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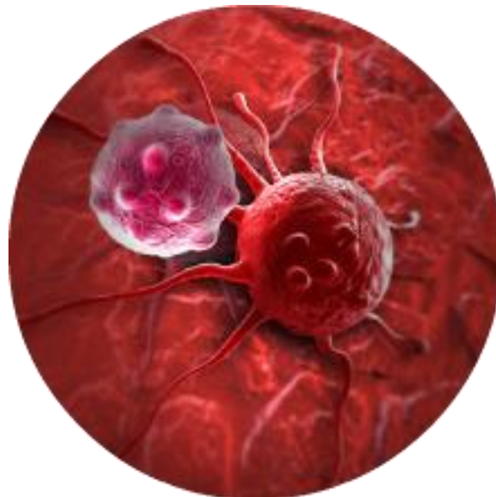
New pathways of protumor “emergency“ granulo/monocytopoiesis



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CHAPTER 1

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 several evidences undoubtedly demonstrated the critical role of inflammation in tumorigenesis, and that some of the underlying molecular mechanisms have been elucidated (1).

In response to tissue injury, a network of chemical signals initiates and maintains a host response 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. A family of chemotactic cytokines, named chemokines, which possess a relatively high degree of specificity for chemoattraction of specific leukocyte populations (2, 3), recruits downstream effector cells and dictates the natural evolution of the inflammatory response.

Inflammation is usually self-limiting; however, disregulation of any of the converging factors can lead to abnormalities and, ultimately, pathologies including cancer. Indeed, in chronically inflamed tissues, a subversion of cell death and/or repair programmes might occur, resulting in uncontrolled proliferation of cells carring DNA mutations. It is estimated that 20% of all cancers is associated with chronic infection and inflammation (4). The connection between inflammation and cancer can be viewed as consisting of two pathways: an extrinsic pathway, driven by inflammatory conditions (such as inflammatory bowel disease) that increase cancer risk; and an intrinsic pathway, driven by genetic alterations (such as oncogenes activation) that cause both inflammation and neoplasia.

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 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 (5). 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 to eliminate nascent tumors (6). 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) (7). In this regard, several studies have emphasized that the “smouldering” inflammation associated with tumors is mainly oriented to tune the adaptive immune response. Indeed, tumor-associated dendritic cells mainly show an immature phenotype (8) and myelomonocytic cells recruited in tumors express an alternative M2 functional phenotype mainly oriented towards the suppression of the adaptive immune response (9, 10). In agreement, clinical studies suggest that an established type-2 “suppressive” immunological profile correlates with poor prognosis, as shown in colorectal, hepatocellular and pancreatic carcinomas and in Hodgkin’s lymphoma (11).

1.1.2 Myeloid cells in cancer

Neoplastic cells condition distant sites, such as the bone marrow and spleen, by releasing soluble factors that drive the accumulation of myeloid cells; these myeloid cells subsequently promote neovascularization and metastasis.

This creates a tumour-driven ‘macroenvironment’. As discussed above, this macroenvironment conditions DCs, macrophages and granulocytes to become immunosuppressive. However, the most prominent effect is the accumulation of highly immunosuppressive, immature myeloid cells (iMCs).

These cells were named myeloid derived suppressor cells (MDSCs) to highlight their common myeloid origin and immunoregulatory properties (12).

The increase in circulating myeloid cells in tumour-bearing hosts, originally termed “reactive neutrophilia” or “emergency granulopoiesis”, was associated with an increased frequency of iMCs. Immature myeloid cells with the same phenotype as MDSCs are continually generated in the bone marrow of healthy individuals and differentiate into mature myeloid cells without causing detectable immunosuppression. However, in cancer, myeloid cell differentiation is diverted from its normal pathway which leads to the terminal differentiation of mature macrophages, DCs and granulocytes towards a pathway that favours the differentiation of pathological MDSCs (Figure 1).

