models of intestinal tumor development and in human stage II/III CRC progression.

Results

p50 NF-κB tunes intestinal inflammation and promotes divergent clinical outcomes in colitis versus colitis associated cancer (CAC)

To investigate the role of p50 NF-κB in inflammation associated with CRC development we adopted the chemical model of colitis associated CRC (CAC) [51] that is based on a single intra-peritoneal injection of the pro-carcinogen azoxymethane (AOM) followed by three rounds of subministration of the chemical irritant dextran sodium sulphate (DSS) in drinking water. We first examined the inflammatory response mounted by wild type (wt) and $p50^{-/-}$ mice, after a single round of DSS-induced colitis. Mice drinking regular water were used as controls. Mice survival was daily monitored until day 15, when mice entered in the resolution phase [51]. No differences in colitis score were observed between wt and $p50^{-/-}$ mice drinking regular water (data not shown). In contrast, in response to DSS administration about 60% of $p50^{-/-}$ mice died by 8 days, while, although with signs of colitis, wt mice were all alive.

At day 15, almost all wt mice (78,6%) had survived to the DSS treatment, whereas only 7,1% of $p50^{-/-}$ mice were still alive (fig S1A). Due to the high sensitivity of $p50^{-/-}$ mice to DSS treatment, we decreased the percentage of the DSS in the drinking water, from 3% to 2%, and shortened the time of treatment from 7 to 5 days. The colitis score was monitored in terms of weight loss, for the entire period of DSS administration and additional 2 weeks (fig S1B). Colon length was monitored at day 8, in 5 mice/group (fig S1C). As results, control wt and $p50^{-/-}$ mice drinking regular water displayed similar body weight (data not shown) and colon length (fig S1C), whereas in response to DSS treatment, lack of p50 resulted in severe body weight loss (fig S1B) and colon shortening (fig S1C). Hence, p50 NF-κB appears to play an essential role in intestinal homeostasis during inflammatory conditions.

To examine the role of p50 NF-κB in colitis-associated cancer, wt and $p50^{-/-}$

mice treated with AOM were either untreated (control) or subjected to three rounds of treatment with DSS and next analyzed for colitis and tumor development. In agreement with the acute model of colitis, lack of p50 was associated with higher grade of intestinal inflammation even in this chronic setting (Fig 1). Indeed, as compared to wt mice, weight loss was more significantly pronounced in $p50^{-/-}$ mice, over the entire experimental period (Fig 1A). In agreement, colon necropsy of $p50^{-/-}$ mice showed significant decrease in colon length, as compared to wt mice (Fig 1B), Moreover, histological analysis showed higher number of ulcers in colon tissues from $p50^{-/-}$ mice (Fig 1C), associated with an overall higher grade of colitis (Fig 1D). Conversely, both macroscopic (fig 1E) and histological (Fig 1F) analysis demonstrated lower numbers of neoplastic lesions, characterized by smaller size, in colons from $p50^{-/-}$ mice, as compared to wt.

Collectively these results indicate that the increased inflammatory response, resulting from p50 NF-κB depletion, impairs CRC development.

Lack of p50 NF-κB impairs survival of colorectal cancer cells

Since the absence of p50 NF-κB impaired tumor multiplicity and growth, we investigated its role in colonic cancer cells proliferation and survival. To this aim, colons were harvested from untreated and AOM/DSS treated mice and immunostained with anti-Ki-67 and anti-active caspase-3 antibodies. Strikingly, while in both untreated and AOM/DSS treated mice lack of p50 generated longer crypts with higher proliferation rate of colonic epithelial cells (Fig S2A), it did not affect cancer cell proliferation (Fig S2A). In agreement, as compared to wt mice, we observed higher expression of genes associated with cell cycle progression in the normal colonic mucosa from either untreated or AOM/DSS treated mice, whereas no differences were found in wt vs $p50^{-/-}$ cancer cells (Fig S2B). Along with NF-κB, STAT3 is a key orchestrator of cell proliferation and survival, which was found up-regulated in AOM/DSS induced CRC [12, 13].

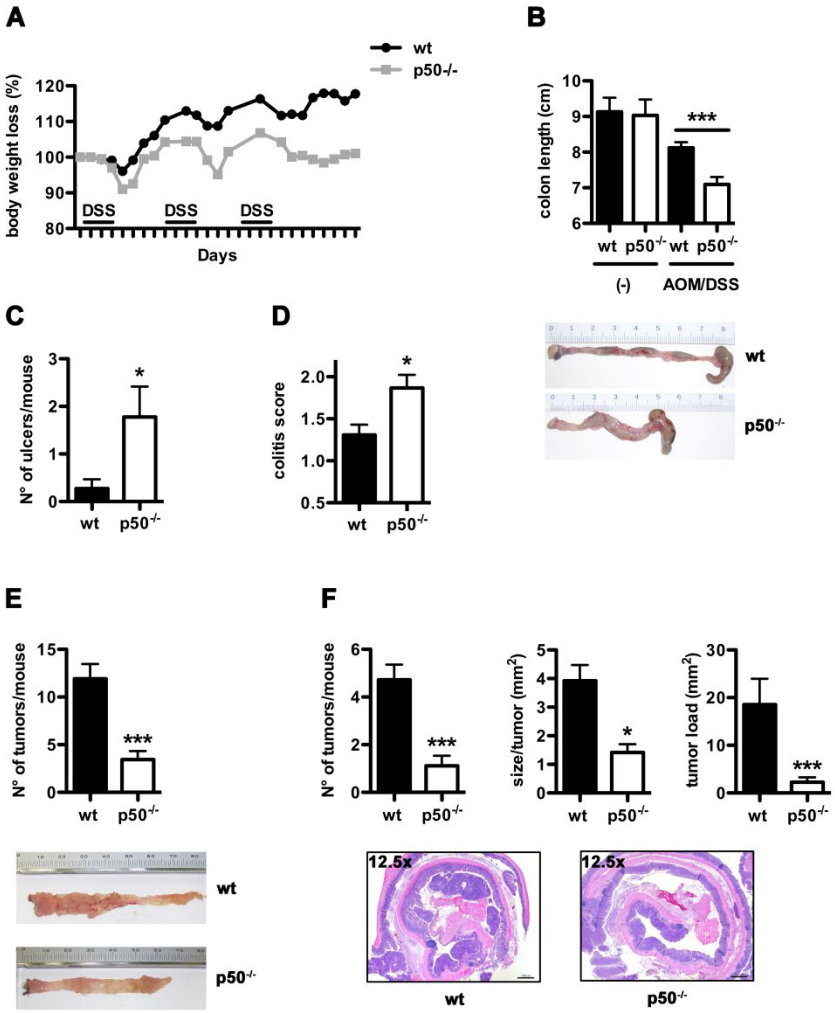

Figure 1: p50$^{-/-}$ mice developed higher colitis but less tumors after AOM/DSS administration. To induce CAC wild type (wt) and p50$^{-/-}$ mice were treated with AOM and DSS. To evaluate colitis, body weight loss was monitored every 2-3 days during the entire experimental period (t test, $P<0.0001$, $N=16$) (A); colon length was measured at the time of harvest (day 100) (B); ulceration (C) and overall degree of inflammation (D) were analyzed on colon swiss rolls sections stained with hematoxylin-eosin (H&E). To evaluate tumor development, colons were longitudinally opened and polyps were counted (E); representative images are shown (E). H&E stained sections of colon swiss rolls were histologically evaluated. The total number and the size of tumors were recorded, thus tumor burden for each mouse were calculated (F); representative images are shown (magnification 12.5x) (F). Data shown are mean±SEM of different mice or tumors. Error bars have been omitted from the weight loss data for clarity of presentation. (*$P<0.05$, **$P<0.01$, ***$P<0.001$, Mann Whitney test, two-tailed, $N\geq9$).

Immunohistochemical analysis of colon tissues showed similar increased levels of P-STAT3 in neoplastic cells from wt and $p50^{-/-}$ mice (Fig S3C) suggesting that in cancer cells, p50 does not alter the activation of STAT3 as well as cancer cells proliferation. In contrast, colon from AOM/DSS treated $p50^{-/-}$ mice showed increased number of cells expressing the activated form of caspase 3, indicating that lack of p50 impairs epithelial and cancer cell survival (Fig 2A). In line, tumors harvested from $p50^{-/-}$ mice expressed higher levels of the pro-apoptotic Bak gene and decreased levels of the pro-survival Bcl-XL gene (Fig 2B). No differences were found in the expression of the antiapoptotic genes Bcl2 and Survivin, as well as the proapoptotic Bax gene (Fig 2B). Colon from untreated wt and $p50^{-/-}$ mice showed similar levels of apoptotic cells (Fig 2A) and survival gene transcripts (Fig 2B).

Overall these results indicate that under inflammatory conditions p50 NF-κB promotes survival of colonic epithelial and cancer cells, thus contributing to tumor development.

p50 NF-κB tunes the balance between two differentially expressed clusters of inflammatory genes, respectively associated with inhibition and promotion of tumor development.

To evaluate the inflammatory profiles associated with CRC progression and resistance we analyzed the expression of several inflammatory genes in total RNA isolated from wt colon, tumors and adjacent healthy tissue in both colitis (9 days after the first DSS administration) and established tumors (after 80-90 days of treatment with DSS). RNA from colon of untreated mice was used as control. By comparing inflammatory transcripts levels in colitis vs cancer, we identified two major clusters of inflammatory genes. Genes cluster 1 were similarly up-regulated in both colitis and in tumors as compared to control colons, whereas cluster 2 was even more expressed in established cancers than in colitis (Fig 3A).

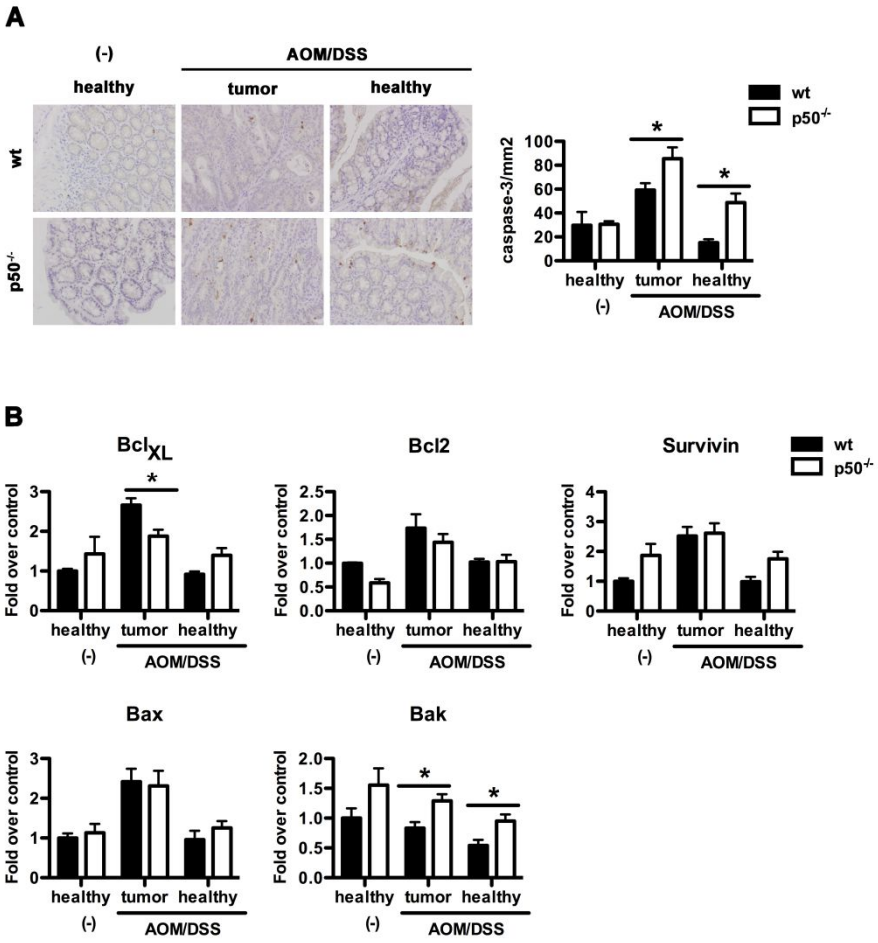

Figure 2: Lack of p50 results in increased apoptosis of both colonic epithelial and tumor cells after AOM/DSS administration. Colon from AOM/DSS treated and untreated mice were formalin fixed and paraffine embedded. Colon sections were evaluated for apoptotic activity through cleaved-caspase 3 immunohistochemistry and digital image analysis. 200x microscopic fields were randomly selected within the neoplastic lesions ("tumor") and the adjacent non-neoplastic mucosa ("healthy"). Representative images are shown (magnification 20x). Data shown are mean±SEM of different fields (*P<0.05, **P<0.01, Mann Whitney test, one-tailed, N≥8) (A). Transcripts levels of survival genes were evaluated in total RNA isolated from colon and tumor of untreated and AOM/DSS-treated mice. Normalized qPCR results are shown as fold induction over healthy untreated wt mice. Data shown are mean±SEM of different mice (*P<0.05, Mann Whitney test, one-tailed, N≥4) (B)

Of note, in the healthy tissue, near the tumor removed, expression levels of most of the analyzed inflammatory genes dropped to levels comparable with those observed in the colon of untreated mice. (Fig 3A). The selective increased expression of cluster 2 in tumor tissues suggested its tumor promoting activity. Of note, cluster 2 includes the well known tumor promoting genes COX2, TNFα and IL-23p19, along with several markers of M2 polarized inflammation (IL-10, TGFβ, ArgI, CCL17, CCL22) [45] (Fig 3A). In contrast, cluster 1 comprises a predominant expression of genes associated with M1/Th1-skewed immune profile (IL-1β, IL-6, IL-12p40, IL-12p35, IL-27p28, Ebi3, CXCL9, CXCL10, iNOS, IFNγ, IL-21, perforin 1, Granzyme B, FasL) (Fig 3A). Since progression from colitis towards cancer is paralleled by the upregulation of cluster 2, our results suggest that a type 1 versus type 2 shift of polarized inflammatory response occurs during the transition from colitis to cancer. To investigate the molecular basis of this event we focused our attention on macrophages, which are considered crucial orchestrators of cancer related inflammation. Since we previously described that nuclear accumulation of p50 in macrophages promotes M2-like transcriptional program [49, 50] , we analyzed the nuclear levels of the p50 and p65 NF-κB subunits in both lamina propria and tumor-associated macrophages. Confocal microscopy analysis showed a selective nuclear accumulation of p50 over p65 in TAM as compared lamina propria macrophages of control mice (Fig 3B)

Next, we examined clusters 1 and 2 in the tumor resistant p50-deficient mice. As compared to the wt counterpart, in $p50^{-/-}$ tumors we observed strong inhibition of IL-23p19 expression, paralleled by significant upregulation of M1/Th1 inflammatory genes (IL-12p40, IL-27p28, Ebi3, CXCL9, CXCL10, iNOS, IFNγ, Perforin 1, Granzyme B, FasL, IL-21) (Fig 3C). These results identify group 2 as the inflammatory profile rising during the transition from colitis to cancer and indicate p50 NF-κB as the main regulator of this transcriptional reprogramming.

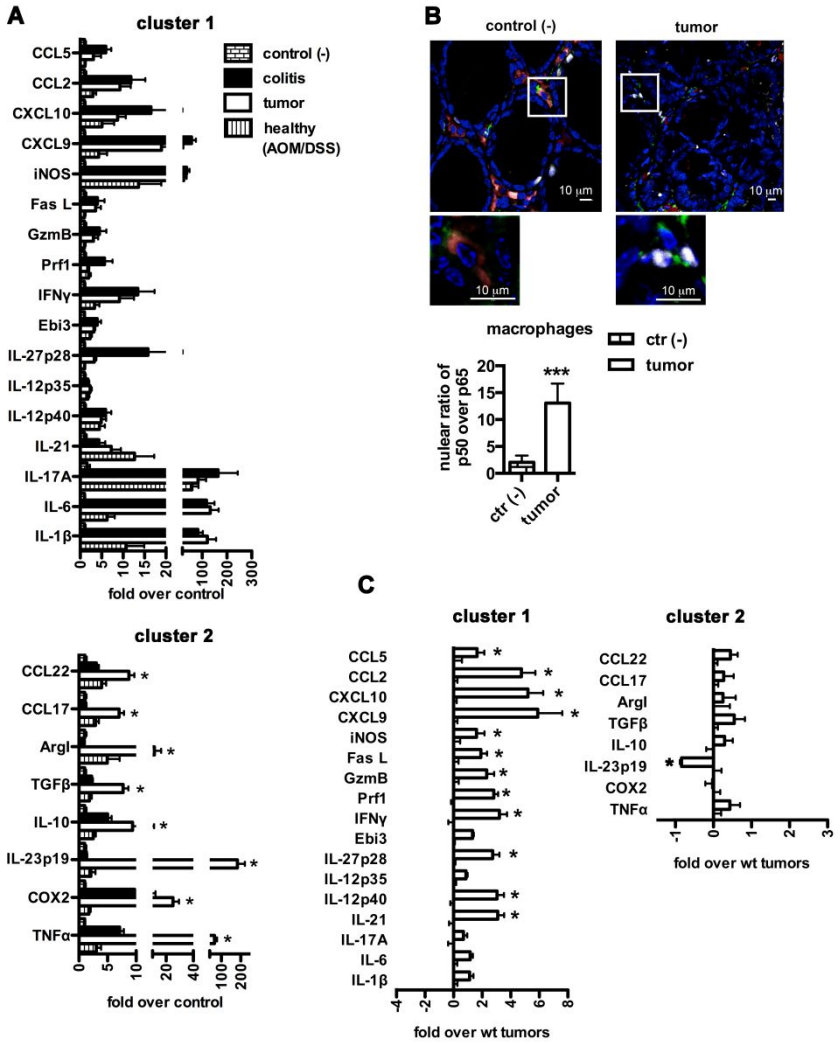

Figure 3: p50 controls the expression of different cluster of inflammatory genes associated with different outcome. The expression of selected inflammatory genes was analyzed in total RNA extracted from colon after 1 cycle of DSS administration (colitis), tumors and adjacent healthy tissue at the end of the AOM/DSS experiment. RNA from colon of untreated mice (control) was used as control. Normalized qPCR results are shown as fold increase over control. Cluster 1 includes genes which were similarly up-regulated in colitis and in tumors as compared to control colons. Cluster 2 includes genes which showed increased levels of expression during progression from colitis towards cancer. Data shown are mean±SEM of different mice (*P<0.05, Mann-Whitney one-tailed N≥5) (A). Immunofluorescent analysis of p50 and p65 nuclear levels in colonic and tumor associated macrophages (B). RNA from tumors of AOM/DSS treated p50-/- mice were analyzed for the expression of the genes clusters 1 and 2. Results are expressed as fold over the levels of wt tumors. Data shown are mean±SEM of different mice (*P<0.05, **P<0.01, ***P<0.001 Mann Whitney test, one-tailed, N≥13).

M1/Th1-skewed inflammation inhibits CAC development

To formally prove the antitumor properties of selected members of cluster 1, AOM/DSS-treated mice underwent systemic (intra-peritoneal) or local (intra-rectal) administration of IL-12 (100ng) and CXCL10 (500ng), once a week (Fig 4A). Control AOM/DSS-treated mice received only vehicle (Fig 4A). Despite control mice (vehicle), IL-12- and CXCL10-treated mice showed similar body weight loss (Fig 4B) and colon length (Fig 4C), a significant reduction of tumor multiplicity was observed in response to IL-12 and CXCL10 treatments (Fig 4D). These results strongly support the concept that activation of the M1/Th1 immune profile may restrains CAC development and disclose the therapeutic potential of IL-12 and CXCL10 in CRC.

Lack of p50 NF-κB shapes tumor-associated immune infiltrate by restraining macrophages and by enhancing cytotoxic effectors cells

In $p50^{-/-}$ mice, increased expression of M1/Th1 genes along with higher rate of colonic and cancer cells apoptosis indicate that p50 activity impairs cytotoxic type 1-skewed inflammatory responses. To further investigate whether p50 could alter the composition of the tumor associated immune infiltrate, colons of untreated and AOM/DSS-treated wt and p50 deficient mice were analyzed for the presence and distribution of the different leukocytes populations.

The results showed a similar number of tumor and lamina propria associated neutrophils ($Ly6G^+$ cells) in wt vs $p50^{-/-}$ mice, while in absence of p50 we observed a reduction of tumor- and lamina propria- (LP) associated monocytes ($Ly6C^+$ cells) and macrophages ($F4/80^+$ cells), along with increased number of T lymphocytes ($CD3^+$ cells), NK ($NKp46^+CD3^-$) and NKT ($NKp46^+CD3^+$) cells, in both tumors and adjacent healthy tissue (fig 5A, B). In absence of p50, the number of mucosal monocytes and macrophages is significantly reduced even in untreated mice, while colonic NK, NKT and T cells are similarly present in wt and $p50^{-/-}$ mice (Fig S3).

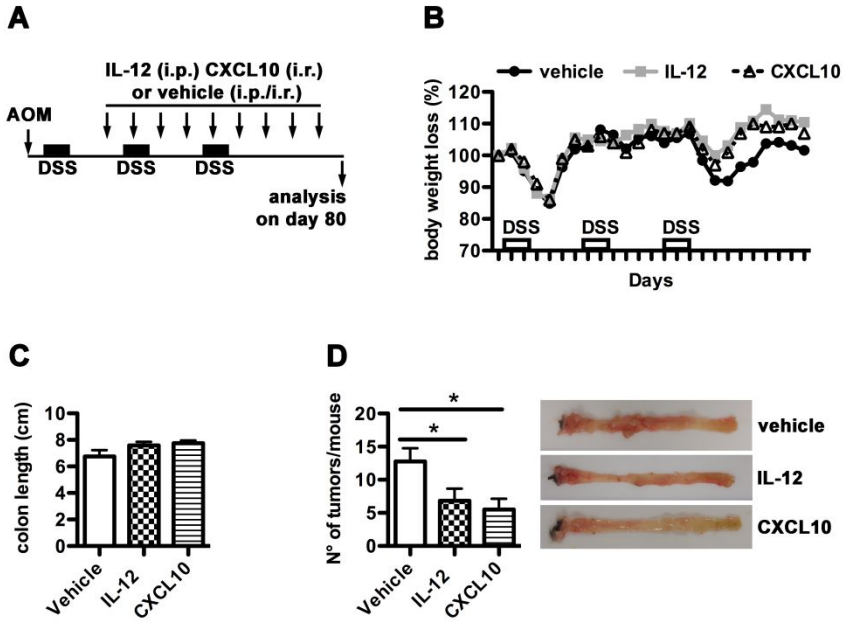

Figure 4: M1 cytokines treatment inhibits CAC development. Schema of treatments with IL-12 and CXCL10 during CAC induction (A) Analysis of body weight loss (B) and colon length (C) of the different groups of mice (vehicle, IL-12, CXCL10). At day 80 tumor development was analyzed by evaluation of longitudinally opened colons for the number of polyps. Representative images are shown (D). Data shown are mean±SEM of different mice (*P<0.05 Mann Whitney test, one-tailed, N≥5)

These data were confirmed by both FACS and expression analysis of genes encoding for markers of the different leukocyte populations (data not shown). Further, in absence of p50, we found an increased expression of CD8 and Tbx-21 (Tbet), while both CD4 and the transcription factors which are typically induced in Th2/ILC2 (GATA-3), Th17/ILC3 (RORc) and Treg (FOXP3) cells, are similarly expressed (Fig 5C).

These results further suggest that ablation of p50 shapes gut associated lymphoid cells towards cytotoxic innate (NK, NKT, ILC1) and adaptive (CD8, Th1) effector cells.

Therefore to rule out whether, in $p50^{-/-}$ mice, T cells responses are needed for tumor resistance, mice were weekly i.p. injected with anti-CD4 and anti-CD8

antibodies, during the entire AOM/DSS treatment. Whereas depletion of $CD4^+$ and $CD8^+$ T cells results in a similar light increased of intestinal inflammation in both wt and $p50^{-/-}$ mice, tumor multiplicity augmented in $p50^{-/-}$ mice reaching the extent of wt mice (Fig 5D). Hence these findings indicate that lack of p50 NF-κB subunit restrains CAC development by enhancing the inflammatory circuits associated with T cells dependent immune surveillance.

p50 NF-κB inhibits tumor development even in a genetic model of intestinal carcinogenesis

As demonstrated for the adaptor protein MyD88, the role played by the molecular determinant of intestinal carcinogenesis developed in presence or absence of a pre-existing chronic inflammation may differ [52-54]. Therefore, we decided to evaluate the role of p50 NF-κB in the spontaneous model of intestinal cancerogenesis. Human germline APC mutations are a cause of familiar adenomatous polyposis, but APC gene is also mutated in over 80% of human cancer, indicating that Apc^{Min} mice represent a useful pre-clinical model to study sporadic cancer [55]. Hence, Apc^{Min} mice were crossed with $p50^{-/-}$ mice, in order to generate $Apc^{Min}/p50^{-/-}$ mice. Next, small intestine and colon were harvested from both Apc^{Min} and $Apc^{Min}/p50^{-/-}$ mice at different ages (12 and 18 weeks) and analyzed for the presence of tumor lesions. As expected, both tumor multiplicity and size increased with mice aging. However, as compared to Apc^{Min} mice, we observed a significant inhibition of both tumor incidence and growth in the $Apc^{Min}/p50^{-/-}$ group (Fig 6A), which was associated with increased survival, from 6 months of age (mean 22 weeks) to 10 months (mean 41 weeks) respectively (fig 6B). The role of p50 was examined at different stages of tumor progression (Fig S4A). As results, the absence of p50 was associated with fewer (Fig S4B) and smaller (Fig S4C, D) tumor lesions, at all stages of tumor development.

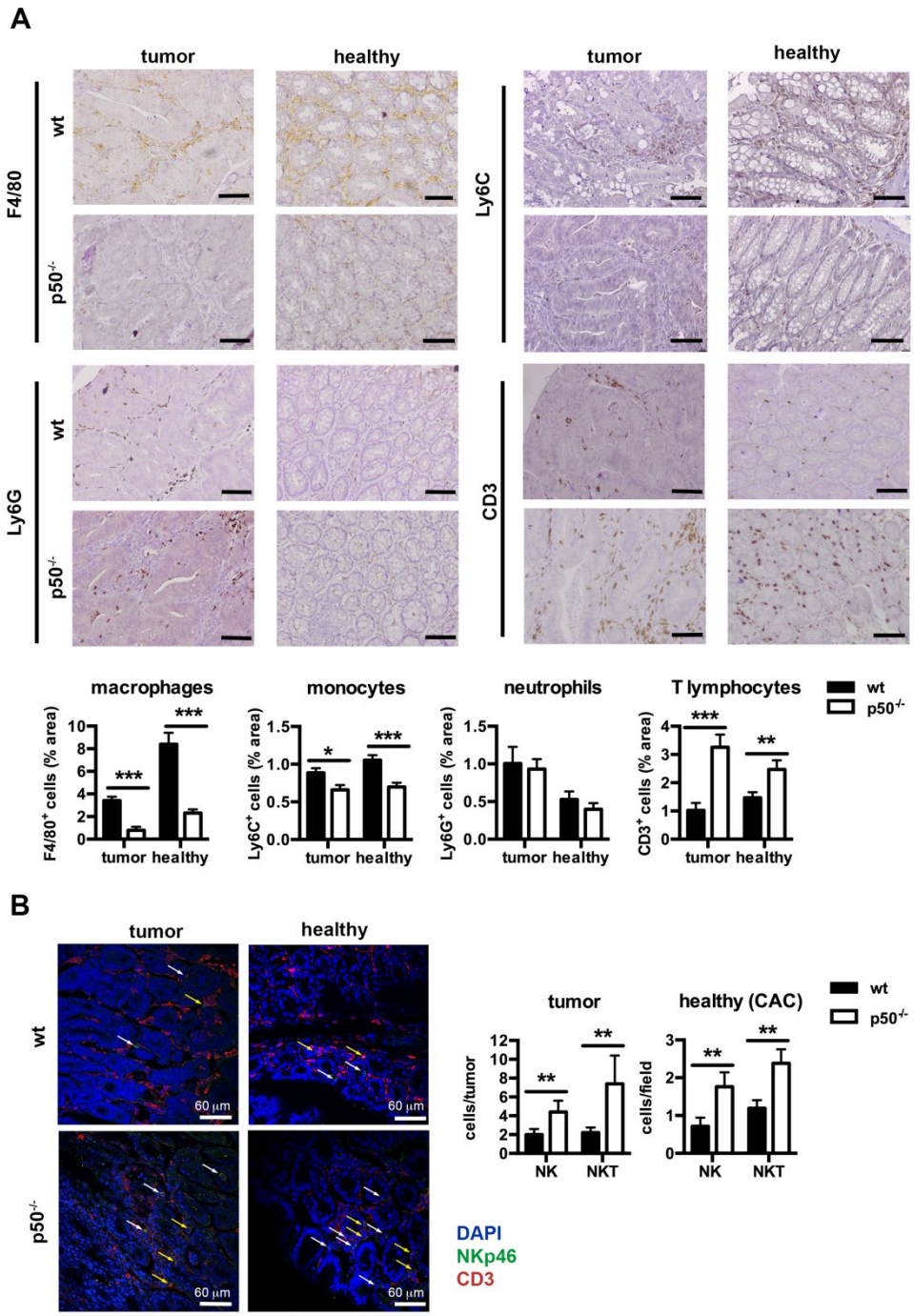

Figure 5 Panels A and B: p50 NF-κB shapes the composition of gut associated immune cells . Colon from AOM/DSS treated and untreated (-) mice were evaluated for the number of both lamina propria and tumor associated immune cells populations. Sections of formalin fixed and paraffine embedded colons were evaluated for the number of macrophages (F4/80), monocytes (Ly6C), neutrophils (Ly6G) and T lymphocytes (CD3) by immunohistochemistry (A). Immunofluorescent staining of frozen colonic samples

with anti-NKp46 (green) and CD3 (red) antibodies. Nuclei were stained with DAPI (blue). White and yellow arrows indicate NK (CD3-NKp46+) and NKT (CD3+NKp46+) cells, respectively (B). Representative images are shown (scale bars are 60 μm). Data shown are mean±SEM of different tumors or fields (**P<0.01, ***P<0.001 Mann Whitney test, one-tailed, N≥5 tumors N≥8 fields).

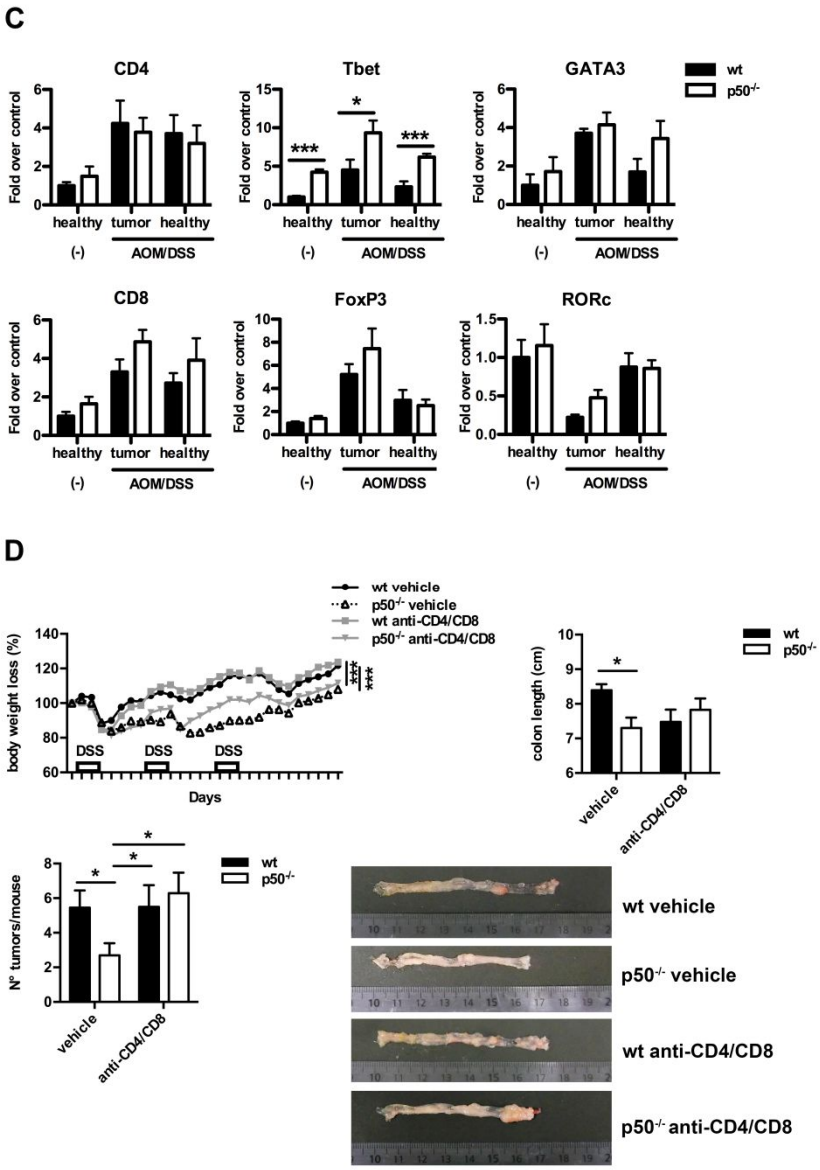

Figure 5 Panels C and D: Transcript levels of genes encoding for markers of different leukocytes populations were evaluated in total RNA isolated from colon and tumors of untreated and AOM/DSS-treated mice. Results are shown as fold induction over healthy untreated wt mice. Data shown are mean±SEM of different mice (*P<0.05, Mann Whitney test, one-tailed, N≥4) (C) T cells were depleted from wt and $p50^{-/-}$ mice during the entire experimental period by i.p. injections of anti-CD4 and anti-CD8

antibodies. Control mice received vehicle only. Body weight loss, colon shortening an tumor development are evaluated. Representative images of longitudinally opened colons at day 80 are shown. Data shown are mean±SEM of different mice (*P<0.05 Mann Whitney test, one-tailed, N≥7) (D).

Our data underlie the relevance of p50 NF-κB activity in the promotion and progression of intestinal cancerogenesis, from the initial events of carcinogenesis (GIN) towards advanced stages (LGA, HGA, C).

Next we investigated whether the inflammatory circuits observed in the CAC tumor model were also expressed during the spontaneous intestinal carcinogenesis occurring in ApcMin mice. Hence, tumors from Apc^{Min} and $Apc^{Min}p50^{-/-}$ mice were analyzed for the density of TAMs and for the expression levels of the gene clusters 1 and 2. In agreement with the CAC model, in the absence of p50 we observed a reduction of TAMs at any stage of tumor development (Fig 6C) thus confirming the importance of p50 in the circuits controlling TAMs accumulation and confirming the low density of TAMs as a favorable prognostic indicator. Next, we analyzed the inflammatory gene profile of CRC lesions. Since Apc^{Min} mice largely develop tumors in the small intestine, CRC lesions were obtained from the colon of 20 weeks old mice, when polyposis is so extended that CRC arises also at the large intestine [56]. Similarly with the CAC model, most of the genes belonging to cluster 1 and 2 were upregulated in tumor tissues as compared to the adjacent healthy mucosa (Fig 6D). Interestingly, lack of p50 confirmed the selective reduction of the tumor-promoting IL-23p19 gene transcript along with enhanced expression of M1/Th1 inflammatory genes (Fig 6E).

Collectively, these results indicate that irrespective of the etiological events triggering CRC development, the p50 NF-κB subunit promotes intestinal cancer development by favoring TAMs accumulation and an M2-type tumor promoting inflammation.

Low levels of nuclear p50 in TAMs and high levels of type 1 gene expression in human CRC are associated with better prognosis.

To explore the potential relevance in human CRC of p50 NF-κB modulation in $CD68^+$ TAMs, we assessed its nuclear expression in tissue specimens from patients with stage II and III tumor. The percentage of nuclear $p50^+CD68^+$ TAMs at the invasion front or within the tumor nests did not differ between stage II and stage III CRCs. As to disease progression, the percentage of nuclear $p50^+CD68^+$ TAMs at the invasive tumor front was significantly higher ($p=0.003$) in patients with than in those without post-surgical progression (Fig. 7A). Besides the frequency of intra-tumoral $p50^+CD68^+$ TAM is directly correlated (Correlation coefficient (r)= 0,733161; r^2 = 0,537524) with those at the invasive tumor front, the rate of nuclear $p50^+$ cells in TAMs within the tumor did not differ significantly according to the outcome (Fig 7A). Consistently, the presence of high $p50^+$ TAMs at the tumor invasion (but not within the tumor), was associated with a significantly worse disease-free survival (Log-rank test, $p=0.004$).

Also the expression levels of the mRNAs coding for type 1 inflammatory genes (IL-12p35, Tbx-21, CXCL9, IL-21, CXCL10, and IL12p40) in tumor tissues did not differ between stage II and III CRCs. However, with respect to patients' outcome, low mRNA levels of inflammatory genes (IL-12p35, Tbx-21, CXCL9, and IL-21) were associated with a significantly worse (all p-values<0.05) or tending to be worse (CXCL10 and IL12p40) disease free survival (Fig. 7B).

Our results indicate that high expression of p50 in TAMs at the invasive margin and low levels of type 1 inflammatory genes, are associated with a significantly worse post-surgical survival, and are a signature of increased metastatic potential in human CRC.