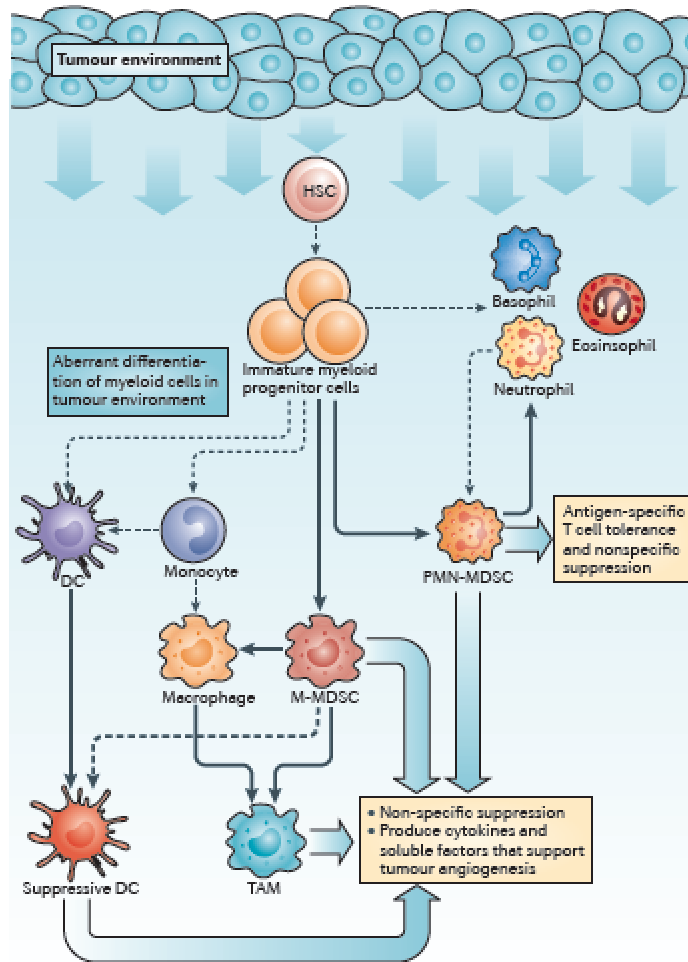

Figure 1- Changes that occur in myeloid cells in cancer.

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 (DCs), 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 myeloid-derived suppressor cells (M-MDSCs), polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), suppressive DCs and tumour-associated macrophages (TAMs) (13).

1.1.3 MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells of myeloid origin that comprises myeloid progenitor cells and immature macrophages, immature granulocytes and immature dendritic cells.

The two major MDSC subsets include the monocyte-like $CD11b^{+}Gr1^{+}Ly6C^{hi}Ly6G^{low}$ (M-MDSCs) and the granulocyte (neutrophil)-like $CD11b^{+}Gr1^{+}Ly6G^{hi}Ly6C^{low}$ (PMN-MDSCs). They are present in an activated state that is characterized by the increased production of reactive oxygen and nitrogen species, and of arginase 1 (14,15). They are potent suppressors of various T-cell functions. In mice, the phenotype of MDSCs is $CD11b^{+}GR1^{+}$, although functionally distinct subsets within this population have been identified (16).

In humans, the phenotype of MDSCs is $LIN^{-}HLA^{-}DR$–$CD33^{+}$ or $CD11b^{+}CD14^{-}CD33^{+}$; human cells do not express a marker that is homologous to mouse GR1 (17,18). MDSCs have also been identified within a CD15+ population in human peripheral blood. MDSCs were first characterized in tumour-bearing mice and in patients with cancer. These cells have been shown to markedly expand systemically when mice are inoculated with transplantable tumour cells and when tumours spontaneously develop in transgenic mice with tissue-restricted oncogene expression. In addition, up to a tenfold increase in MDSC numbers was detected in the blood of patients with different types of cancer (17,18).

In several mouse tumour models, as many as 20–40% of nucleated splenocytes are MDSCs (in contrast to the 2–4% seen in normal mice). In addition, MDSCs are found in tumour tissues and in the lymph nodes of tumour-bearing mice.

Although initial observations and most of the current information on the role of MDSCs in immune responses has come from studies in the field of cancer research**,** accumulating evidence has shown that MDSCs also regulate immune responses during bacterial and parasitic infections, acute and chronic inflammation, traumatic

stress, sepsis and transplantation. Recent studies (19) indicate that MDSCs have characteristics of both M1 and M2 macrophages (see paragraph "TAM").

Indeed, it was described in tumor-bearing mice a population of circulating CD11b+Gr-1+ inflammatory monocytes expressing IL-4Rα and able to release both IL-13 and IFN-γ (19), characteristics that are compatible with a function intermediate between those of M1 and M2 macrophages.

Cooperation between IL-13 and IFN-γ led to sustained activation of both ARG1 and NOS2 in MDSC populations, causing dysfunctional T cell responses.

These results suggest that MDSCs and TAMs respond with an M2 macrophage–oriented program to classic signals driving macrophage activation in tumor-bearing hosts.

1.1.4 MDSCs expansion

Factors that induce MDSC expansion can include cyclooxygenase 2 (also known as PTGS2), prostaglandins (20-22), stem-cell factor (SCF) (20), macrophage colony-stimulating factor (M-CSF), IL-6 (23), granulocyte/macrophage CSF (GM-CSF) (22) and vascular endothelial growth factor (VEGF) (23).

Most of these factors trigger signaling pathways in MDSCs that converge on Janus kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3), which are signalling molecules that are involved in cell survival, proliferation, differentiation and apoptosis (24). STAT3 is arguably the main transcription factor that regulates the expansion of MDSCs.

MDSCs from tumour-bearing mice have markedly increased levels of phosphorylated STAT3 compared with IMCs from naïve mice (25).

Exposure of haematopoietic progenitor cells to the supernatant from tumour-cell cultures resulted in the activation of JAK2 and STAT3, and was associated with an expansion of MDSCs *in vitro*. However, this expansion was abrogated when STAT3 expression in haematopoietic progenitor cells was inhibited (26).

Moreover, ablation of STAT3 expression through the use of conditional knockout mice or selective STAT3 inhibitors markedly reduced the expansion of MDSCs and increased T-cell responses in tumour-bearing mice (25,27).
STAT3 activation is associated with increased survival and proliferation of myeloid progenitor cells, probably through the upregulation of the expression of
B-cell lymphoma XL, cyclin D1, MYC and survivin. One such pathway involves the calcium-binding pro-inflammatory proteins S100A8 and S100A9 (29). STAT3-mediated upregulation of these proteins in myeloid progenitors inhibits DC differentiation and promotes MDSC accumulation (30).
In addition, STAT3 regulates the transcription factor CCAAT/enhancer-binding protein-β (C/EBPβ). C/EBPβ regulates myelopoiesis in healthy individuals and has a crucial role in controlling the differentiation of myeloid progenitors to functional MDSCs (28). STAT3 is responsible, at least in part, for inducing the expansion of MDSCs populations via upregulation of C/EBPβ and also has an indirect role in myeloid cell mobilization, accumulation and survival (31).
So, abnormal and persistent activation of STAT3 in myeloid progenitor cells prevents their differentiation into mature myeloid cells and thereby promotes MDSC expansion.

1.1.5 TAMs

Tumor Associated Macrophages (TAMs) represent the major population of leucocytes infiltrating tumors and several studies indicate that TAMs express crucial tumor-promoting functions (e.g. induction of tumor cell proliferation and angiogenesis, incessant matrix turnover, repression of adaptive immunity), which ultimately have an important impact on disease progression (32).
According clinical studies have demonstrated a correlation between high frequency of TAMs and the poor prognosis for many different human tumors including lymphoma, cervix, bladder, breast and lung cancers (33).

Macrophages are highly plastic cells able to finely modulate their programs in response to different microenvironmental conditions (34).
In response to diverse signals, macrophages undergo polarized activation (35). Classically activated (M1) macrophages are pro-inflammatory and elicit tissue destructive reactions. Alternatively activated (M2) macrophages are oriented to tissue repair and remodelling, immunoregulation, and tumor promotion.
Despite their potential anti-tumor activities several evidence indicate that tumors co-opt macrophages to promote their own development and invasion through the surrounding stroma (32, 36). Indeed, in many cancers TAMs express an M2 like phenotype which supports immune escape, tumor growth and malignancy (34, 37).
TAMs express high levels of M2 macrophage markers (IL-10, TGF-β, ARG1, and the mannose receptor) and low levels of mediators of M1 macrophage–mediated inflammation (IL-12, TNF-α, and IL-6) (32).

1.2 IL-17 cytokine family

Interleukin 17 (IL-17) family is a recently identified group of cytokines sharing homology in amino acid sequences with highly conserved cysteine residues critical to their 3-dimensional shape [38]. So far, six members, IL-17A (commonly referred as to IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and
IL-17F, have been identified [39-42]. Murine IL-17A is a 21 kDa glycoprotein containing 147 amino acid residues that shares 63% amino acid identity with human IL-17A (155 amino acids), and both mouse and human IL-17A are secreted as disulfide-linked homodimers. Each member of the IL-17 family shares
16%–50% amino acid identity with IL-17A (43,44). Among the family members, IL-17A and IL-17F share the highest amino acid sequence identity (50%), whereas IL-17E is the most divergent, with 16% identity to IL-17A. The IL-17 receptor (IL-17R) family includes five members (IL-17RA to IL-17RE).

Functional receptors for IL-17 family cytokines are thought to consist of homodimers or heterodimers. For example, the heterodimer of IL-17RA and IL-17RC is a receptor for homodimers and heterodimers of IL-17A and IL-17F, whereas the heterodimer consisting of IL-17RA and IL-17RB serves as a receptor for IL-17E. Of note, IL-17A, IL-17B, IL-17C, and IL-17F, but not IL-17E, can induce the expression of proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-1β from fibroblasts and peritoneal exudate cells and promote neutrophil migration, suggesting that these family members play similar roles in the development of certain diseases. Alternatively, IL-17E appears to be involved in promoting Th2 cell-type immune responses. However, the functional roles of other members of the IL-17 family have not been as well characterized as for IL-17A.