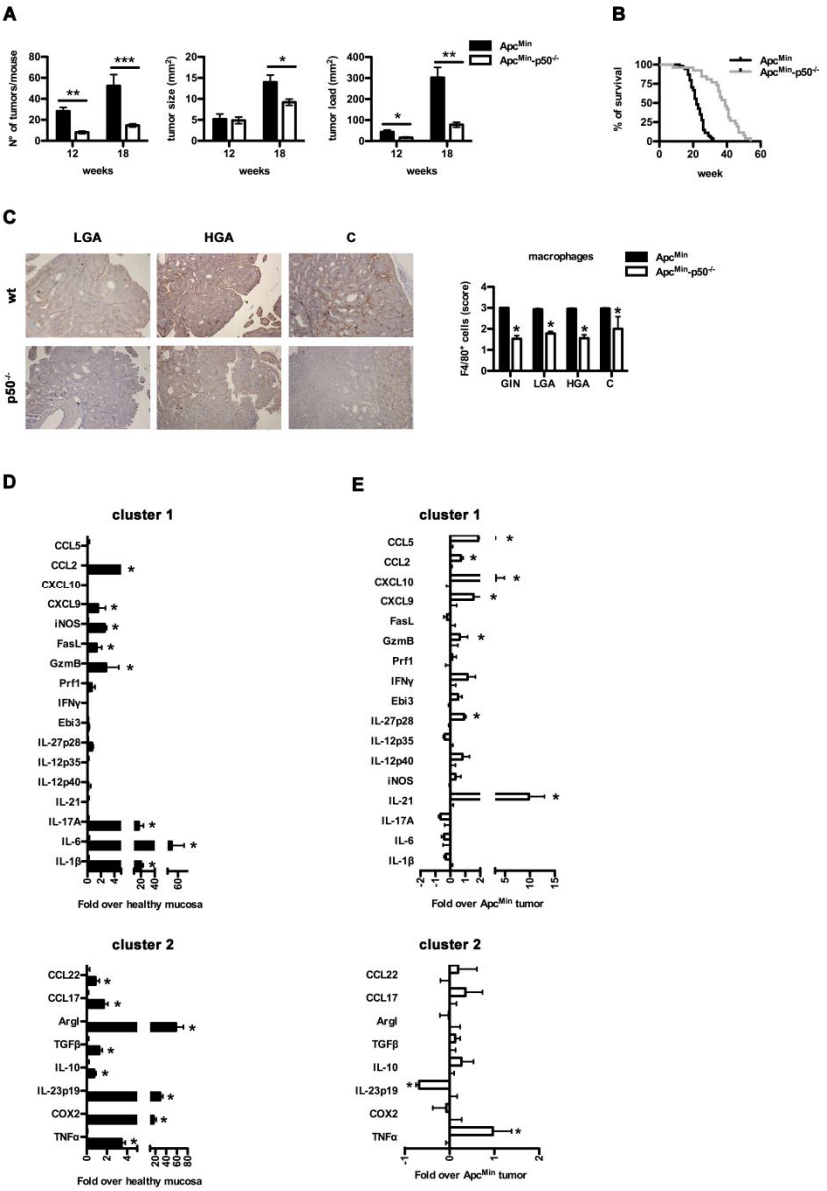

Figure 6: lack of p50 inhibits spontaneous intestinal tumor development. Histological analysis of small gut and colon harvested from Apc^{Min} and Apc^{Min}-$p50^{-/-}$ mice at 12 or 18 weeks of age. Number and size of tumors was recorded, then tumor burden was calculated for each mouse. Data shown are mean±SEM of different mice or tumors (*P<0.05, **P<0.01, ***P<0.001 Mann Whitney test, one-tailed, N≥7). (A). Apc^{Min} and Apc^{Min}-$p50^{-/-}$ mice were passively monitored for their survival (**P<0.01; N≥ 27). (B). To evaluate tumor associated macrophages gut sections were immunohistochemical stained with anti-F4/80 antibody. Semi-quantitative analysis of $F4/80^{+}$ cells were performed on the entire gut sections. Representative images are shown (magnification 20x) Data shown are the mean±SEM of different tumor lesions. Low grade adenoma (LGA), high grade adenoma (HGA) and carcinoma (C) (*P<0,05; Mann

Whitney test, one-tailed, LGA, N≥44; HGA, N≥18; K, N≥3). (C). Analysis of the expression of the above identified gene cluster 1 and 2 in CRC isolated from Apc^{Min} mice as compared to adjacent healthy colonic mucosa (D) Analysis of CRC from Apc^{Min} and Apc^{Min}-$p50^{-/-}$ mice for the genes clusters identified as previously described (E) . Data shown are the mean±SEM of different mice (*P<0,05; Mann Whitney test, one-tailed, N=6).

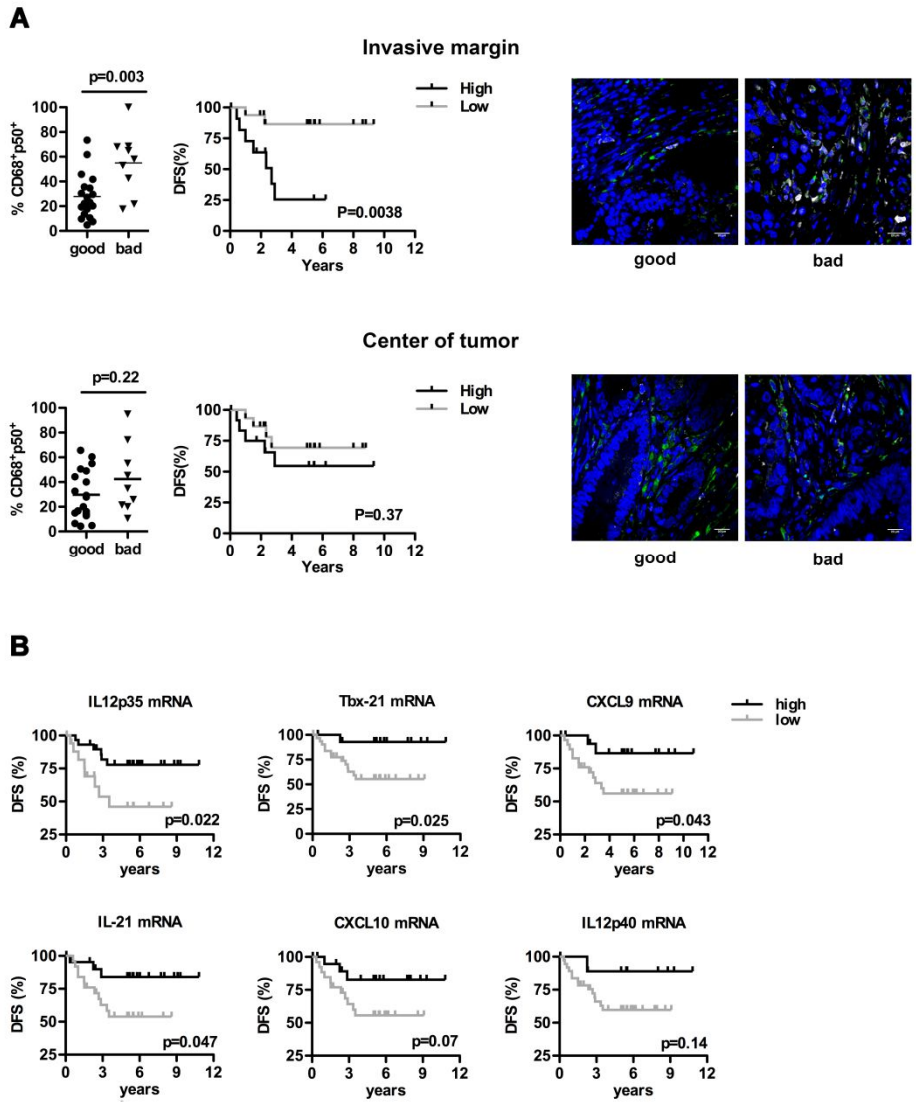

Figure 7: Both nuclear levels of p50 in TAMs and expression levels of type 1 inflammatory genes could predict better CRC outcome. Immunofluorescent staining of 28 stage II and III CRC specimens for CD68 and p50. Analysis of the percentage of p50+ TAM at both invasive margin and tumor center in relation to tumor relapse. Good or bad outcome are defined as tumor recurrence or not by 5 years follow up. Kaplan-Meier curves show disease free survival (DFS) of CRC patients in relation of high (>mean) or low (<mean) p50+ TAM. Representative images are shown (A) Analysis of selected type 1 inflammatory genes

expression in total RNA from 47 stage II and III CRC specimens. Results are normalized over β-actin as housekeeping gene. For each gene transcript, cut–off value was extrapolated by ROC curve analysis (IL-12p35 4.28e-5; IL-12p40 7.88e-5; iNOS 1.56e-2; CXCL9 3.72e-3; CXCL10 8.32e-3; IL-21 1.13e-4) than Kaplan-Meier curves show disease free survival (DFS) of CRC patients in relation of the expression levels of the selected type 1 inflammatory genes in tumor samples. (B).

Discussion

Owing the importance of inflammatory tumor microenvironment on the development and progression of colorectal cancer [3], type density and location of immune infiltrate, the so called "immunoscore", has been recently suggested as a better predictor of CRC patients outcome than other methods for staging [33]. Among inflammatory cells, tumor associated macrophages (TAMs) has long been recognized as the major orchestrators of cancer related inflammation [1, 57] and predictors of poor prognosis for many different human tumors [36, 58, 59]. However, the impact of macrophages on colorectal cancer (CRC) outcome is controversial, likely because of its anatomical site. In addition to tumor-derived signals, CRC-associated macrophages activation can be influenced by unique local microenvironmental signals (e.g. microbial products) to whom they are exposed [28] and consequently multiple macrophages populations with either pro- or anti-tumoral activities could co-exist in CRC. In vivo evaluation of macrophages polarized activation is challenging. To dissect the complexity of functional macrophages heterogeneity, a panel of genes which are typically induced in response to M1 and M2 signals, should be evaluated [46]. Alternatively, macrophages polarized activation could be easier and more feasible investigated by addressing the activation of transcription factors that selectively control the expression of M1 and M2 transcription programs. We have previously demonstrated that p50 NF-κB is a key orchestrator of M1 versus M2 macrophages polarized activation, in different pre-clinical models of infections (sepsis, helminth infections), allergic inflammation (asthma) and

cancer [49, 50]. Here we originally find that high accumulation of p50 NF-κB in the nuclei of TAMs located at the invasive margin of human stage II/III CRC could be successfully used to predict disease recurrence. Despite the percentage of nuclear $p50^+$ TAMs in the stroma is significantly associated with those at the invasive margin, the predictive value of p50 in TAM situated at the center of tumor did not reach statistic significance. This results suggest that macrophages located at the invasive margin are more potent than those situated in the center in inducing immune reactions against neoplastic cells. Accordingly, a positive association between the number of FasL expressing macrophages at the invasive margin of CRC and apoptotic cancer cells has been reported [60].

Whereas a type 1 inflammatory profile is predictor of beneficial outcome for CRC patients (fig 7 B) [61], the unfavorable prognostic significance of p50 nuclear accumulation in TAM is likely associated with its M2-skewing ability, as we previously demonstrated in murine established tumors [49] and we confirmed, here in both a chemical model of colitis associated CRC (AOM/DSS) and in a genetic model of intestinal tumorigenesis (Apc^{Min} mice).

Although an M1 towards M2 switch of TAM functions has been suggested during tumor development [26] the molecular basis and the clinical relevance of this dynamic "reprogramming" of macrophage polarization have not been fully elucidated. In particular, whereas in established cancers M2-skewed activation has been mainly associated with tumor growth and progression, at tumor initiation stage, the impact of M1 macrophages seems to be dual: through the release of genotoxic molecules (e.g. ROS and NOS) and growth factors (e.g. IL-6) they could support both neoplastic transformation and proliferation of initiated cells [11, 62] while as effector cells, they likely contribute to immune surveillance by eliminating pre-malignant cells [14, 63]. Since both AOM/DSS treatment in C57BL6 mice strain and Apc^{Min} mice mainly results in the development of multiple benign polyps [55, 64, 65] both models are suitable to investigate how p50-driven inflammation support CRC initiation. Our results

coherently support a model in which, independent on their triggers, an M1 towards M2 switch of polarized inflammation is driven by p50 accumulation in macrophages and associated with intestinal tumors development. Indeed, exploring the molecular mechanisms linking p50-driven inflammation with colitis associated CRC development we identified two clusters of inflammatory genes, which are differentially modulated by p50 and whose relative expression impact on disease outcome. In particular, whereas both gene clusters are up-regulated in early inflamed colons, during disease progression, nuclear accumulation of p50 in TAM is associated with the selective increased expression of gene cluster 2 which includes several M2-related genes (e.g. IL-10, TGFβ, ArgI, CCL17, CCL22) along with other intestinal tumor promoting genes (e.g. COX2, IL-23p19, TNFα). In contrast, enhanced expression of gene cluster 1, which includes M1/Th1 inflammatory genes only, occurred in $p50^{-/-}$ tumors and is associated with tumor resistance. Hence, despite $p50^{-/-}$ mice showed an increased gut inflammation, both tumor multiplicity and size are strongly reduced. In absence of p50, also Apc^{Min} mice showed a reduced tumor development associated with selective increased expression of M1/Th1 inflammatory genes. Hence our findings indicate that in both models, intestinal cancer development is promoted by an M2 shift of polarized inflammation and identify p50 NF-κB as the crucial molecule that skews polarized inflammation towards pro-tumoral functions.

Lack of p50 profoundly modifies immune cells infiltration too. Besides $p50^{-/-}$ tumors express higher levels of monocytes chemoattractants (e.g. CCL2, CCL5 and MCSF) (Fig 3 and data not shown) TAM accumulation is strongly reduced in both intestinal tumor models. Lamina propria macrophages and monocytes are also less present in $p50^{-/-}$ than in wt mice, both untreated and AOM/DSS treated. Since, gut macrophages are constantly replenished by bone marrow-derived monocytes [66], overall these results suggest that p50 are required for monocytes/macrophages recruitment. This hypothesis is also supported by the

observation that, ex-vivo, migration of $p50^{-/-}$ peritoneal macrophages towards several different chemoattractants is severely impaired (unpublished data). In contrast, innate and adaptive lymphoid cells migration seems to be functional in absence of p50. Indeed, at steady state NK, NKT and T cells are similarly present in the lamina propria of wt and $p50^{-/-}$ mice and are increased in $p50^{-/-}$ colons and tumors of AOM/DSS treated mice. According to the accumulation of cytotoxic lymphoid cells, in $p50^{-/-}$ tumors we observed an increased expression of type 1-specific chemoattractants (e.g. CXCL9, CXCL10), cytokines (IL-12p35, IL-12p40, IL-27p28, IL-21) and effector molecules (IFNγ, Prf 1, Gzm B, FasL). Although IL-21 can support CAC by promoting Th17-driven inflammation [67, 68], IL-21 can also exert anti-tumor effects [69-72]. Indeed, IL-21 drives CD8+ CTL differentiation via the actions of the transcription factor T-bet [73] and stimulates multiple other lymphocyte subsets, including follicular Th cells, B cells and NK cells [74]. In our $p50^{-/-}$ mice, IL-21 induction does not correlate with increased expression of IL-17 or RORc, but with augmented levels of genes (e. g. IFNγ, Prf 1, Gzm B, FasL, Tbx-21/Tbet) expressed by NK and CTL cells. Hence we can speculate that ablation of p50 selectively enhanced anti-tumor effects of IL-21 without engaging pro-tumoral Th17 driven inflammation. Indeed, in absence of p50, both bone-marrow-derived dendritic cells and peritoneal macrophages showed a defective LPS-induced expression of IL-23p19 [75]. Noteworthy IL-23 drives Th17 cells expansion and functions [76] but also exert other pro-tumoral effects including inhibition of NK cells effector functions [77]. In response to gut microbial products, tumor associated myeloid cells are recognized as the major orchestrators of pro-tumoral IL-23 and IL-17 responses [28]. Accordingly IL-23p19 expression increased during progression from colitis to tumor, while it is strongly inhibited in $p50^{-/-}$ CRC. Consistently, in tumors from $Apc^{Min}p50^{-/-}$ mice, IL-23p19 transcript levels are also significant reduced further suggesting that, lack of p50 contributes to tumor resistance by inhibiting IL-23-driven inflammatory circuits.

In contrast lack of p50 enhances cytotoxic immune responses that in turn restrain tumor development by eliminating neoplastic cells. Indeed, $p50^{-/-}$ tumor cells are more apoptotic than the wt counterpart. Although ablation of p50 can impair colonic cancer cell survival also in a cell autonomous manner [78] adaptive immune-surveillance seems to be crucial for $p50^{-/-}$ mice anti-tumor activities, because depletion of CD4/CD8 abolished colitis-associated CRC resistance.

Overall our results in mice and human indicate that p50-driven M2 inflammation promotes both CRC development and progression. Hence p50 could be exploit both as novel prognostic indicator and therapeutic target for CRC patients. Although different human [79, 80] and mouse [81] studies suggests a link between decreased levels of p50 and an higher risk to develop colitis, at steady state, both $p50^{-/-}$ mice and $Apc^{Min}p50^{-/-}$ mice did not shown any signs of intestinal inflammation. In contrast, DSS treatment exacerbated inflammatory response in p50-/- mice indicating that for the small percentage (1%-4%) of human CRC cases that are associated to colitis [82] the anti-tumor efficacy of p50 targeting approaches could be limited by serious intestinal adverse drug reaction and alternative strategies to enhance type 1 inflammation should be adopted. In this regards our preclinical studies identified both IL-12 and CXCL10 as a potential immunotherapeutic drugs capable to limit CRC development without worsening colitis.

Materials and Methods

Mice

p50 NF-κB-deficient mice were generated by Prof. Michael Karin (46), whereas Apc^{Min} mice were from Jackson Laboratories (Bar Harbor, Maine, USA). The Apc^{Min} mice will be crossed with $p50^{-/-}$ mice in order to generate $Apc^{Min}p50^{-/-}$ mice. All mice were on the same C57BL6 background. In all experiments mice bred in the same SPF animal facility were used.

The study was designed in compliance with principles set out in the following laws, regulations and policies governing the care and use of laboratory animals: 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). The study was approved by the scientific board of Humanitas Clinical and Research Center. Humanitas Clinical and Research Center Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments. Animals were maintained in a specific-pathogen-free environment and tested negative for pathogens in routine screening. Mice have been monitored daily and euthanized when displaying excessive discomfort. To assess overall survival, Apc^{Min} and $Apc^{Min}p50^{-/-}$ mice were continuously monitored for a period up 40 weeks.

Azoxymethane (AOM)/ Dextran sodium sulfate (DSS)-induced colorectal cancer

6-8 weeks old wt and $p50^{-/-}$ mice were injected intraperitoneally (i.p.) with a single dose (10 mg/kg) of the mutagenic agent azoxymethane (Sigma) and maintained on regular diet and water for 5 days. After 5 days, mice received

water with 1,5-2% dextran sodium sulfate (DSS) (MP Biomedicals molecular mass, 40 kDa) for 5 days. After this, mice were maintained on regular water for 14 days and subjected to two more DSS treatment cycles.

When specified, starting from day 15 (e.g. recovery phase of the first DSS cycle), wt mice underwent i.p. administration of IL-12 (100ng) or intra-rectal injection of CXCL10 (500ng), once a week. As control mice received vehicle only.

When specified, starting from the day before the first DSS treatment, wt and $p50^{-/-}$ mice received an i.p injection of 0,3mg anti-mouse CD4 (clone GK1.5; BioXcell) and 0,3mg anti-mouse CD8 (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.

The clinical course of colitis was evaluated by monitoring mice body weight during the course of the experiment and by measuring colon length at necroscopy. At the time of harvest mice were euthanized, colons were resected, flushed with PBS, opened longitudinally and macroscopically evaluated for tumors number.

Histologic analysis

At the end of AOM/DSS experiments, mice were euthanized, colons were resected, flushed with PBS, opened longitudinally and rolled up. At the indicated age, Apc^{Min} and $Apc^{Min}p50^{-/-}$ mice were euthanized, both small gut and colons were harvested, flushed with PBS and prepared according to swiss and roll technique. Gut samples were fixed in 10% neutral buffered formalin for 24h and paraffin embedded, next 4 μm H&E-stained serial tissue sections were used for pathologic evaluation in a blinded fashion by a pathologist. Intestinal lesions were classified as gastrointestinal intraepithelial neoplasia (GIN), low-grade (LGA) and high-grade adenoma (HGA) and adenocarcinoma (C). Histological evaluation of grade of colitis was performed according to the score of Cooper et

al. (1993), and Suzuki et al. (2005), only slightly modified to adapt it to the findings of present study. The scoring of colitis was made at 40x magnification on the entire colon swiss roll with or without proliferative lesions and expressed as mean score/mouse. Additionally, the number of ulcers, and total number and size of the neoplastic lesions was recorded.

Analysis of immune cell infiltrate by immunohistochemistry or immunofluorescence

10 µm colonic slides were deparaffinized and rehydrated. Antigen unmasking was carried out by incubation in a decloaker chamber at 125°C for 3 minutes and 90°C for 10 minutes in Diva Decloaker retrieval solution (#902-2004C-012611 Biocare). Immunohistochemistry was done with the following antibodies: rat anti-mouse F4/80 (clone CI:A3-1, AbD Serotec), rat anti-mouse Ly6G (clone 1A8 BD Bioscences), rat anti-mouse Ly6C (clone ER-MP20, ThermoFisher), Rabbit anti-human CD3 (#A0452, DAKO). Secondary antibody used was Rat or Mouse HRP-Polymer Kit (Biocare medical). Sections were stained with the chromogen 3,3'- diaminobenzidine (DAB) and nuclei were counterstained with hematoxilin. Next slides were mounted with Eukitt and analyzed.

The total antigen^{+} area (μm^2) and fraction area (total antigen+ area/total area of field at 200x) were evaluated using the ImageJ analysis program (http://rsb.info.nih.gov/ij/) in 200x microscopic fields selected within the neoplastic lesions ("tumor") and the adjacent non-neoplastic mucosa ("non-tumor").

8 µm of cryostat colonic sections were fixed for 3 min in cold Aceton:Cloroform 3:1. Immunostaining were carried out with Rabbit anti-human CD3 (#A0452, DAKO) and goat anti-mouse NKp46 (#AF2225 R&D). Donkey anti Rabbit AlexaFluor 647 conjugated and Donkey anti Goat

AlexaFluor 488 conjugated were used as secondary antibodies. 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). Slides were analyzed with Olympus Fluoview FV1000 laser scanning confocal microscope with 60X (N.A.0.4) and single cell count was performed (n≥5 field for every sample; n≥3 for every condition).

Analysis of p50 nuclear accumulation in murine and human TAM:

10 µm murine and 3 µm of human colonic sections were deparaffinized and rehydrated. Human slides were priory exposed to UV radiation over night. Antigen unmasking was carried out by incubation in a decloaker chamber at 125°C for 3 minutes and 90°C for 10 minutes in Diva Decloaker retrieval solution (#902-2004C-012611 Biocare). Unspecific binding sites were blocked with BSA 2% + Triton X-100 0,1% in PBS+/+ Tween20 0,05% for 1h. For murine samples, mouse monoclonal anti-p65 NF-κB antibody (#6956 Cell Signaling), rabbit monoclonal anti-p50 NF-κB antibody (#AB32360 Abcam) and rat monoclonal anti-mouse F4/80 (clone CI:A3-1, AbD Serotec), was used. Goat anti mouse AlexaFluor 555 conjugated, Goat anti Rabbit AlexaFluor 647 conjugated and Goat anti rat AlexaFluor488 conjugated were used as secondary antibodies. For human samples, primary Monoclonal Mouse Anti-Human CD68 (clone M0814, Dako), rabbit monoclonal anti human p50 NF-κB (clone E381 Abcam) was used. Goat anti mouse AlexaFluor 488 conjugated and Goat anti Rabbit AlexaFluor 647 conjugated were used as secondary antibodies. 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). Slides were analyzed with Olympus Fluorview FV1000 confocal microscope with 60X (N.A.0.4) and single cell count was performed (n≥8 field for every sample; n≥3 for every condition).

Immunohistologic analysis of colonic cancer cells apoptosis

To assess the number of apoptotic cells, 4 μm paraffin-embedded sections of colon swiss roll were immunostained with a primary rabbit polyclonal antibody against cleaved-caspase 3 antigen (Cell Signaling, #Asp175). The number of apoptotic cells were counted using the ImageJ analysis program (http://rsb.info.nih.gov/ij/) in 200x microscopic fields selected within the neoplastic lesions ("tumor") and the adjacent non-neoplastic mucosa ("non-tumor").

Real-Time PCR Analysis

At the end of AOM/DSS treatment colons were washed with ice-cold saline then macroscopic tumors and the adjacent healthy tissue were harvested and maintained in RNA stabilization solution (RNAlater, Ambion). Colons from untreated mice were used as control. Similarly, macroscopic colonic tumors and the adjacent healthy tissue were harvested from 20-24 weeks old Apc^{Min} and $Apc^{Min}p50^{-/-}$ mice. Total RNA was extracted from tissues through the TissueLyser II (Qiagen) and RNeasy Lipid Tissue Mini Kit (Qiagen). RNA was reverse transcribed by the cDNA Archive kit (Applied Biosystem), amplified usingGOTAQ qPCR Master Mix (Promega), and detected by the CFX96 Real-Time System (Biorad). Expression data were normalized to Actin or 18S mRNA expression.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance between groups was assessed by unpaired one- or two-tailed Student's t test or Mann Whytney (Prism software) as specifies. $P \leq 0.05$ was considered significant. Experiments were repeated at least two times.

Supplementary materials and methods

Dextran sodium sulfate (DSS)-induced colitis

To induce acute colitis, 6-8 weeks old wt and p50-/- mice were treated for 5 days with 2-3% of dextran sodium sulfate (DSS; MP Biomedicals molecular mass, 40 kDa) in drinking water followed by 2 weeks of regular water. The clinical course of colitis was evaluated by monitoring mice body weight during the course of the experiment and by measuring colon length at necroscopy. For survival studies, mice were treated for 7 days with 3% of DSS in drinking water followed by 5 days of regular water to allow recovery. Mice were daily monitored for their survival until day 12, when mice start the resolution phase.

Immunohistochemistry

To assess the extent of the proliferative activity, 4 μm paraffin-embedded sections of colon swiss roll were immunostained with a primary rabbit monoclonal antibody against Ki-67 antigen (LabVision; #RM-9106-S). The number of Ki67-positive and Ki67- negative nuclei were counted using the ImageJ analysis program (http://rsb.info.nih.gov/ij/) in 400x microscopic fields randomly selected within the neoplastic lesions ("tumor") and in 200x microscopic fields randomly selected within the adjacent non-neoplastic mucosa ("non-tumor"), where only entirely visible crypts were considered.

To evaluate STAT3 activation, immunohistochemistry with primary rabbit polyclonal antibody against murine pSTAT3 Tyr705 (#9145S Cell Signaling) was performed. Analysis was performed with randomized double blind immunoscore from (-) to (+++) according to less to more observed positivity ($n \geq 3$).

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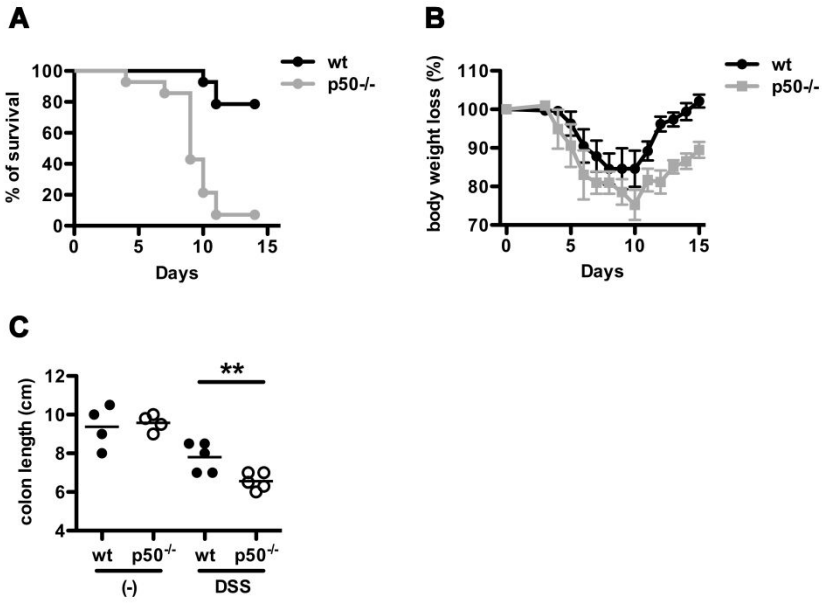
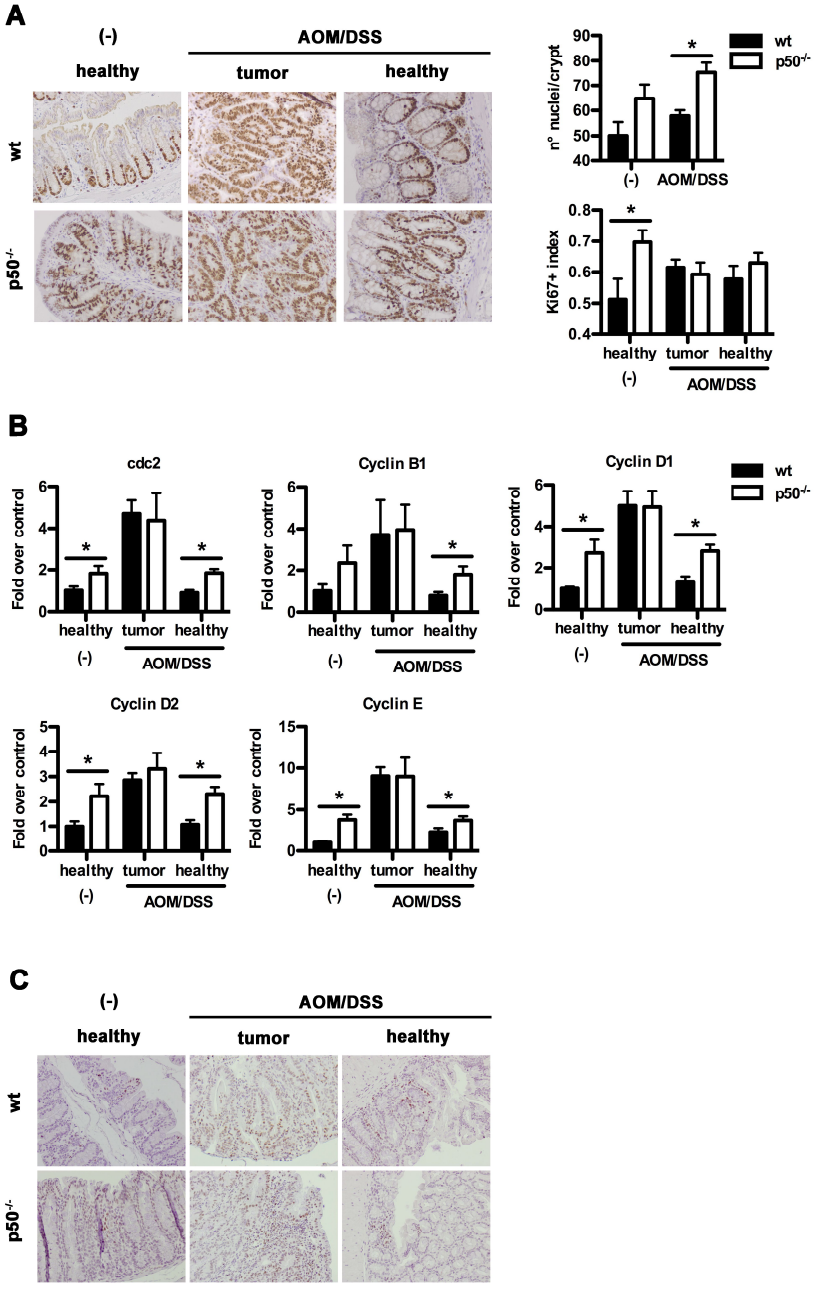
Supplemental data

Supplemental figure 1. $p50^{-/-}$ display exacerbated DSS-induced colitis. Mice survival upon 3% of DSS administration in drinking water for 7 days. One of three different experiments is shown. (P<0.0001, N=14) (A). Mice received 1.8% of DSS in drinking water for 5 days and regular water for the following 14 days. Weight loss was monitored every 2-3 days. (B). At day 9, DSS-treated and sham (saline) mice were euthanized; colons were resected and measured (C). Data shown are mean±SEM of different mice (t test, P<0.01, P<0.0001, N= 5)



Supplemental figure 2. Wt and $p50^{-/-}$ mice showed similar levels of proliferative colonic cancer cells. Colon from AOM/DSS treated and untreated mice were formalin fixed and paraffine embedded. Colon sections were evaluated for proliferative index through ki-67 immunohistochemistry. The number of Ki67-positive and Ki67-negative nuclei were counted in 400x microscopic fields randomly selected within the neoplastic lesions ("tumor") and in 200x microscopic fields randomly selected within the adjacent non-neoplastic mucosa ("healthy"), where only entirely visible crypts were considered. Data shown are mean±SEM of different fields (*$P<0.05$, Mann Whitney test, one-tailed, $N\geq8$) (A). Transcripts levels of proliferative genes were evaluated in total RNA isolated from colon and tumor of untreated and AOM/DSS-treated mice. Normalized qPCR results are shown as fold induction over healthy untreated wt mice. Data shown are mean±SEM of different mice (*$P<0.05$, Mann Whitney test, one-tailed, $N\geq4$) (B). Colon sections were immunostained with anti-P-Stat-3 antibodies. Data shown are mean±SEM of different fields (magnification 20x) (C).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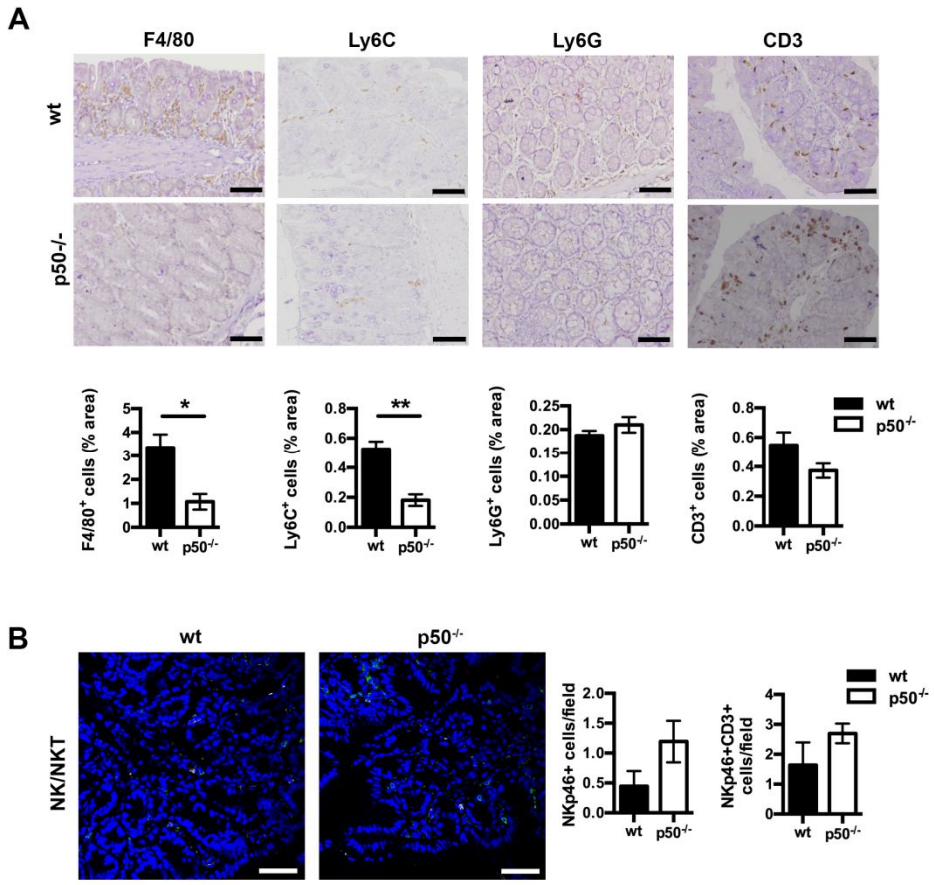
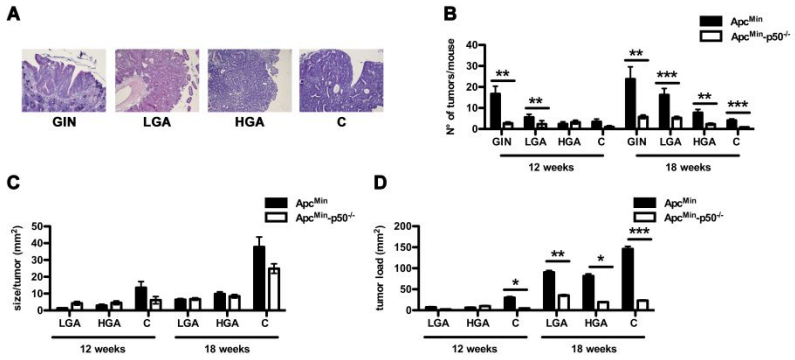
Supplemental figure 3. At steady state, lack of p50 selectively impairs lamina propria monocytes/macrophages accumulation. Immunohistochemical analysis of paraffine embedded colon sections from untreated wt and $p50^{-/-}$ mice were evaluated for monocytes ($Ly6C^{+}$), macrophages (F4/80), T lymphocytes ($CD3^{+}$). (A) Confocal microscopy analysis of NK ($CD3^{-}NKp46^{+}$) and NKT ($CD3^{+}Nkp46^{-}$) cells in slides from frozen wt and $p50^{-/-}$ control colons (B). Data shown are mean±SEM from 3 different mice. Representative images are shown.



Supplemental figure 4. Lack of p50 impairs tumor development and advancement. Tumors were classified as: gastrointestinal neoplasia (GIN), low grade adenoma (LGA), high grade adenoma (HGA) and carcinoma (C) (A), next tumor incidence (B), size (C) and burden (D) were evaluated in relation to the tumor stage. The results shown are the mean±SEM of different tumor lesions (*P<0,05 N≥13)



CHAPTER FOUR

Tumors promote p50-driven transcriptional reprogramming to divert Interferon-γ-mediated myeloid cell functions towards immunosuppression

Unpublished results

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Running title: p50 NF-κB promotes M-MDSC suppressive function

Abstract

Myeloid-derived suppressor cells (MDSC) comprise monocytic (Ly6C$^+$) and polymorphonuclear (Ly6G$^+$) populations, sharing an immature state and the ability to suppress adaptive immunity. Surprisingly, monocytic Ly6C$^+$MDSC express the immunosuppressive molecule nitric oxide (NO) in response to Interferon-γ (IFNγ), a "macrophage-activating factor" also able to induce inflammatory and antitumor responses. By investigating the mechanisms controlling these opposing activities elicited by IFNγ on myeloid cells, we demonstrate that mouse and human tumors induce selective nuclear accumulation of p50 NF-κB in the monocytic MDSC subset, diverting their response towards NO-mediated immunosuppression. Genomic and epigenetic studies demonstrated that p50 NF-κB promotes chromatin changes necessary for IFNγ-induced binding of STAT1 onto regulatory regions of several IFNγ-dependent genes, including inducible nitric oxide synthase (iNOS). In agreement, ablation of p50 in Ly6C$^+$MDSC abolished their NO-mediated suppressive activity and restored IFNγ-mediated antitumor activity in vivo. Thus, tumors alter the epigenetic gene regulation of myeloid cells through increased p50 nuclear levels, promoting differentiation of suppressive Ly6C$^+$MDSC and limiting the anticancer properties of IFNγ.