1.2.1 IL-17A and IL-17F

IL-17A and IL-17F are highly homologous and bind to the same receptor. Furthermore, IL-17A and IL-17F can both be secreted as disulfide-linked homodimers or heterodimers. Thus, these two molecules are likely to have similar biological activities (45,46). Indeed, both IL-17A and IL-17F are involved in the development of inflammation and host defense against infection by inducing the expression of genes encoding proinflammatory cytokines (TNF, IL-1, IL-6, G-CSF, and GM-CSF), chemokines (CXCL1, CXCL5, CXCL8, CCL2 and CCL7), antimicrobial peptides (defensins and S100 proteins), and matrix metalloproteinases (MMP1, MMP3, and MMP13) from fibroblasts, endothelial cells, and epithelial cells. IL-17A also promotes stem cell factor (SCF-) and G-CSF-mediated granulopoiesis and recruits neutrophils to the inflammatory sites. IL-17A also induces the expression of intercellular cell adhesion molecule 1 (ICAM-1) in keratinocytes as well as iNOS and cyclooxygenase-2 in chondrocytes.

However, IL-17F is a weaker inducer of proinflammatory cytokine expression and is produced by a wider range of cell types, including innate immune cells and epithelial cells. Moreover, the tissue distribution of IL-17RA and IL-17RC is different. These differences may result in some functional specialization of these cytokines.

1.2.2 IL-17A- and IL-17F-producer cells

There are several type of hematopoietic cells known to produce IL-17.

IL-17A and IL-17F were initially reported to be predominantly expressed in Th 17 cells. In addition to IL-17A and IL-17F double-positive cells, populations that are only IL-17A or IL-17F positive have been identified. The mechanisms that regulate IL-17A and IL-17F production also differ; IL-17F is expressed earlier than IL-17A during Th17 cell development (47). In addition to Th17 cells, a wide variety of T cells also produce IL-17A and IL-17F. These cytokines are produced by cytotoxic CD8+ T cells (Tc17) under conditions that are similar to those required by Th17 cells, but different from those required by IFN-γ producing CD8+ T cells (Tc1). Similarly, distinct populations of γfT (γf-17) cells and NKT (NKT-17) cells produce IL-17A and IL-17F (48). However, IL-23 and IL-1 can directly induce γδ-17 cell development in the absence of IL-6 and TCR ligation because, unlike naïve CD4+ and CD8+ T cells, these cells constitutively express IL-23R, IL-1R, and RORγt. Likewise, NKT cells produce IL-17A in the presence of IL-1 and IL-23 in combination with TCR stimulation. These two T cell populations (γδ-17 and NKT-17) can rapidly produce IL-17A and IL-17F in response to proinflammatory cytokine stimulation and may therefore provide an essential initial source of these two cytokines. More recently, innate lymphoid populations of neutrophils, monocytes, natural killer cells, and lymphoid tissue inducer (LTi)-like cells have been shown capable of rapidly producing IL-17A and IL-17F (48).

IL-17 producing cells with macrophage morphology have been described in patient with breast cancer (8). In addition, IL-17A is produced by intestinal Paneth cells (49), whereas IL-17F mRNA, but not IL-17A mRNA, is expressed in colonic epithelial cells (50), suggesting that IL-17A and IL-17F from nonlymphoid cells may also regulate immune responses. Substantial efforts are underway to clarify the mechanisms that control IL-17A and IL-17F production in these cell types, and the relative contributions of the resulting cytokines in immune response.

1.3 Nuclear receptor (NR) superfamily

The nuclear receptor (NR) superfamily is a highly conserved family of transcription factors. Humans, mice, and rats have respectively 48, 49, and 47 nuclear receptors each. NRs function as ligand-dependent transcription factors and share considerable amino-acid sequence homology (51).

General structural characteristics of NRs are a variable amino-terminal A/B region, a central, highly conserved DNA binding domain (DBD), also termed C region, a hinge region (D), and a carboxy-terminal ligand binding domain (LBD, or E region). The LBD is responsible for the recognition and binding of the receptor's ligand as well as ligand-dependent transcriptional activity.

Some receptors contain an additional C-terminal region (F region) whose function is poorly understood. Approximately half of the NR superfamily have well characterized natural ligands whereas the remaining receptors are considered "orphan" receptors and remain the focus of intense research (52).

The majority of NRs with identified natural ligands are also validated targets for clinical purposes and are a rich source of therapeutics aimed at the treatment of a great number of diseases, including inflammation, cancer, and metabolic disorders. Orphan NRs are an active area of research due to the potential for identification of ligands that may be used to modulate these receptors with the goal of developing targeted therapeutics for various diseases (53).

Over the past few years, there have been significant breakthroughs in the identification of novel ligands, both natural and synthetic, for several orphan NRs.

1.4 Retinoid-related orphan receptors (RORs)

The cloning of several steroid hormone receptors in the 1980s led to an intense search by many laboratories for additional, novel members of the steroid hormone superfamily (54,55). This resulted in the identification of a number of orphan receptors, including members of the retinoid-related orphan receptor (ROR) subfamily, which consists of RORα (NR1F1, RORA or RZRα) (56), RORβ (NR1F2, RORB or RZRβ) (57) and RORγ (NR1F3, RORC or TOR) (58). ROR genes have been cloned from several mammalian species and zebrafish.

1.4.1 ROR structure and activity

1.4.2 ROR gene structure

The *ROR*α human gene spans a relatively large 730 kb genomic region.
The *ROR*β and *ROR*γ genes cover approximately 188 and 24 kb, respectively.
As a result of alternative promoter usage and exon splicing, each ROR gene generates several isoforms that differ only in their amino-terminus (57,59)
(Figure 2).

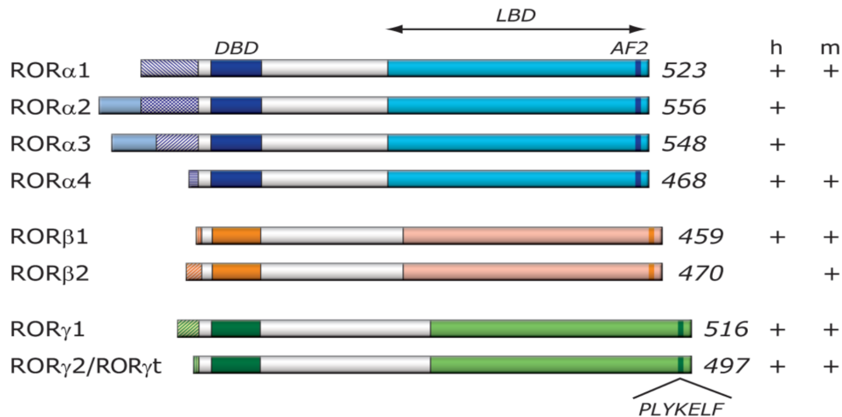

Figure 2- Schematic representation of ROR family members.
Schematic structure of the various ROR isoforms.
The different ROR isoforms identified in human and mouse are shown on the right (+/-) (109)

Four human RORα isoforms, referred to as RORα1-4, have been identified, while only two isoforms, α1 and α4, have been reported for mouse.

The mouse *ROR*β gene generates two isoforms, β1 and β2, while humans appear to express only the RORβ1 isoform (57). Both the mouse and human *ROR*γ gene generate two isoforms, γ1 and γ2 (60). Most isoforms exhibit a distinct pattern of tissue-specific expression and are involved in the regulation of different physiological processes and target genes. For example, human RORα3 is only found in human testis (61). RORα1 and RORα4 are both prominently expressed in mouse cerebellum, while other mouse tissues express predominantly RORα4 (62). In the mouse, expression of RORβ2 is restricted to the pineal gland and the retina, while RORβ1 is the predominant isoform in cerebral cortex, thalamus, and hypothalamus (57). RORγ2, most commonly referred to as RORγt, is exclusively detected in a few distinct cell types of the immune system, while RORγ1 expression is restricted to several other tissues (63).

Expression of the nuclear hormone receptor RORC has been reported in Th17 cells, Th22 cells, γδ T cells, NKT cells and CD4+CD8+ thymocytes.

In addition, RORC is expressed by cells that do not belong to the T or B
cell lineage, are thymus and recombinase activating geneindependent, yet have a lymphoid-like morphology (64,65). These cells are currently referred to as RORC expressing innate lymphoid cells (RORC+ ILC) (66).
Although most ROR isoforms are under the control of different promoters, little is known about the transcriptional regulation of their tissue-specific expression.

1.4.3 ROR protein structure

The ROR genes encode proteins of 459 to 556 amino acids (Figure 1).
RORs exhibit a typical nuclear receptor domain structure consisting of four major functional domains: an N-terminal (A/B) domain followed by a highly conserved DNA-binding domain (DBD), a hinge domain, and a C-terminal ligand-binding domain (LBD). RORs regulate gene transcription by binding to specific DNA response elements (ROREs), consisting of the consensus RGGTCA core motif preceded by a 6-bp A/T-rich sequence, in the regulatory region of target genes (67). RORs bind ROREs as a monomer and do not form heterodimers with retinoid-X receptors (RXRs) (57) (Figure 3).