Introduction

Microenvironmental signals are sensed by myeloid cells through specific cytokine and/or innate immune receptors, whose activation leads to the expression of different polarized programs of inflammation [1, 2]. This functional plasticity is exemplified in the M1 *vs* M2 extremes of macrophage polarization[3] and is considered to have a major impact on the orchestration of cancer-related inflammation[4-6]. Myeloid cell plasticity goes beyond the nature of the encountered signal, as a long exposure to inflammatory signals (e.g. LPS-tolerance) promotes a time-dependent reprogramming from M1 to M2 polarized programs, with relevance in the onset and resolution of inflammation [7, 8]. Interestingly, LPS-induced tolerance in macrophages is characterized by increased nuclear levels of the p50 NF-κB subunit [8] and results in altered macrophage responses to cytokines (eg. IFNγ, IL-4) [8]. This observation implies that exposure to chronic inflammatory conditions, such as those associated with infection and cancer, may functionally reprogram myeloid cells, thereby affecting their responses to different agonists, including cytokines.

Dynamic changes in myeloid cell functions have been reported to parallel tumor progression [4, 9] and different populations of myeloid cells have been found in various tumors [10]. Cancer fuels this heterogeneity by promoting sustained myelopoiesis and accumulation of myelomonocytic cells, which support the angiogenesis and stroma remodeling needed for their growth [9, 10]. Eventually, these events may affect the efficacy of cytokine-mediated immunotherapy, as heterogeneous polarized populations may respond with different functional outcomes. Indeed, in both preclinical and clinical cancer settings, divergent outcomes have been reported in response to cytokines [11-14]. IFNγ, originally termed “macrophage activating factor” [15], was paradoxically shown to be equally necessary for melanoma development and rejection [11]. IFNγ has pleiotropic effects on the tumor microenvironment, including anti-angiogenic activities, suppression of pro-tumorigenic properties

and enhancement of tumoricidal activity of macrophages, and processing and presentation of tumor antigens to T lymphocytes [14, 16]. However, IFNγ also promotes immunosuppressive functions in myeloid cells associated with cancer [10], mainly through the induced expression of the immunosuppressive enzymes indoleamine 2,3 dioxygenase (*Ido*) and inducible nitric oxide synthase (iNOS, encoded by the *Nos2* gene), respectively involved in the catabolism of L-tryptophan [17] and L-arginine [9]. IFNγ also induces the expression of the ligand programmed-death receptor-ligand 1 (PD-L1, B7-H1) [18]. Strikingly, such puzzling scenarios have been confirmed in the clinic, where mixed responses to IFNγ treatment were reported in different malignancies[13, 14, 19]. Hence, it is necessary to clarify whether reprogramming of myeloid cell functions occurring in cancer progression may contribute to failure of cytokine-mediated immunotherapy.

Tumors alter myeloid cells and convert them into potent immunosuppressive cells, MDSC in particular, which are considered new anticancer targets[10]. A major NO-dependent pathway of immunosuppression is promoted by IFNγ in the M-MDSC subset[10, 20], which results in enhanced production of reactive nitrogen species that suppress $CD8^+$ T cells through promotion of signaling defects[9]. M-MDSC share common myeloid precursors and a similar M2-like gene profile with tumor-associated macrophages (TAM)[9, 21]. Since we previously reported that accumulation of nuclear p50 NF-κB plays an essential role in the orientation of M2 polarized functions[8, 22], we investigated its role in M-MDSC differentiation.

Results

p50 NF-κB controls M-MDSC suppressive function in response to IFNγ and regulates differentiation of monocytic and granulocytic precursors.

We have previously shown that p50 NF-κB nuclear accumulation, occurring in tumor-associated macrophages (TAM) and LPS-tolerant macrophages, impairs M1 polarization, as well as antitumor properties[8, 22]. Since tumor growth is supported by the expansion of suppressive leukocyte populations, among which MDSC play a major role[20], we first investigated whether p50 NF-κB could promote their suppressive phenotype in a mouse model of fibrosarcoma (MN/MCA1). According to our previous findings[22], C57BL/6 $p50^{-/-}$ mice injected with MN/MCA1 cells displayed both reduced tumor growth and metastasis number, as compared to Wt mice (Figure 1A). As MDSC accumulate preferentially in secondary lymphoid organs[9, 10], we evaluated their number in the spleen of tumor-bearing mice, at different times of tumor growth (i.e. 11 and 21 days, corresponding to tumor volumes of 2 and 3 cm^3). In apparent contrast with the reduced tumor growth, the number of $CD11b^+Gr1^+$ splenic MDSC (21 days) was significantly higher in $p50^{-/-}$ tumor-bearing mice, (Figure 1B). IFN-γ is a major inducer of NO-mediated suppressive activity by MDSC [9, 23], therefore we measured the level of *Nos2* mRNA expression in IFNγ-treated Wt and $p50^{-/-}$ MDSC, isolated at different times of tumor growth (11 and 21 days). IFNγ treatment induced a sharp increase of *Nos2* mRNA levels in splenic Wt, but not in $p50^{-/-}$ $CD11b^+Gr1^+$ MDSC, isolated 21 days after tumor cells injection (Figure 1C). Consistently, IFNγ did not induce NO production in $p50^{-/-}$ MDSC (Figure 1D). The arginase I (Arg1) enzyme is involved in the metabolism of the amino acid L-Arg[10] and promotes the suppressive function of G-MDSC[9]. Analysis of the mRNA expression levels of Arg1 did not reveal consistent differences between Wt and $p50^{-/-}$ MDSC (data not shown) and therefore it was not further investigated. Next, we determined

the nuclear levels of the p50 and p65/RelA NF-κB subunits in magnetically sorted splenic $CD11b^{+}Gr1^{+}$ MDSC. Similarly to tumor free mice (day 0), p50 and p65 were poorly or not detectable in splenic $CD11b^{+}Gr1^{+}$ MDSC isolated at early stages of tumor development (11 days) (Figure 1E). However, a striking and selective increase in nuclear p50 was observed in $CD11b^{+}Gr1^{+}$ MDSC isolated at later stages (21 days), which correlated with the IFNγ-mediated induction of *Nos2* mRNA (Figure 1C).

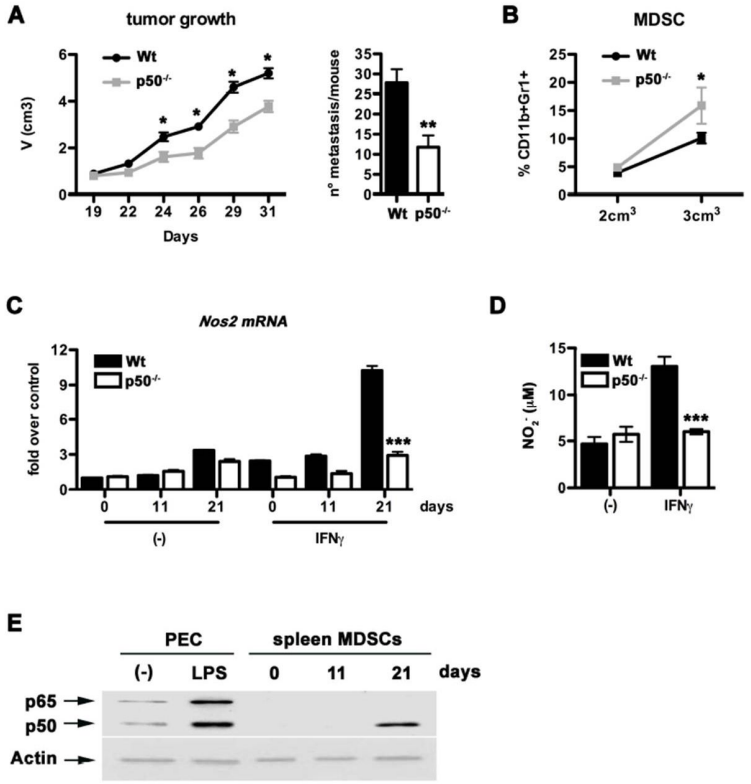

Figure 1. Role of p50 NF-κB in the IFNγ-dependent production of NO by MDSC. (A) Left, inhibition of both tumor growth (MN/MCA1) and metastasis formation in p50 NF-κB–deficient mice (n=7 mice/group; *P<.05, **P<.01). (B) Increased number of splenic $CD11^{+}Gr1^{+}$ MDSC in $p50^{-/-}$ tumor-bearing mice (n=5 mice/group; *P<.05). (C) Lack of p50 in MDSC impairs *Nos2* mRNA expression in response to IFNγ. (D) Lack of p50 in MDSC impairs NO production in response to IFNγ(n=5; ***P<.001) (E) Western blot showing the time-dependent nuclear accumulation of p50 in splenic $CD11^{+}Gr1^{+}$ MDSC isolated from tumor-bearing mice.

The granulocytic G-MDSC($Ly6G^{+}Ly6C^{low}$) and monocytic M-MDSC ($Ly6G^{-}Ly6C^{high}$) subsets can separately or synergistically act to alter T cell functions[9]. Analysis of both populations in Wt and $p50^{-/-}$ mice showed a marked expansion of M-MDSC in the spleen of tumor bearing $p50^{-/-}$ mice, but not of G-MDSC (Figure 2A). Consistent with these results, p50 nuclear accumulation, as evaluated by confocal microscopy, selectively occurred in M-MDSC and only at advanced stages of tumor development (day 21; Figure 2B). Conversely, the p65 NF-κB subunit was either poorly or not detectable (Figure 2B). In agreement with the selective accumulation of nuclear p50 in M-MDSC, only this subset produced significant levels of NO in response to IFNγ, and this was robustly and significantly reduced in the absence of p50 NF-κB (Figure 2C). Figure S1 shows the degree of purity of the monocytic and granulocytic MDSC subsets, following cell sorting from the spleen of tumor-bearing mice. To establish the actual role of p50 NF-κB in the suppressive activity of M-MDSC, cells were activated with IFNγ, loaded with ovalbumin and then co-cultured for three days with total splenocytes purified from either spleen or lymph nodes of OT-1 transgenic mice, expressing the T cell receptor specific for the ovalbumin antigen. In keeping with the data above, $p50^{-/-}$ M-MDSC displayed reduced suppressive activity (Figure 2D), estimated as proliferation of co-cultured OT1 splenocytes, which correlated with decreased levels of NO in the co-culture supernatants (Figure 2E). Inhibition of T cell proliferation was NO-dependent, as addition of the nitric oxide synthase inhibitor L-NMMA in the co-culture abolished both T cell suppression (Figure 2D) and NO production (Figure 2E). These data suggest that the NO-dependent suppressive capacity of M-MDSC in response to IFNγ is gradually acquired during tumor development and that it occurs in a p50-dependent manner.

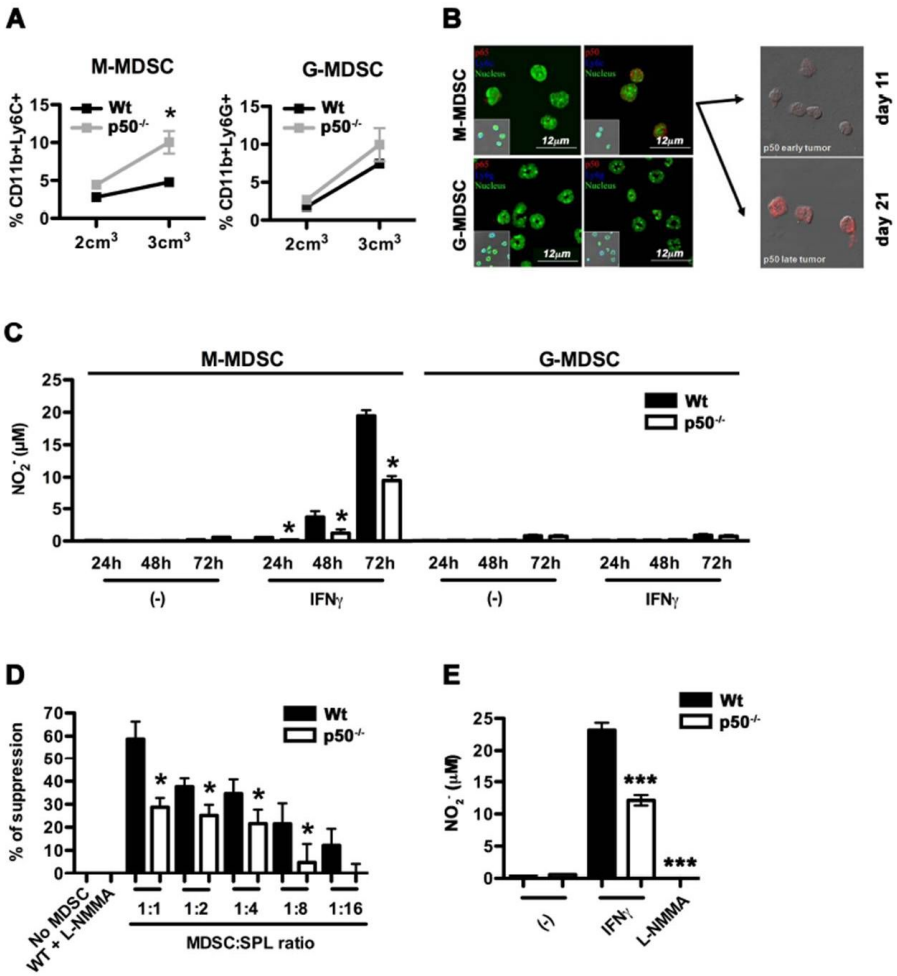

Figure 2. Selective up-regulation of nuclear p50 NF-κB in M-MDSC correlates with their IFNγ-dependent NO production and suppressive activity. (A) Increased number of M-MDSC in the spleen of $p50^{-/-}$ tumor-bearing mice(n=5 mice/group; *P<.05). (B) Confocal microscopy showing selective up-regulation of nuclear p50 NF-κB in M-MDSC during tumor growth (day 21). (C) Reduced IFNγ-mediated NO production in $p50^{-/-}$ M-MDSC. (D) Decreased antigen-specific suppressive activity of $p50^{-/-}$ M-MDSC in response to IFN-γ, at different MDSC:OT1 splenocytes ratio. (E) 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. (n=3; *P<.05, ***P<.001).

The predominance of the M-MDSC subset in the spleen of tumor-bearing mice might result from their accelerated proliferation rate or preferential skewing of their 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. HSC are immunophenotipically 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 allow to quantify their derived progenitors *in vitro*. We observed striking differences in BM myelopoietic potential between $p50^{-/-}$ and control Wt mice. As shown (Figure 3A and B), hematopoietic cells from the BM of $p50^{-/-}$ mice were enriched in LSK progenitors as compared with Wt counterpart. The clonogenic potential of HSC from $p50^{-/-}$ was significantly higher than from Wt BM (Figure 3C), specifically: both GM-CFU and M-CFU progenitors were significantly increased in the BM of $p50^{-/-}$ than Wt mice, whereas G-CFU did not varied significantly (Figure 3C). These results indicate in the BM of $p50^{-/-}$ mice 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, associated with increased number of immature myeloid precursors (Figure 3D). Actually, 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 (Figure 3D). Monocytic differentiation was 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 (Figure S2). Similarly, no impairment was observed in the erythropoiesis and megakaryocitopoiesis of $p50^{-/-}$ mice (not shown). Consistent with the contraction of the granulocytic compartment and expansion of the myeloid blasts observed by 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 (Figure 3E). In

addition, morphologic analysis of peripheral blood smears of 12 weeks-old $p50^{-/-}$ and Wt mice (6 mice per group) showed that circulating granulocytes from $p50^{-/-}$ mice were enriched in immature and blast-like forms compared with circulating granulocytes from Wt controls (Figure 3F). Overall, these results demonstrate that p50 deficiency is associated with defective granulocytic differentiation in favor of the myeloid lineage, that in tumor bearing mice might foster the accumulation of splenic M-MDSC.

Tumor-derived factors prime M-MDSC for higher IFNγ-mediated NO production.

To establish whether the expression of p50 NF-κB in the hematopoietic compartment was uniquely responsible for the suppressive activity of M-MDSC, BM cells from either Wt or $p50^{-/-}$ mice were transplanted into sub-lethally irradiated C57BL/6 Wt and $p50^{-/-}$ mice. Chimeric mice were next implanted with the MN/MCA1 fibrosarcoma and then monitored for MDSC functions. As shown (Figure 4A), M-MDSC isolated from the spleen of mice transplanted with $p50^{-/-}$ bone marrow cells, and subsequently activated with IFNγ, were strongly impaired in their capacity to suppress T cell proliferation, as well as in NO production. In the attempt to identify tumor-derived signals controlling nuclear p50 levels, the levels of IL-10, TGFβ, GM-CSF, G-CSF and M-CSF were estimated by ELISA in the tumor supernatants (TSN) (Figure 4B). Next, BM-MDSC were cultured for 48 h in the presence of either tumor supernatant (TSN) or colony growth factors driving myeloid cell differentiation (GM-CSF, G-CSF). Confocal microscopy confirmed the induction of p50 by TSN, whereas neither GM- or G-CSF were able to induce this event (Figure 4C).

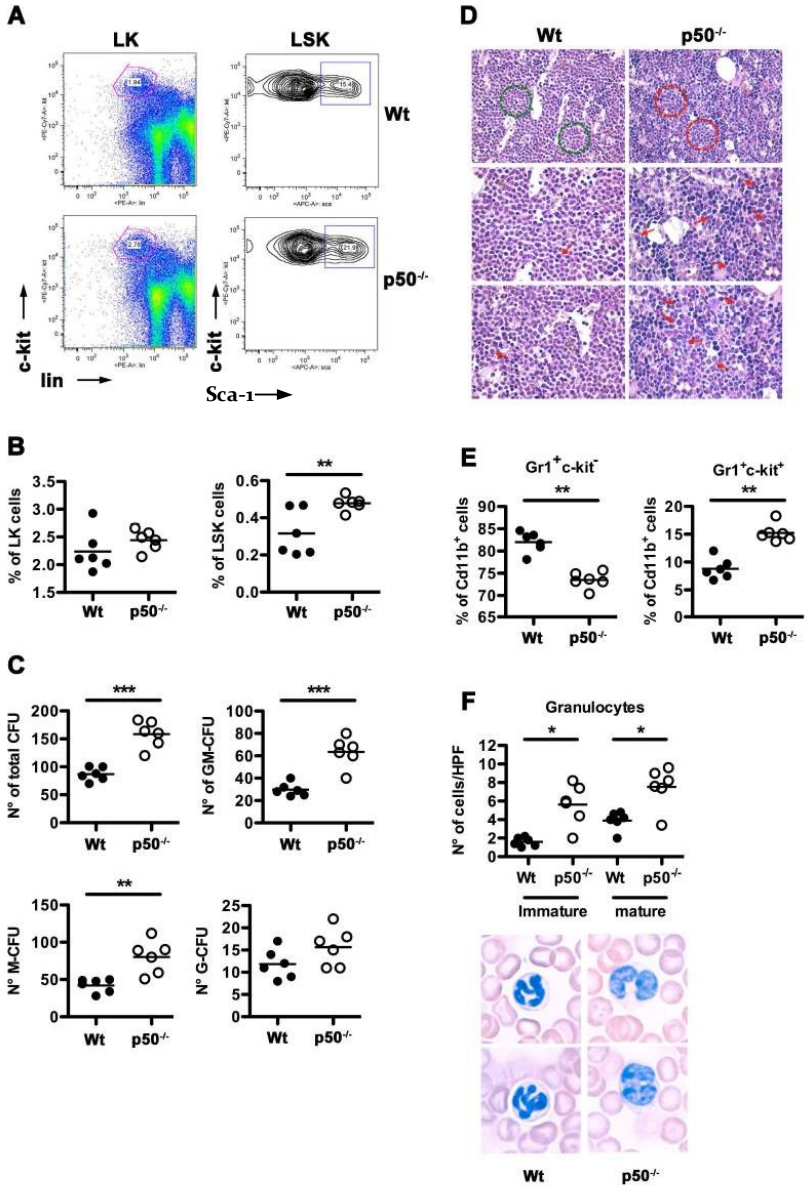

Figure 3. p50 deficiency in the BM stroma associates with enhanced myelopoiesis. BM cells from Wt and p50$^{-/-}$ mice were stained with mAb to c-Kit, Sca-1 and lineage- specific markers (CD3, CD11b, CD11c, Gr-1, B220, ter119). LSK progenitors were defined as Sca1^{+} cells within the gate of lin^{-}c-Kit^{+} 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 (n=6 mice/group; $^{**}P < 0.001$). (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. $^{**}P < .01$. $^{***}P < .001$. (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, x400; lower panels, x630. (E) 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. $^{**}P < 0.001$. (F) Morphologic analysis (left) and quantification (right) of Giemsa-stained PB smears from 12 weeks-old p50$^{-/-}$ and Wt mice (6 mice per group) showing that circulating granulocytes from p50$^{-/-}$ mice are enriched in immature and blast-like forms.