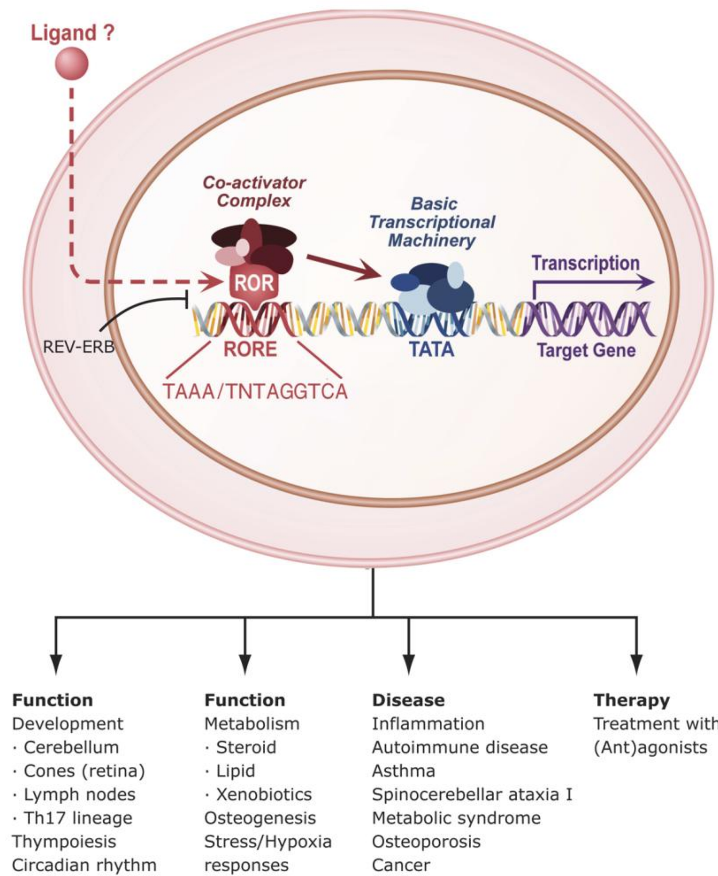

Figure 3-Mechanism of action of RORs, physiological functions and roles in disease. RORs bind as a monomer to ROREs consisting of the GGTCA consensus core motif preceded by a 6A/T rich region. RORs interact with coactivators or corepressors to positively or negatively regulate gene transcription. RORs are critical in the regulation of many physiological processes and may have a role in several pathologies.
[109]

Although RORα-γ and their different isoforms recognize closely-related ROREs, they exhibit distinct affinities for different ROREs. The amino-terminus (A/B domain) has been shown to play a critical role in conferring DNA binding specificity to the various ROR isoforms. In addition to the RORE sequence and the amino terminus, the promoter context may play an important factor in determining which ROR is recruited to a particular RORE. The LBDs of nuclear receptors are multifunctional and play a role in ligand binding, nuclear localization, receptor dimerization, and provide an interface for the interaction with coactivators and corepressors. X-ray structural analysis demonstrated that RORs have a secondary domain structure that is characteristic of that of nuclear receptors (68).

The LBDs of RORs contain, in addition to the typical 12 canonical α-helices (H1-12), two additional helices, H2’ and H11’. The activation function 2 (AF2) in H12 consists of PLYKELF, which is 100% conserved among RORs (Figure 1). Deletion of the H12 or point mutations within H12 causes loss of the ROR transactivation activity and results in a dominant-negative ROR (69).
It is believed that H10 plays a critical role in the homo- and heterodimerization of nuclear receptors. Structure analyses revealed the presence of a link in H10 of the LBD of RORα and RORβ that would greatly affect the dimerization capability of RORs (68). This is consistent with the conclusion that RORs do not form homodimers or heterodimers with other RORs or RXRs.

1.4.4 RORs: ligand-dependent transcription factors

The ligand binding domains of NRs are multifunctional.
Typically, ligand binding induces a conformational change in the receptor resulting in dissociation of corepressors and recruitment of co-activators (62).
However, RORs are constitutively active meaning that they are in an active conformation in the absence of ligand and that ligand binding might actually repress receptor activity (inverse agonist). While identification of the endogenous ligands for RORs has been controversial, recent evidence suggests that, similar to the liver X receptor’s (LXRs), oxygenated sterols may function as high affinity ligands. Indeed, 7- oxygenated sterols (7α-OHC, 7β-OHC, and 7-ketocholesterol) function as inverse agonists to both RORs. The 7-oxygenated sterols bind to both RORα and RORγ isoforms with an affinity significantly greater than the affinity for cholesterol and cholesterol sulfate, and suppress their transactivation properties.
It was also shown that both RORα and RORγ are constitutively active in the absence of ligand, able to bind co-activator peptides, and activate transcription. Several other endogenous RORα and RORγ ligands have been described recently. 24Shydroxycholesterol (24S-OHC) is a high affinity ligand for RORα and RORγ,

and similar to the 7-oxygentated sterols, 24S-OHC acts as an inverse agonist and dose dependently reduces the RORα and RORγ constitutive activity (70).
X-ray structure analysis of the RORβ(LBD) identified stearic acid as a fortuitously-captured ligand that appeared to act as a stabilizer by filling the ligand-binding pocket, rather than as a functional ligand (71). Subsequently, several retinoids, including all-*trans* retinoic acid (ATRA) and the synthetic retinoid ALRT 1550 (ALRT), were identified as functional ligands for RORβ (72).
ATRA and ALRT 1550 were able to bind RORβ(LBD) reversibly and with high affinity and reduced RORβ-mediated transcriptional activation, suggesting that they act as partial antagonists. These retinoids were also able to bind RORγ and inhibit RORγ-mediated transactivation, but did not bind RORα or affect RORα-induced transactivation (72). Although future research needs to determine whether *in vivo* ROR activity is regulated by endogenous ligands, these crystallographic and structural studies do support the concept that ROR activity can be modulated by specific endogenous and/or synthetic (ant)agonists.
This conclusion is highly relevant to the emerging roles of RORs in several pathologies, including inflammation, various autoimmune diseases, obesity, and asthma, and the promise that these receptors might serve as potential targets for pharmacological intervention in these diseases (Figure 3).

1.4.5 Role of RORγ in the development of secondary lymphoid tissues

RORγ1 and RORγt (RORγ2) exhibit distinct patterns of tissue-specific expression. RORγ1 is expressed in many tissues, including liver, adipose, skeletal muscle, and kidney, while the expression of RORγt is exclusively expressed in a few distinct cell types of the immune system [73,74]. Mice deficient in RORγ expression lack lymphnodes and Peyer's patches (PPs), suggesting that RORγ is indispensable for lymph node organogenesis and development of PPs [73,74].

Lymphoid tissue inducer (LTi) cells play a critical role in the development of lymph nodes and PPs. LTi cells originate from hematopoietic precursor cells in the fetal liver and have been recently characterized as CD45int$CD4^+CD3^-$CD127 (IL-7Rα)$^+$Lin$^-$ cells in mice and in humans as lineage-negative CD45int$CD4^-CD3^-$ CD127hiLin$^-$ cells [73]. LTi cells are absent from spleen, mesentery, and intestine of RORγ$^{-/-}$ E18.5 embryos, indicating that RORγt plays a critical role in the generation and/or survival of LTi cells [73]. As a consequence, the lack of lymph nodes and PPs in RORγt- or RORγ-deficient mice is due to the absence of LTi cells.Besides PPs and mesenteric lymph nodes, the intestinal immune system contains several other lymphoid cell compartments, including cryptopatches and isolated lymphoid follicles (ILFs) [75]. ILFs develop from cryptopatches in response to inflammatory innate immune signals generated by the colonization of the intestine by bacteria. Lin-cKit$^+$CD127$^+$CD44$^+$ cells in cryptopatches and in ILFs express high levels of RORγt and appear to constitute the adult counterpart of LTi cells [74,75]. The deficiency in cryptopatches and ILFs observed in RORγ$^{-/-}$ mice indicate that RORγt is essential for the development of these cell compartments and is due to the absence of these LTi-like cells in these mice.

1.4.6 Critical functions of RORγ in thymopoiesis

Several studies have demonstrated that RORγt plays a critical role in the regulation of thymopoiesis [76]. During thymopoiesis, T cell precursor $CD25^{-}CD44^{+}CD4^{-}CD8^{-}$ cells (DN1) differentiate successively via two intermediate stages, $CD25^{+}CD44^{+}$ (DN2) and $CD25^{+}CD44^{-}$ (DN3), into $CD44^{-}CD25^{-}$ (DN4) thymocytes. These cells then differentiate via immature single positive (ISP) cells ($CD3^{-}CD4^{-}CD8^{low}$) into double positive CD4+CD8+ (DP) thymocytes.

After successful T cell receptor α (TCRα) gene rearrangement, DP cells expressing TCRαβ receptor undergo a careful selection process to eliminate thymocytes expressing nonfunctional or autoreactive TCR. The positive selected DP thymocytes mature into single positive (SP) $CD4^{+}CD8^{-}$ helper and $CD4^{-}CD8^{+}$ cytotoxic T cells that then colonize the secondary lymphoid organs, including the spleen, lymph nodes, and PPs. RORγt is transiently expressed during thymopoiesis [76]. It is undetectable in DN thymocytes and highly induced when ISP cells differentiate into DP thymocytes and again down-regulated when DP thymocytes differentiate into mature T lymphocytes. At birth and during early stages of life, RORγ null mice have a significantly smaller thymus compared to wild type mice as a result of a drastic reduction in the number of double positive DP and SP thymocytes, while the percentage of ISP thymocytes is greatly increased [73].

The accumulation of ISP cells in $ROR\gamma^{-/-}$ mice appears to be due to a delay in the differentiation of ISP into DP cells, suggesting a role for RORγt in the regulation of the ISP-DP transition [76]. In addition, DP thymocytes undergo massive apoptosis *in vivo* and *in vitro* [78]. The accelerated apoptosis of $ROR\gamma^{-/-}$ DP thymocytes is related to reduced expression of the anti-apoptotic gene *Bcl-XL.*

This repression is an early and key event in accelerated apoptosis in $ROR\gamma^{-/-}$ thymocytes [78]. Thus, RORγ functions as a positive regulator of *Bcl-XL* expression and, as a result, promotes the survival of DP thymocytes, thereby enabling gene rearrangement

1.4.7 RORs and cellular metabolism

Accumulating evidence indicates that RORs play an important role in the regulation of several metabolic pathways, particularly lipid and steroid metabolism [79]. Initial characterization of RORγ$^{-/-}$ mice revealed that they display normal cholesterol and triglyceride levels, with slightly lower blood glucose levels than their wt counterparts [80]. However, recent evidence suggests that RORγ may indeed have a role in metabolism through regulation of adipogenesis and insulin sensitivity. Meissburger et al. demonstrate that RORγ is a negative regulator of adipocyte differentiation in vitro. When overexpressed during adipocyte differentiation, RORγ decreases the amount of differentiated adipocytes.