Next, we tested the capacity of TSN to prime MDSC for IFNγ-induced NO production (Figure 4D). Noteworthy, when M-MDSC were primed with TSN and subsequently treated with IFNγ, they showed higher levels of both *Nos2* mRNA and NO production. In addition, BM-MDSC were treated with IL-10, as this cytokine was significantly secreted in the TSN (Figure 4B) and previously demonstrated to promote nuclear accumulation of p50 in macrophages[22]. As results, IL-10 induced a significant induction of nuclear p50, with poor effect on p65 (Figure 4E). Moreover, similarly with TSN, IL-10 primed IFNγ-treated MDSC for enhanced expression of *Nos2* mRNA expression and NO production(Figure 4E). In spite of this, incubation of TSN with neutralizing anti-IL-10 antibodies only marginally reduced its priming activity (not shown), suggesting that additional tumor-derived signals may cooperate to this event.

To establish whether nuclear accumulation of p50 in M-MDSC was common to other tumor types, we investigated a murine model of spontaneous hepatocellular carcinoma (HCC), due to the absence of the Mdr2 hepatocyte membrane transporter (mdr2$^{-/-}$ mice)[24] (Figure S3). Only M-MDSC isolated from the spleen of 14 months-old, tumor bearing mdr2$^{-/-}$ mice, showed accumulation of nuclear p50 NF-κB, while no p50 accumulation was observed in the nucleus of G-MDSC cells (Figure 5A).

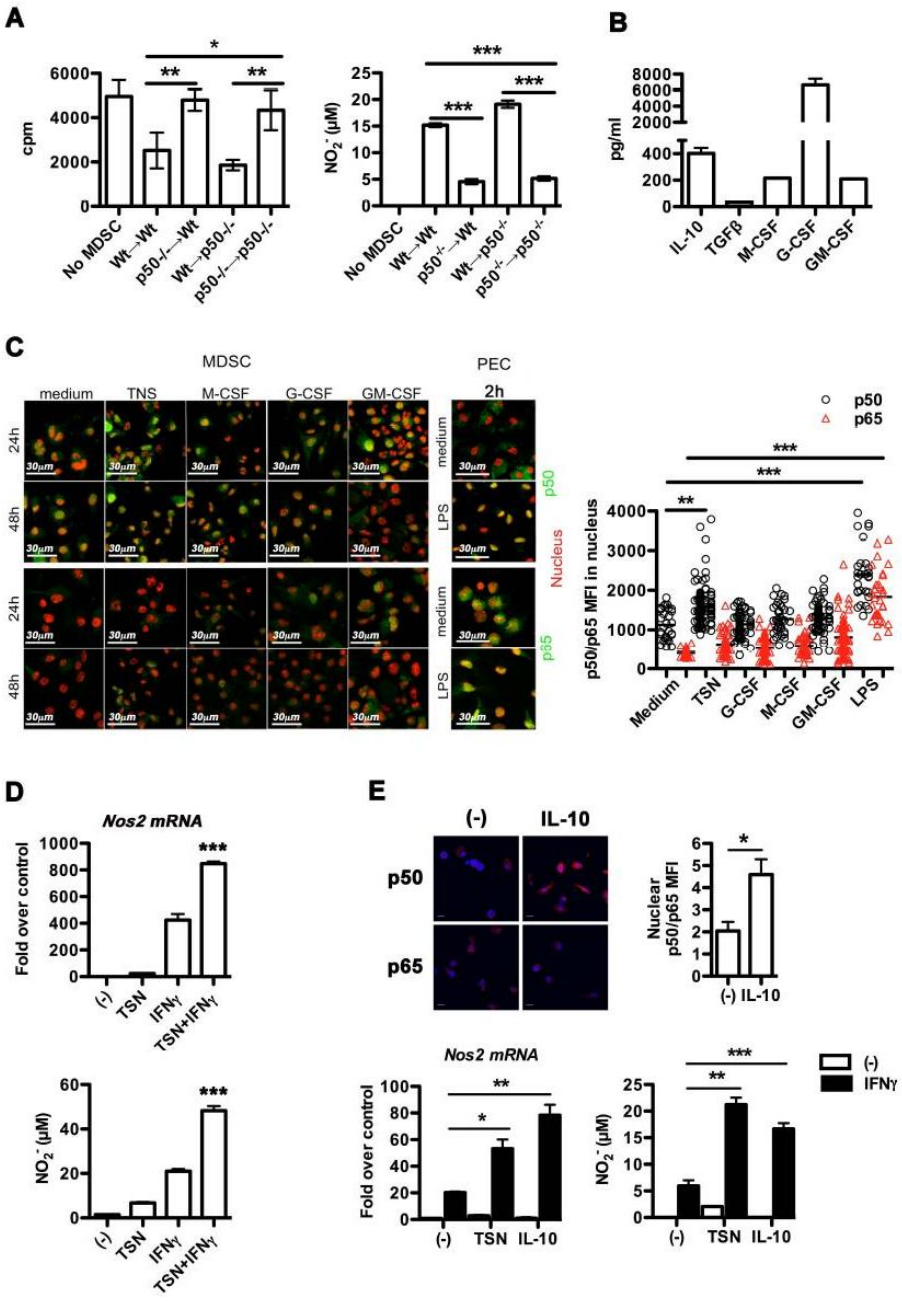

Figure 4. Priming of M-MDSC with tumor supernatant (TNS) enhances NO production in response to IFNγ. (A) Splenic M-MDSC from $p50^{-/-}$ BM-transplanted mice display reduced suppressive activity (left) and NO production (right) in response to IFNγ. Proliferation was assessed by ^{3}H-thymidine incorporation and expressed in cpm (n=3; *P<.05; **P<.01; ***P<.001). (B) Levels of IL-10, TGFβ, GM-CSF, G-CSF and M-CSF estimated by ELISA in TSN. (C, left) Confocal microscopy of p50 and p65/RelA NF-κB in BM-derived M-MDSC conditioned with either TSN, M-CSF, G-CSF or M-CSF plus G-CSF (M/G-CSF).

(C, right) Mean fluorescence intensity (M.F.I.) of nuclear p50 and p65 in BM-derived M-MDSC conditioned with either TSN, M-CSF, G-CSF, M-CSF plus G-CSF (M/G-CSF) or LPS, as indicated. PEC stimulated for 2h with LPS were used as positive controls. (D) TSN primes IFNγ-treated MDSC for enhanced expression of *Nos2* and NO production (n=3; ***P<.001). (E, top-left) Confocal microscopy of p50 and p65/RelA NF-κB in untreated (-) and IL-10-conditioned BM-derived M-MDSC. (E, top-right) Nuclear p50/p65 ratio in BM-derived M-MDSC conditioned with IL-10. (E, low) IL-10 primes IFNγ-treated MDSC for enhanced expression of *Nos2* and NO production (n=3; ***P<.001).

In addition, we determined the p50 nuclear levels in human peripheral blood $CD14^{+}HLA^{-}DR^{low/-}$ M-MDSC cells[10, 25], from colorectal cancer (CRC) patients. Compared to healthy donors, we observed an increased number of blood $CD14^{+}HLA^{-}DR^{low/-}$ cells in CRC patients (Figure 5B, left). Furthermore, confocal microscopy analysis, demonstrated higher levels of nuclear p50 NF-κB in $CD14^{+}HLA^{-}DR^{low/-}$ cells, purified from peripheral blood of cancer patients (Figure 5B, center and right), as compared to peripheral $CD14^{+}$ mononuclear cells from healthy donors.

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

Overall, these results indicate that accumulation of p50 NF-κB in monocytic MDSC drives their capacity to elicit suppressive activity, through an IFNγ-mediated NO-dependent mechanism. This observation also suggests that increased nuclear p50 NF-κB in M-MDSC could limit the antitumor activity of IFNγ *in vivo*. Hence, we tested the antitumor activity of IFNγ *in vivo*, both in Wt and p50-deficient tumor-bearing mice. As shown in figure 6A, while IFNγ treatment of Wt tumor-bearing mice did not result in tumor inhibition, $p50^{-/-}$ tumor-bearing mice treated with IFNγ displayed significant inhibition of both tumor growth and metastasis formation, paralleled by an increased number of both $CD4^{+}$ and $CD8^{+}$ T cells in both spleen and primary tumor tissues, as well as by increased IFNγ production by tumor infiltrating $CD4^{+}$ and $CD8^{+}$ T cells (Figure 6B).

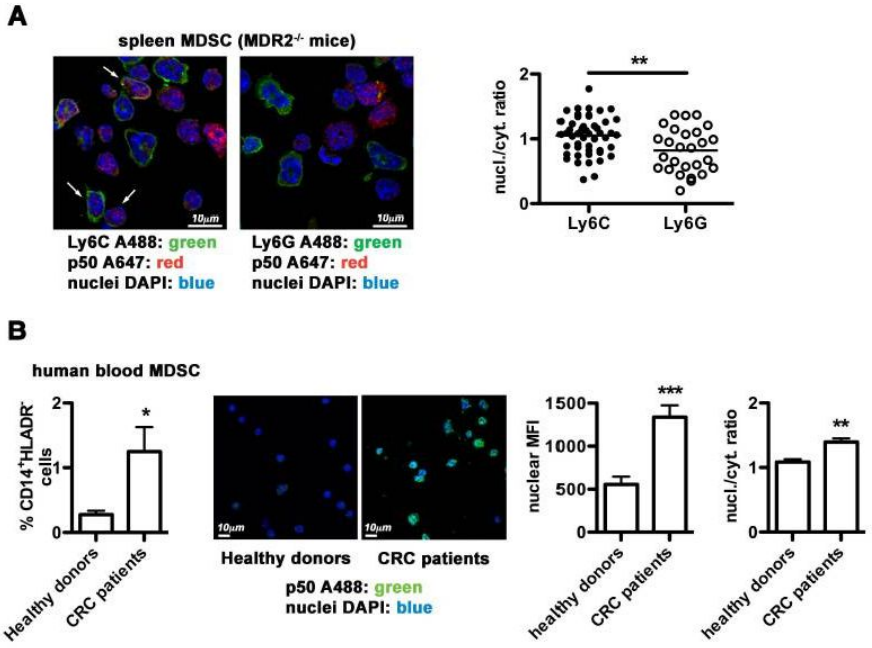

Figure 5. Increased nuclear levels of p50 in M-MDSC from mouse and human tumor bearers. (A, left) Confocal analysis of nuclear p50 in splenic M-MDSC isolated from hepatocellular carcinoma (HCC) tumor-bearing mice (mdr2$^{-/-}$ mice). (A, right) Dot plot representation of nucleus *vs* cytoplasm ratio of p50 in M-MSDC vs G-MDSC from mdr2$^{-/-}$ mice (**P<.01). (B, left) Increased number of blood CD14^{+}HLA-DR$^{low/-}$ cells from colorectal carcinoma (CRC) patients (n=4 *P<.05). (B, center) Representative confocal microscopy on nuclear p50 in peripheral blood CD14^{+}HLA-DR$^{low/-}$ cells from CRC patients, as compared to peripheral blood mononuclear CD14^{+} cells from healthy donors. (B, right). M.F.I. of nuclear p50 and nuclear vs cytoplasmic ratio in CD14^{+}HLA-DR$^{low/-}$ cells from CRC patients (n=20 **P<.01; ***P<0,001).

Finally, we investigated whether lack of IFNγ antitumor activity observed in Wt tumor-bearing mice was mediated by p50 NF-κB expressing M-MDSC. To address this issue, p50$^{-/-}$ tumor-bearing mice were adoptively transferred with Wt M-MDSC (1x10^{6}) and treated daily with IFNγ (10U). Transfer of Wt M-MDSC in p50$^{-/-}$ tumor-bearing mice inhibited IFNγ antitumor activity, resulting in restoration of both tumor growth and metastasis formation, as well as in decreased production of IFNγ by CD8^{+} T cells, both in the spleen and primary tumor (Figure 6C). These data demonstrate that tumor-mediated induction of

nuclear p50 in M-MDSC is a key event driving their suppressive functions and limiting the antitumor activity of IFNγ *in vivo*.

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

Along with MDSC, macrophages produce NO in response to IFNγ, which mediate either their immunosuppressive[4] or tumoricidal capacity[26]. Hence, we investigated whether p50 NF-κB could modulate NO-production in IFNγ-treated thioglycollate elicited macrophages (PEC). Similarly to M-MDSC, IFNγ-treated PEC expressed high levels of inducible *Nos2* mRNA and increased NO production (Figure S4A), both of which were strongly decreased in $p50^{-/-}$ cells. Furthermore, as compared to IFNγ-treated PEC, LPS-tolerant PEC (L/M), characterized by increased nuclear levels of p50 NF-κB[8] (Figure S4B), expressed higher levels of *Nos2* mRNA in response to IFNγ (L/IFNγ), but not to LPS (L/L) (Figure S4C), further suggesting the

role of nuclear p50 in controlling the magnitude of IFNγ-mediated NO production. In analogy, TSN-primed PEC produced higher level of NO in response to IFNγ, in a p50-dependent manner (Figure S4D). Based on these results, as well as on the high number of cells required for mechanistic studies, we addressed the epigenetic events by which p50 controls IFNγ-dependent responses in PEC. We initially tested if p50 modulates IFNγ-induced STAT1 activation. Wt and $p50^{-/-}$ PEC were activated with IFNγ for different times and STAT1 phosphorylation determined by western blot. As shown in figure 7A, lack of p50 did not prevent or reduce STAT1 phosphorylation, suggesting that downstream events occurring in $p50^{-/-}$ cells could be responsible for defective IFNγ-mediated *Nos2* expression. To directly assess this hypothesis in macrophages, we generated mRNA sequencing (mRNA-Seq) data sets of wild-type and $p50^{-/-}$ macrophages (PEC) treated with IFNγ for 4 hours.

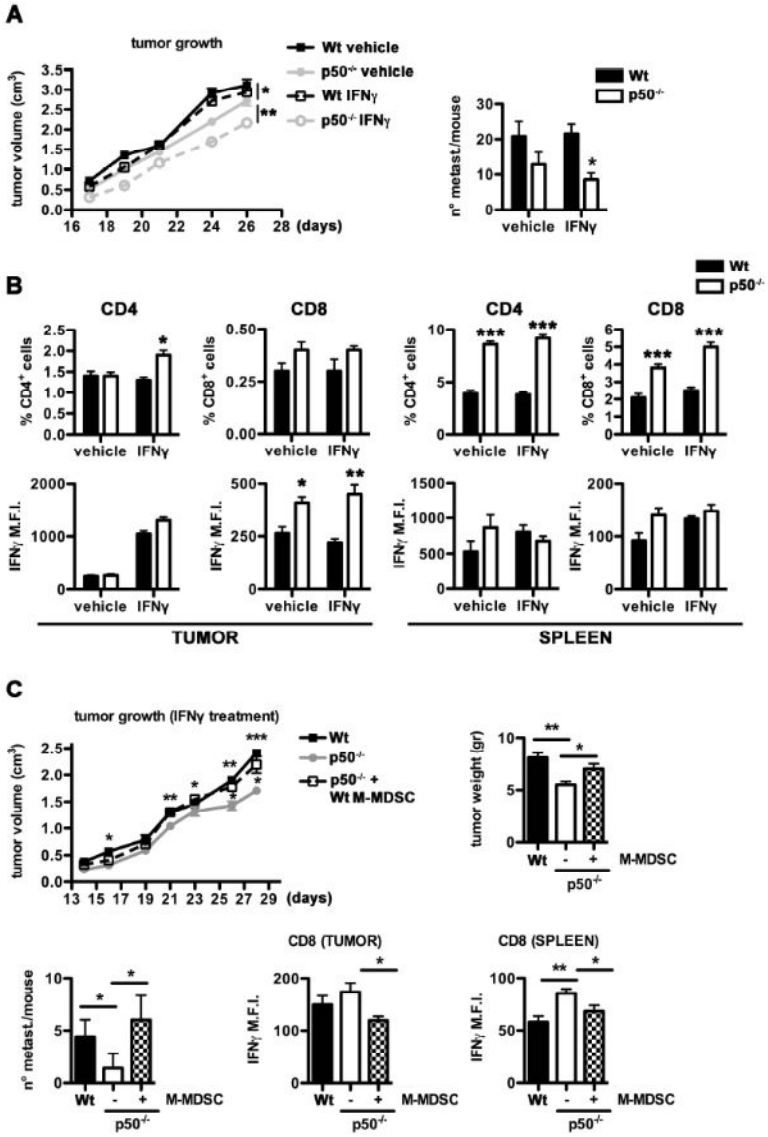

Figure 6. Ablation of p50 NF-κB in M-MDSC restores IFNγ-mediated antitumor activity *in vivo*. (A) Antitumor effects of IFNγ in Wt vs $p50^{-/-}$ mice (n=7 mice per group, *P<.05, **P<.01). (B) Increased number of $CD4^+$ and $CD8^+$ T cells and increased IFNγ production, in both spleen and tumor tissues of $p50^{-/-}$ mice (n=7, *P<.05, **P<.01, ***P<.001). (C) Adoptive transfer of Wt M-MDSC in $p50^{-/-}$ tumor-bearing mice abolishes the antitumor activity of IFNγ *in vivo*, promoting tumor growth and metastasis formation. The panel also shows the M.F.I. of IFNγ expression determined by FACS in both spleen and tumor-associated $CD8^+$ T cells. Wt, tumor growth in Wt mice; $p50^{-/-}$, tumor growth in $p50^{-/-}$ mice; $p50^{-/-}$ + Wt M-MDSC, tumor growth in $p50^{-/-}$ mice adoptively transferred with Wt M-MDSC (n=7 mice per group, *P<.05, **P<.01, ***P<.001).

Data obtained with two biological replicates were highly correlated (r^2>0.96) (Figure S4E), indicating high reproducibility between samples. Overall, $p50^{-/-}$ macrophages showed selective gene expression defects in response to IFNγ stimulation as compared to wild-type controls (Figure 7B and supplementary table 1). Using as cutoffs a log2(fold change) ≥ 1 and a FDR ≤ 0.01, of the 681 genes induced by IFNγ in wild-type macrophages, 85 (12.5%, cluster 2) were impaired in $p50^{-/-}$ cells, whereas 27 (3.9%, cluster 1) were hyperinduced. Genes whose maximal induction required p50 included known regulators of inflammatory responses and M1 macrophage marker genes such as *Nos2*, *Cxcl9*, *Ptgs2* and *Ciita.* On the other hand, p50 deficiency resulted in an increased induction of *Irf8* (encoding the monopoiesis-inducing transcription factor IRF8)[27] (supplementary table 1) and *Socs3* (which encodes for a negative regulator of the Jak-Stat pathways). Gene repression in response to IFNγ was also affected in $p50^{-/-}$ macrophages, with 47 genes (7.2% of all repressed genes, cluster 5) showing a defective down-regulation that was often associated with basally higher expression levels (Figure 7B). 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 explored the possibility that p50 may selectively influence Stat1 recruitment to a subset of p50-dependent genes. To this aim, we stimulated wild-type and $p50^{-/-}$ macrophages with IFNγ for 2 hours and then performed Chromatin Immunoprecipitation coupled to next-generation sequencing (ChIP-Seq) using a validated antibody directed against Stat1[28]. Almost all DNA binding events occurred only after Stat1 activation, with ≈ 30,000 Stat1 peaks detected in IFNγ-treated cells (supplementary table 2). Furthermore, Stat1 binding positively correlated with IFNγ-induced gene expression in a statistically significant manner (Figure 7C), highlighting the prominent role of Stat1 as a transcriptional activator.

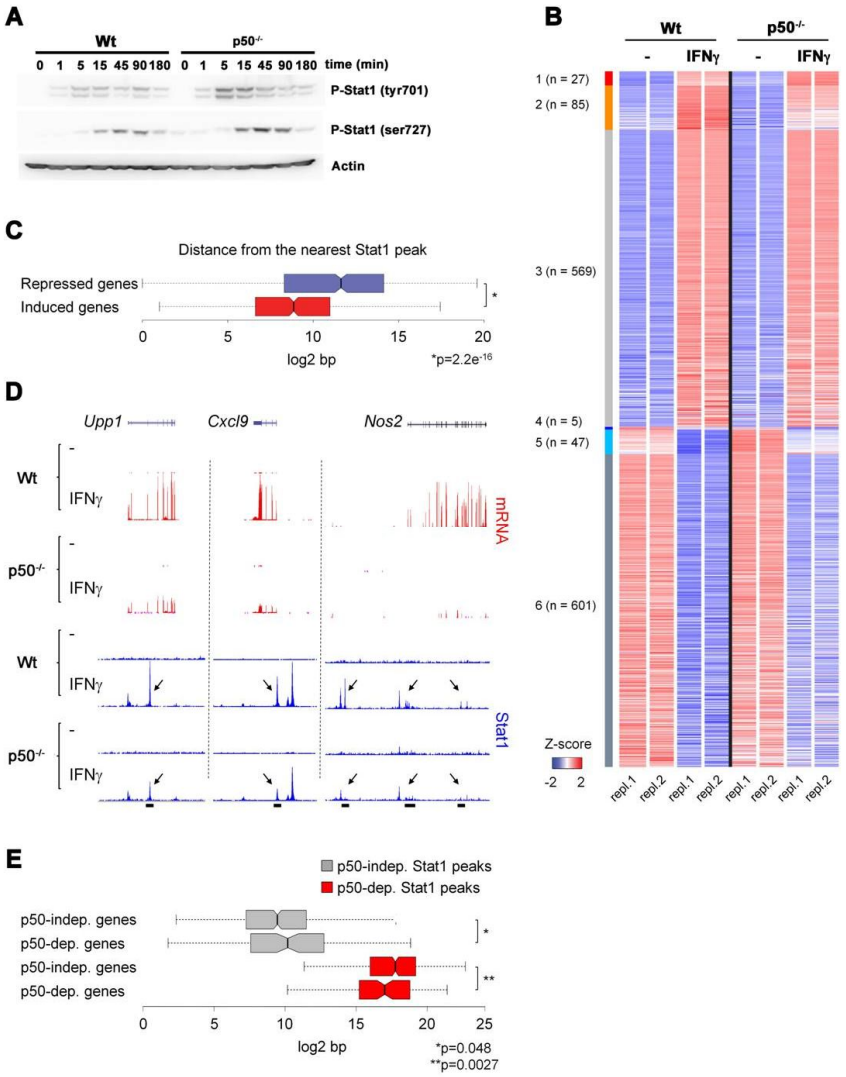

Figure 7. Effects of p50 deficiency on gene expression and Stat1 binding in macrophages treated with IFNγ. (A) STAT1 phosphorylaiton in Wt vs p50-/- PEC in response to IFNγ treatment. (B) Heatmap showing selective gene expression defects in $p50^{-/-}$ macrophages (PEC) stimulated with IFNγ for 4 hours (two biological replicates). The number of genes belonging to each of the six clusters is indicated on the left. (C) Genomic distances between genes induced and repressed by IFNγ and the nearest Stat1 peak. (D) A representative snapshot of three genes (*Upp1*, *Cxcl9* and *Nos2*) with impaired mRNA induction and Stat1 recruitment at their regulatory elements in $p50^{-/-}$ macrophages stimulated with IFNγ. Arrows and black bars indicate sites of defective Stat1 occupancy. (E) Box plot of the genomic distances between p50-dependent (or p50–independent) genes and p50-dependent (or p50-independent) Stat1 peaks, showing a direct correlation between transcriptional defects and reduced Stat1 binding in $p50^{-/-}$ macrophages treated with IFNγ. Wilcoxon test was used to calculate *p* values in C and E.

A discrete fraction of the Stat1 cistrome was selectively affected by p50 deficiency, with an abrogation or reduction of Stat1 occupancy at 2573 sites (8.3% of all inducible peaks) in $p50^{-/-}$ macrophages relative to wild-type controls. Loss of Stat1 binding in $p50^{-/-}$ macrophages frequently occurred at regulatory elements of p50-dependent genes. For instance, Stat1 was not efficiently recruited to either promoters or enhancers of *Upp1*, *Cxcl9* and *Nos2* genes in

$p50^{-/-}$ macrophages, and this was associated with the reduced induction of these genes in response to IFNγ (Figure 7D). These observations were then validated at a genomic scale by computationally integrating our ChIP-Seq and mRNA-Seq datasets (supplementary table 3). As shown in figure 7E, 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. Altogether, these findings identify a role for p50 in controlling IFNγ-induced gene expression, and are consistent with a model of p50-dependent assistance of Stat1 recruitment to selected p50-dependent genes in response to IFNγ treatment in macrophages.

Discussion

Activation and resolution are highly integrated phases of the inflammatory response that in a dynamic fashion requires concerted differentiation, maturation and actions of innate immune cells, including neutrophils, monocytes and macrophages [29]. In cancer, aberrant expansion of myeloid cells takes place, resulting in the generation of the tumor-supporting populations TAM and MDSC, respectively accumulating at the tumor site and in secondary lymphoid organs [4, 9, 30]. Moreover, MDSC dramatically increase under different inflammatory conditions, including autoimmune diseases, trauma, burns and sepsis [10], to promote resolution of both inflammation and immunity. MDSC and TAM share common myeloid precursors[9, 10] and phenotypic traits, including the expression of M2 polarized genes[21]. Here we demonstrate, *in vitro* and *in vivo*, that the differentiation of functionally suppressive M-MDSC during cancer growth is controlled by nuclear accumulation of the p50 NF-κB subunit, a key event in the resolution phase of the inflammatory response[8]. We show that accumulation of nuclear p50 NF-κB results in a selective transcriptional reprogramming, diverting the response of IFNγ-activated myeloid cells towards enhanced NO-mediated suppressive functions. We also indicate that this event is likely to represent a major impairment for successful cytokine-mediated cancer immunotherapy, as ablation of p50 NF-κB reinstates both IFNγ-mediated antitumor activity *in vivo* and the expansion of both $CD4^+$ and $CD8^+$ IFNγ producing T cells, in both spleen and tumor tissues. We also observed increased nuclear localization of p50 in blood $CD14^+HLA^-DR^{low/-}$ MDSC from colorectal cancer patients. This observation may be clinically relevant, as IFNγ is currently under evaluation in immunotherapeutic protocols against various human tumors, including colorectal cancer, soft tissue sarcoma, melanoma and plasma cell neoplasms[13]. Despite a number of studies previously reported either moderate or poor success in the clinical use of IFNγ[11, 14, 31], studies in tumor mouse models (fibrosarcoma) demonstrated

the requirement of both IFNγ and IFNγR for the control of tumor development and progression[16, 32, 33]. This ambiguous scenarios is reminiscent of the dual controversial immunological activities of IFNγ, which from one side promotes transcription of STAT1-dependent genes involved in the activation of the immune response (eg. MHC class I and class II, IL-12)[34] and, in contrast, induces immunosuppressive pathways, including expression of the inhibitory molecule B7-H1 in antigen-presenting cells[35, 36] and expression of immunosuppressive enzymes IDO[17] and iNOS[9] [10]. The biologically active form of IFNγ promotes auto-phosphorylation of the receptor subunits IFNγR1and IFNγR2 through the non-receptor tyrosine kinases Janus activated kinase (JAK)1 and JAK2, leading to phosphorylation and nuclear translocation of the homodimer STAT1, which eventually binds to gamma activated sequence (GAS) sites on the promoters of downstream target genes[37], including *Nos2*[38]. Our results indicate that accumulation of p50 NF-κB does not interfere with IFNγ-dependent STAT1 phosphorylation, but rather controls the chromatin landscape of myeloid cells to promote binding of STAT1 onto specific gene regulatory elements of IFNγ-responsive genes, including *Nos2*. Our observation that lack of p50 results in increased M-MDSC numbers in the spleen of tumor-bearing mice, with low NO production capacity, as well as in the preferential skewing of HSC towards the monocytic branch in the bone marrow, is in agreement with the $Irf8^{high}/Nos2^{low}$ profile observed in p50 deficient macrophages, since IRF8 is considered a cell fate switching factor driving terminal differentiation of macrophages[27]. Collectively our data indicate the tumor-induced nuclear p50 NF-κB accumulation in myeloid cells as a tumor-escaping strategy promoting immunosuppression through the induction of epigenetic alterations associated with enhanced IFNγ/STAT1-dependent induction of *Nos2*.

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); 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. p50 NF-κB deficient mice were available in the laboratory. OT-I mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). *Mdr2*-knockout mouse were kindly donated by Dr. Pikarsky E (Hadassah-Hebrew University Medical Center, Jerusalem, Israel).

Cell culture and reagents. Bone marrow MDSC (BM MDSC) were derived from bone marrows of C57BL/6 mice as previously described[39]. Bone marrow cells were cultured for 4 days in RPMI1640 containing 10% FBS, supplemented with 40ng/ml of murine recombinant IL6, GM-CSF and G-CSF (Peprotech). Peritoneal Exudate Macrophages (PEC) and Tumor Associated Macrophages (TAM) were obtained as previously described[21]. The concentration for the different treatments were as follows: MN/MCA1 tumor supernatant, dilution ratio 1:1 to medium, PGE_2 (Sigma) 10^{-5} mol/L, murine recombinant IL10 (Peprotech) 20ng/ml, TGFβ (Peprotech) 20ng/ml,, M-CSF (Peprotech) 20ng/ml, GM-CSF (Peprotech) 20ng/ml, GCSF (Peprotech) 20ng/ml.

Tumor. 8 weeks old $p50^{-/-}$ mice and Wt littermate controls were injected intramuscularly in the left leg with 10^5 cells of murine fibrosarcoma (MN/MCA1). Tumor growth was monitored 3 times a week with a caliper.

In vivo treatment with IFNγ. Mice were injected daily with 10U of murine recombinant IFNγ (Peprotech) or vehicle intramuscularly, starting from day 3 after tumor injection.

MDSC purification. $CD11b^+$ $GR1^+$ cells were purified by magnetic separation (MACS Miltenyi) from the spleens of tumor-bearing mice. In details, myeloid suppressor 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. The purity of the cell populations evaluated by flow cytometry exceeded 90%.

Nitrite production. $2*10^5$ MDSC or PEC were plated and stimulated with IFNγ (200U/ml) for indicated time points. NO was estimated by Griess reagent system (Promega).

Suppression assay. Cells were then stimulated with IFNγ (200U/ml), in the presence or absence of 500μM of L-NG-monomethylarginine (L-NMMA, Calbiochem). At day 1 (PEC) or day 3 (MDSC), 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 OVA antigen (Sigma). [^{3}H] thymidine was added for the last 16 hours of culture and its incorporation was analyzed by MicroBeta plate counter (Perkin Elmer).

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.

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

LPS-Tolerance in PEC. To induce LPS tolerance, PEC were treated as previously described[8].

Real-time PCR. Real-time PCR was performed as previously described[21]. The sequences of the murine iNOS gene-specific primers are: (Fw: gccaccaacaatggcaaca Rev: cgtaccggatgagctgtgaatt).

Western Blot Analysis. For NF-κB, nuclear and cytosolic extracts were analyzed by SDS-PAGE (10% acrylamide) as described [22]. Immunoblotting was performed with rabbit anti-p50 (no. 1141) and anti-p65 (no. 1226) antisera[40], anti-phospho-STAT1 (tyr701 or ser727) (Cell Signaling Technologies) and anti-actin antibody (Santa Cruz Biotechnologies).

Flow cytometry and sorting. $1*10^6$ cells/ml were re-suspended in HBSS (Hank's balanced salt solution, Lonza) supplemented with 0.5% BSA (Sigma). Staining was performed at 4°C for 20 minutes, with the following antibodies: anti-mouse Ly6G and Ly6C from Miltenyi Biotec; anti-mouse/human CD11b (clone M1/70), anti-mouse CD45 (clone 30-F11), anti-human CD45 (clone HI30), anti-mouse CD8, anti-mouse CD4, anti-mouse IFN-γ (Biolegend San

Diego, CA), anti-human HLA-DR, anti-human CD14 (BD biosciences, San Diego, CA). For intracellular staining Cytofix/Cytoperm and Permwash staining kit (BD Pharmigen) were used. Cells were detected using the BD FACS Canto cytofluorimeter and analyzed with BD FACS Diva Software. Cell sorting was performed using a BD FACS Aria cell sorter.

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 $Ly6C^+$ MDSC, that were immune magnetically purified from the spleen of tumor-bearing mice.

Isolation of MDSC from colorectal cancer (CRC) patients. 30 ml of peripheral blood were collected from either healthy donors or CRC patients and stratified on Percoll gradient to separate peripheral blood mononuclear cells (PBMC). Cells were stained and sorted to obtain $CD14^+$ HLA-DR^{neg} population and subsequently analyzed by confocal microscopy.

Confocal Microscopy. Cells were prepared as previously described[21] and stained with the following primary antibodies: rabbit policlonal anti-p50 (#C-20, 1μg/ml; Santa Cruz Biotech) and anti-p65 (#NLS, 1μg/ml; Santa Cruz Biotech), rat monoclonal anti-Ly6C (#AL-21, 2μg/ml; BD Biosciences) and anti-Ly6G (#1A8, 2μg/ml; BD Biosciences). After 1h of incubation, the detection antibodies goat anti-rabbit IgG Alexa® 488 and goat anti-rat IgG Alexa® 647 (Invitrogen, Molecular Probes) were used. For DNA detection DAPI (Invitrogen, Molecular Probes) was used.

Enzyme-linked Immunosorbent Assay (ELISA). Murine GM-CSF, G-CSF, M-CSF, IL6, IL-10, IL1β and TGF-β were tested by ELISA kits purchased from R&D Systems.

Chromatin Immunoprecipitation and Sequencing (ChIP-Seq). ChIP was carried out with a previously described high-throughput protocol[28, 41]. Illumina-compatible ChIP-Seq libraries were generated starting from from 1-5 ng of DNA [41] and sequenced on a HiSeq2000 (Illumina).

mRNA Sequencing (mRNA-Seq)

Total RNA was extracted from 1-5* 10^6 macrophages (RNeasy kit, Quiagen), 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).

Computational Methods:

ChIP-Seq analysis. After quality filtering according to the Illumina pipeline, short reads (51 bp), were mapped to the mm9 genome using Bowtie v0.12.7[42]. Only uniquely mapping reads with two or fewer mismatches (-m 1 –v 2) were retained. Peak calling was performed using MACS v1.4[43] with default parameters and bw = 100. Each ChIP was compared to input DNA derived from bone marrow-derived macrophages (GEO accession: GSM499415). We defined as IFNγ-inducible those peaks induced in treated vs. untreated samples (threshold of 1e−10 for peak calling) that in at least one of the two samples were enriched relative to the input genomic DNA (threshold of 1e−5). p50-dependent Stat1peaks were called using a threshold of 1e−5.

RNA-Seq analysis. After quality filtering according to the Illumina pipeline, paired-end reads (51 bp) were mapped to the mm9 reference genome (Ensembl build 63,) and to the Mus musculus transcriptome (Illumina's iGenomes) using

TopHat[44]. We allowed up to two mismatches and specified a mean distance between pairs (-r) of 250 bp. FPKMs (fragments per kilobase of exon per million fragments mapped) and fragment counts of multiple reads were scaled via upper-quartile normalization using Cuffdiff from Cufflinks v2.1.1[45]. Differential expression analysis between Wt and $p50^{-/-}$ samples was evaluated with an exact test for the negative binomially distributed counts using EdgeR (Bioconductor package)[46, 47]. Differentially expressed genes were selected using an FDR $\leq$ 0.01, FC (fold change) >=1, fpkm >= 0.1.Tracks for the UCSC genome browser[48] were generated using the uniquely alignable reads. Tracks were linearly rescaled to the same sequencing depth.

Acknowledgments

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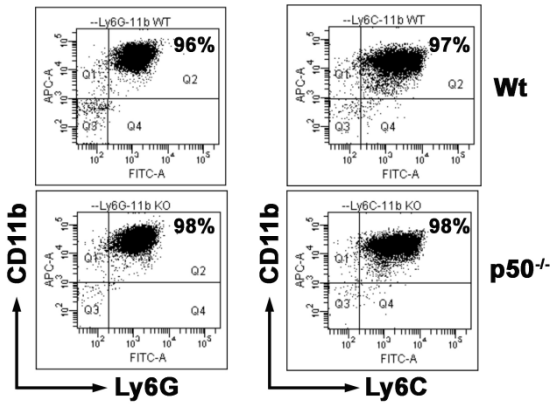
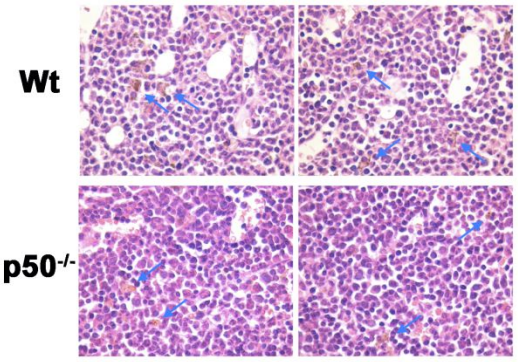
Supplemental data

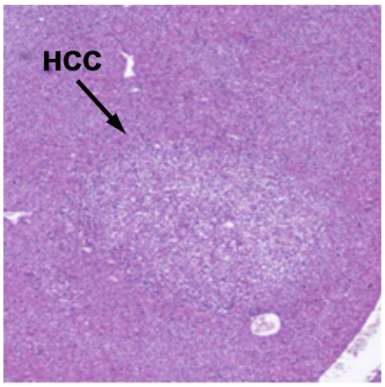
Supplemental figure 1. The scatterplot shows the purity of the monocytic and granulocytic MDSC subsets, following cell sorting from the spleen of tumor-bearing mice.



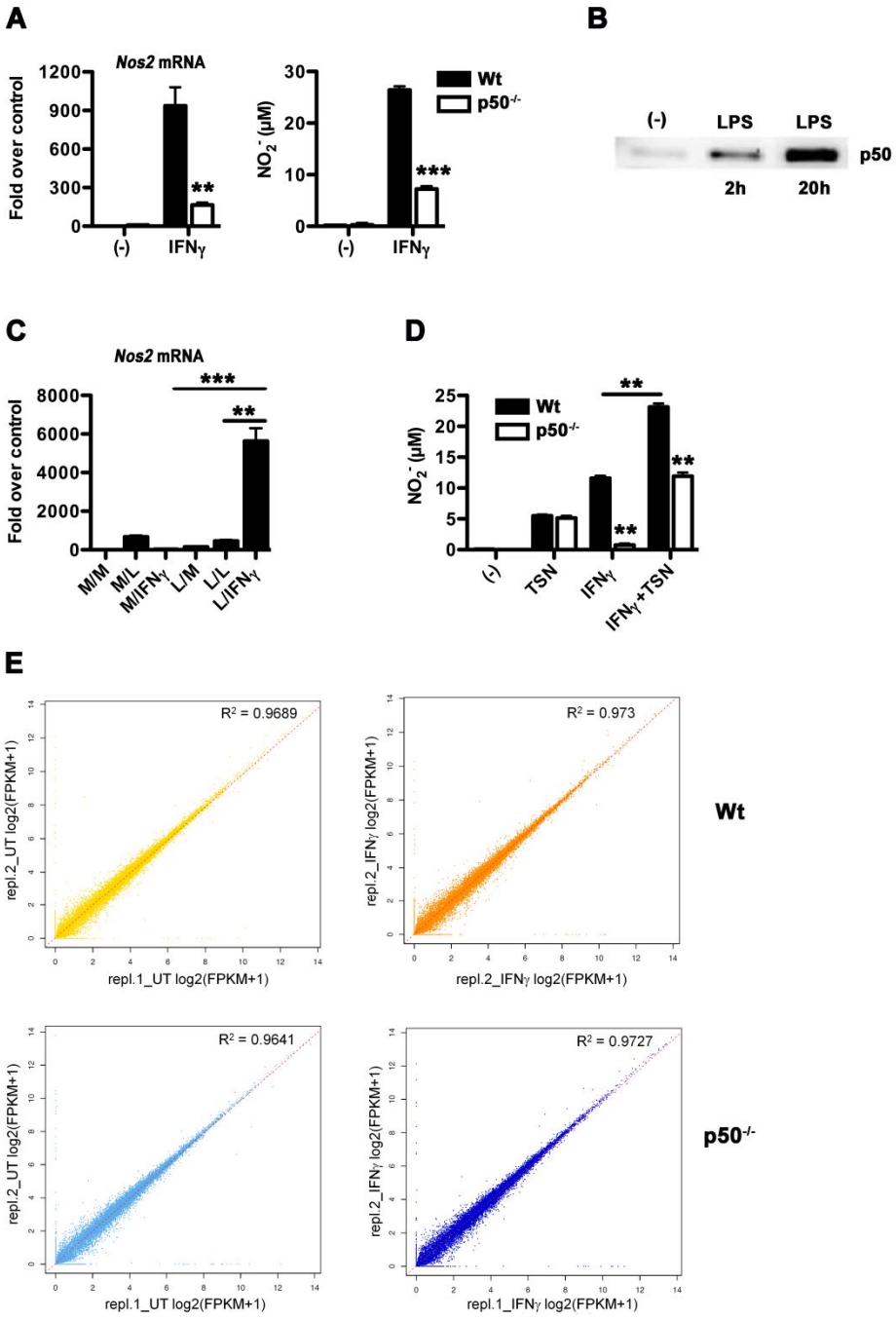
Supplemental figure 2. Monocytic differentiation is preserved in the BM of $p50^{-/-}$ mice, as testified by the presence of cells with mature monocytic morphology and by the normal density of hemosiderin-laden macrophages



Supplemental figure 3. H&E-stained liver's section from livers of mdr2$^{-/-}$ knockout mouse, that developed hepatocellular carcinoma (HCC) lesions (arrow).



Supplemental figure 4. Lack of p50 NF-κB in PEC inhibits NO production in response to IFNγ. (A) Decreased *Nos2* mRNA expression (left) and NO production (right) by IFNγ-treated p50$^{-/-}$ PEC. (B) Increased nuclear levels of p50 NF-κB in LPS-tolerant PEC (LPS 20 hrs). (C) LPS-tolerant PEC (L/M) display enhanced *Nos2* mRNA expression in response to IFNγ (L/IFNγ). M/M, medium; M/L, PEC activated 4hrs with LPS; L/L, LPS-tolerant PEC rechallenged 4 hrs with LPS. (D) TNS primes IFNγ-treated PEC for higher production of NO, in a p50 dependent manner (n=3 **P<.01; ***P<0,001). (E) Reproducibility of RNA-Seq biological duplicates. Pearson's correlation plots between replicates for each experimental conditions and calculated R^2.


A
Nos2 mRNA
Fold over control
1200
900
600
300
0
**
(-)
IFNγ
NO2- (μM)
30
20
10
0
***
Wt
p50-/-
B
(-)
LPS
LPS
p50
2h
20h
C
Nos2 mRNA
Fold over control
8000
6000
4000
2000
0
***
**
M/M
M/L
M/IFNγ
L/M
L/L
L/IFNγ
D
NO2- (μM)
25
20
15
10
5
0
Wt
p50-/-
**
**
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(-)
TSN
IFNγ
IFNγ+TSN
E
R2 = 0.9689
repl.2_UT log2(FPKM+1)
repl.1_UT log2(FPKM+1)
R2 = 0.973
repl.2_IFNγ log2(FPKM+1)
repl.2_IFNγ log2(FPKM+1)
Wt
R2 = 0.9641
repl.2_UT log2(FPKM+1)
repl.1_UT log2(FPKM+1)
R2 = 0.9727
repl.2_IFNγ log2(FPKM+1)
repl.1_IFNγ log2(FPKM+1)
p50-/-

CHAPTER FIVE

5. Discussion

Activation and resolution are highly integrated phases of the inflammatory response that in a dynamic fashion requires concerted differentiation, maturation and actions of innate immune cells, including neutrophils, monocytes and macrophages [1]. Inflammatory cells abundantly infiltrate human cancers where depending on their functional activation may exert beneficial or detrimental activities [12]. Indeed type density and location of immune infiltrate, the so called "immunoscore", has been recently suggested as a better predictor of CRC patients outcome than other methods for staging [13]. Among inflammatory cells, tumor associated macrophages (TAMs) has long been recognized as the major orchestrators of cancer related inflammation [4, 14] and predictors of poor prognosis for many different human tumors [15-17]. However, the impact of macrophages on colorectal cancer (CRC) outcome is controversial, likely because of its anatomical site. In addition to tumor-derived signals, CRC-associated macrophages activation can be influenced by unique local microenvironmental signals (e.g. microbial products) to whom they are exposed [12] and consequently multiple macrophages populations with either pro- or anti-tumoral activities could co-exist in CRC. In vivo evaluation of macrophage polarized activation is challenging. To dissect the complexity of functional macrophage heterogeneity, a panel of genes which are typically induced in response to M1 and M2 signals, should be evaluated [18]. Alternatively, macrophage polarized activation could be easier and more easily investigated by addressing the activation of transcription factors that selectively control the expression of M1 and M2 transcription programs. Here we originally find that high accumulation of p50 NF-κB in the nuclei of TAMs located at the invasive margin of human stage II/III CRC can be successfully used to predict disease recurrence. Despite the percentage of nuclear $p50^{+}$ TAMs in the stroma is

significantly associated with those at the invasive margin, the predictive value of p50 in TAM situated at the center of tumor did not reach statistic significance. This results suggest that macrophages located at the invasive margin critically control immune reactions against neoplastic cells. Accordingly, a positive association between the number of FasL expressing macrophages at the invasive margin of CRC and apoptotic cancer cells has been reported [19].

Whereas a type 1 inflammatory profile is predictor of beneficial outcome for CRC patients [20], the unfavourable prognostic significance of p50 nuclear accumulation in TAM is likely associated with an M2-skewing ability, as we previously demonstrated in murine tumors [10] and confirmed here in both a chemical model of colitis associated CRC (AOM/DSS) and in a genetic model of intestinal tumorigenesis (Apc^{Min} mice).

Although an M1 towards M2 switch of TAM functions has been suggested during tumor development [3] the molecular basis and the clinical relevance of this dynamic "reprogramming" of macrophage polarization have not been fully elucidated. In particular, whereas in established cancers M2-skewed activation has been mainly associated with tumor growth and progression, during the tumor initiation stage the impact of M1 macrophages seems to be dual: through the release of genotoxic molecules (e.g. ROS and NOS) and growth factors (e.g. IL-6) they can support both neoplastic transformation and proliferation of initiated cells [21, 22], while as cytotoxic effector cells they can contribute to the elimination of pre-malignant cells [23, 24]. Since both AOM/DSS treatment of both C57BL6 and Apc^{Min} mice mainly results in the development of multiple benign polyps [25-27] both models are suitable to investigate how p50-driven inflammation support CRC initiation. Our results coherently support a model in which, independent of the triggering signals, an M1 towards M2 switch of polarized inflammation is driven by p50 accumulation in macrophages and associates with intestinal tumors development. Indeed, exploring the molecular mechanisms linking p50-driven inflammation with colitis associated CRC

development we identified two clusters of inflammatory genes, which are differentially modulated by p50 and whose relative expression impact on disease outcome. In particular, whereas both gene clusters are up-regulated in early inflamed colons, during disease progression nuclear accumulation of p50 in TAM was associated with the selective increased expression of gene cluster 2, which includes several M2-related genes (e.g. IL-10, TGFβ, ArgI, CCL17, CCL22) along with other intestinal tumor promoting genes (e.g. COX2, IL-23p19, TNFα). In contrast, enhanced expression of gene cluster 1, which includes M1/Th1 inflammatory genes only, occurred in $p50^{-/-}$ tumors and was associated with tumor resistance. Hence, despite $p50^{-/-}$ mice showed an increased gut inflammation, both tumor multiplicity and size are strongly reduced. In absence of p50, also Apc^{Min} mice showed reduced tumor development associated with selective increased expression of M1/Th1 inflammatory genes. Hence our findings indicate that intestinal cancer development is promoted by an M2 shift of polarized inflammation and identify p50 NF-κB as the crucial molecule that skews polarized inflammation towards pro-tumoral functions.

We aslo observed that lack of p50 also modifies immune cells infiltration. Besides $p50^{-/-}$ tumors express higher levels of monocytes chemoattractants (e.g. CCL2, CCL5 and MCSF) TAM accumulation was strongly reduced in both intestinal tumor models. Lamina propria macrophages and monocytes were also decreased in $p50^{-/-}$ than in wt mice, both in untreated and AOM/DSS treated conditions. Since, gut macrophages are constantly replenished by bone marrow-derived monocytes [28], these results suggest that p50 is required for monocytes/macrophages recruitment. This hypothesis is also supported by the observation that, ex-vivo, migration of $p50^{-/-}$ peritoneal macrophages towards several different chemoattractants is severely impaired (unpublished data). In contrast, innate and adaptive lymphoid cells migration seems to be functional in absence of p50. Indeed, in steady state conditions the number of lamina propria

NK, NKT and T cells observed in wt and $p50^{-/-}$ mice was similar, while they increased in $p50^{-/-}$ colons and tumors of AOM/DSS treated mice. According to the accumulation of cytotoxic lymphoid cells, in $p50^{-/-}$ tumors we observed an increased expression of type 1-specific chemoattractants (e.g. CXCL9, CXCL10), cytokines (IL-12p35, IL-12p40, IL-27p28, IL-21) and effector molecules (IFNγ, Prf 1, Gzm B, FasL). Although IL-21 can support CAC by promoting Th17-driven inflammation [29, 30], IL-21 can also exert anti-tumor effects [31-34]. Indeed, IL-21 drives $CD8^+$ CTL differentiation via the actions of the transcription factor T-bet [35] and stimulates multiple other lymphocyte subsets, including follicular Th cells, B cells and NK cells [36]. In our $p50^{-/-}$ mice, IL-21 induction does not correlate with increased expression of IL-17 or RORc, but with augmented levels of genes (e.g. IFNγ, Prf 1, Gzm B, FasL, Tbx-21/Tbet) expressed by NK and CTL cells. Hence, we can speculate that ablation of p50 selectively enhanced anti-tumor effects of IL-21 without engaging pro-tumoral Th17 driven inflammation. In agreement, in the absence of p50 both bone-marrow-derived dendritic cells and peritoneal macrophages showed defective LPS-induced expression of IL-23p19 [11]. Noteworthy IL-23 drives Th17 cells expansion and functions [37] but also exert other pro-tumoral effects including inhibition of NK cell effector functions [38]. In response to gut microbial products, tumor associated myeloid cells are recognized as the major orchestrators of pro-tumoral IL-23 and IL-17 responses [39]. Accordingly IL-23p19 expression increased during progression from colitis to tumor, while it is strongly inhibited in $p50^{-/-}$ CRC. Consistently, in tumors from $Apc^{Min}p50^{-/-}$ mice IL-23p19 transcript levels were also significantly reduced, further suggesting that lack of p50 contributes to tumor resistance by inhibiting IL-23-driven inflammatory circuits.

In contrast, lack of p50 enhances cytotoxic immune responses that in turn restrain tumor development by eliminating neoplastic cells. Indeed, $p50^{-/-}$ tumor cells are more apoptotic than the wt counterpart. Although ablation of p50 can

impair colonic cancer cell survival, also in a cell autonomous manner [40], adaptive immune-surveillance seems to be crucial for $p50^{-/-}$ mice anti-tumor activities, as depletion of CD4/CD8 abolished colitis-associated CRC resistance.

Overall our results in mice and human indicate that p50-driven M2 inflammation promotes both CRC development and progression. Hence p50 could be exploited both as novel prognostic indicator and therapeutic target for CRC patients. Although different human [41, 42] and mouse [43] studies suggests a link between decreased levels of p50 and an higher risk to develop colitis, at steady state both $p50^{-/-}$ mice and $Apc^{Min}p50^{-/-}$ mice did not show signs of intestinal inflammation. In contrast, DSS treatment exacerbated the inflammatory response in $p50^{-/-}$ mice, indicating that for the small percentage of human CRC cases associated to colitis (1%-4%) [44] the anti-tumor efficacy of p50 targeting approaches could be limited by serious intestinal adverse drug reactions. In this regards our preclinical studies identified both IL-12 and CXCL10 as a potential immunotherapeutic drugs capable to limit CRC development without worsening colitis.

In addition to TAM, cancer triggers an aberrant expansion of an heterogenous population of immature and suppressive myeloid cells, called Myeloid Derived Suppressor Cells (MDSC), which mainly accumulate in secondary lymphoid organs [2-4]. MDSC and TAM share common myeloid precursors [2, 5] and phenotypic traits, including the expression of M2 polarized genes [6].

Here we demonstrate, *in vitro* and *in vivo*, that the differentiation of functionally suppressive M-MDSC during cancer growth is controlled by nuclear accumulation of the p50 NF-κB subunit, a key event in the resolution phase of the inflammatory response [8]. We show that accumulation of nuclear p50 NF-κB results in a selective transcriptional reprogramming, diverting the response of IFNγ-activated myeloid cells towards enhanced NO-mediated suppressive functions. We also indicate that this event is likely to represent a

major impairment for successful cytokine-mediated cancer immunotherapy, as ablation of p50 NF-κB reinstates both IFNγ-mediated antitumor activity *in vivo* and the expansion of both $CD4^+$ and $CD8^+$ IFNγ producing T cells, in both spleen and tumor tissues. We also observed increased nuclear localization of p50 in blood $CD14^+HLA^-DR^{low/-}$ MDSC from colorectal cancer patients. This observation may be clinically relevant, as IFNγ is currently under evaluation in immunotherapeutic protocols against various human tumors, including colorectal cancer, soft tissue sarcoma, melanoma and plasma cell neoplasms [46]. Despite a number of studies previously reported either moderate or poor success in the clinical use of IFNγ [47-49], studies in tumor mouse models (fibrosarcoma) demonstrated the requirement of both IFNγ and IFNγR for the control of tumor development and progression [50-52]. This ambiguous scenarios is reminiscent of the dual controversial immunological activities of IFNγ, which from one side promotes transcription of STAT1-dependent genes involved in the activation of the immune response (eg. MHC class I and class II, IL-12) [53] and, in contrast, induces immunosuppressive pathways, including expression of the inhibitory molecule B7-H1 in antigen-presenting cells [54, 55] and expression of immunosuppressive enzymes IDO [56] and iNOS [2, 5]. The biologically active form of IFNγ promotes auto-phosphorylation of the receptor subunits IFNγR1and IFNγR2 through the non-receptor tyrosine kinases Janus activated kinase (JAK)1 and JAK2, leading to phosphorylation and nuclear translocation of the homodimer STAT1, which eventually binds to gamma activated sequence (GAS) sites on the promoters of downstream target genes [57], including *Nos2* [58] Our results indicate that accumulation of p50 NF-κB does not interfere with IFNγ-dependent STAT1 phosphorylation, but rather controls the chromatin landscape of myeloid cells to promote binding of STAT1 onto specific gene regulatory elements of IFNγ-responsive genes, including *Nos2*. Our observation that lack of p50 results in increased M-MDSC numbers in the spleen of tumor-bearing mice, with low NO production capacity, as well

as in the preferential skewing of HSC towards the monocytic branch in the bone marrow, is in agreement with the *Irf8*^{high}/*Nos2*^{low} profile observed in p50 deficient macrophages, since IRF8 is considered a cell fate switching factor driving terminal differentiation of macrophages [59].

Nowadays anticancer therapies are not only directed against cancer cells, but they are associated with strategies intending to reduce cancer-mediated immunosuppression. Different approaches have been explored to harness the potency of the immune system to target cancer. These have been essentially focused on enhancing the immunogenicity of the tumour or on the induction and expansion of immune effectors to potentially target and eradicate the tumour. However, till now, efforts to actively stimulate the immune system against tumours in patients have been largely disappointing despite substantial evidence that peripheral immune responses against tumour antigens can be generated. Moreover, immune-modulating activities of chemotherapeutic agents are often very complex to understand, in fact the same molecules may play opposite roles depending on tumour type, immune contexture, and/or precise therapeutic strategy. To overcome this limitation, a possible approach might be the combination of chemo/radiotherapy with specific immunostimulatory agents. These complexities underscore the need for an ever more profound comprehension of the dynamic changes in the tumour microenvironment and in systemic immune response as tumours evolve, progress and respond to therapy. An improved knowledge of these aspects will facilitate the rational design of highly efficient, synergistic regimens that combine anticancer agents and immunotherapies.

In this scenario, our studies are intended to clarify some of the molecular mechanisms that underlie the cross-talk between cancer and myeloid cells and collectively our data indicate the tumor-induced nuclear p50 NF-κB accumulation in myeloid cells as a tumor-escaping strategy promoting

immunosuppression through the induction of both M2-like polarization of TAM and alterations of the epigenetic landscape of MDSC resulting in enhanced IFNγ/STAT1-dependent expression of the suppressive enzyme *Nos2*.

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CHAPTER SIX

6. List of publications

Molecular and epigenetic basis of macrophage polarized activation.

Porta C, Riboldi E, Ippolito A, Sica A

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Non so come il mondo potrà giudicarmi ma a me sembra soltanto di essere un bambino che gioca sulla spiaggia, e di essermi divertito a trovare ogni tanto un sasso o una conchiglia più bella del solito, mentre l'oceano della verità giaceva inesplorato davanti a me.

(Sir Isaac Newton)