However, in vivo differentiation of adipocyte precursors in RORγ$^{-/-}$ mice was enhanced but showed decreased size. The smaller adipocytes were insulin sensitive and protected the mice from obesity induced hyperglycemia and insulin resistance [81]. Moreover, analysis of adipose stromal-vascular fractions from obese human subjects demonstrated a positive correlation between RORγ expression and adipocyte size that was negatively correlated with adipogenesis and insulin sensitivity. These findings suggest that RORγ may be a novel target for the treatment of obesity-associated insulin resistance [80]. The deletion of both RORα and RORγ exhibit similar changes in cholesterol, triglyceride, and blood glucose levels as the single knockout mice. Gene expression analysis from livers of double knock out (DKO) mice suggests a degree of functional redundancy between RORα and RORγ which is most likely due to the similarities in RORE binding affinities.

However, the recent evidence regarding obesity and insulin resistance in the RORγ$^{-/-}$ mice highlights the differences between the two NRs in metabolic processes.

1.4.7 RORs and T cell lineage specification

1.4.8 Role of RORs in Th17 cell differentiation

Recent studies identified a critical role for RORs in the regulation of lineage specification in helper T cells (82). Differentiation into different effector CD4+ T cell lineages, T helper (Th) 1, Th2, Th17, and T regulatory (Treg or Th3) cells is initiated through an interaction of dendritic cells with uncommitted (naïve) CD4+ T helper cells (Thp). Th1, Th2, Treg, and Th17 are characterized by their synthesis of specific cytokines and their immuno-regulatory functions (Figure 4).

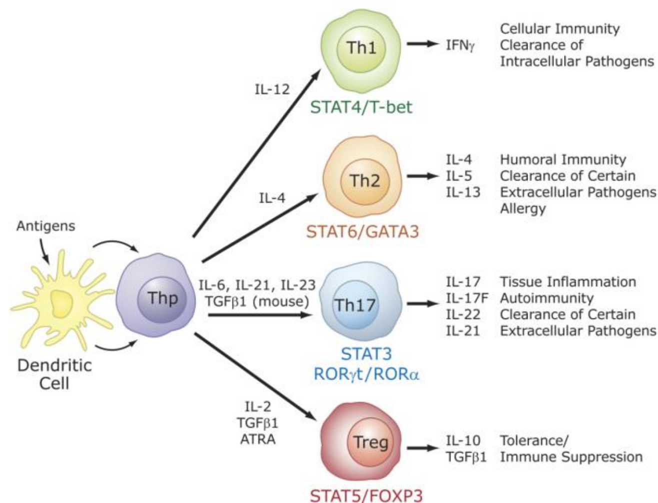

Figure 4- Specific role for RORs in T cell lineage specification.
Differentiation into different effector CD4+ T cell lineages, T helper (Th) 1, Th2, Th17, and T regulatory (Treg) cells. [109]

Interferon γ is the signature cytokine produced by Th1 cells, while IL-4, IL-5 and IL-13 are major cytokines produced by Th2 cells. The recently discovered Th17 cells produce IL-17A (IL-17), IL-17F, IL-21, and IL-22 as major cytokines, while Treg cells synthesize IL-10 and TGFβ1 (83,84). T helper and Treg cells play a critical role in several inflammatory responses, including adaptive immune responses to various pathogens.

Host defense is coordinated by the proinflammatory Th1, Th2, and Th17 cells, while Treg cells are involved in the down regulation and contraction of an immune inflammatory response. Th17 cells are believed to be the major proinflammatory cells involved in autoimmunity, while Treg cells protect against autoimmunity (84). Differentiation into Treg cells and Th17 cells is often reciprocal, involving several positive and negative regulatory networks that favor one or the other lineage (74). This includes a yin-yang relationship between RORγt and FOXP3 expression and their regulation by several cytokines, transforming growth factor β (TGFβ), and ATRA. Littman, Cua, and their colleagues were the first to report that RORγt is required for the differentiation of naïve CD4+ T cells into Th17 cells (85). This was supported by findings from several other laboratories (84).

RORγt is induced during differentiation of antigen-stimulated Thp cells along the Th17 lineage in response to IL-6 or IL-21 and TGFβ. Cells deficient in IL-6 do neither express RORγt nor IL-17F and IL23R (86). IL-6 mediates its action through activation of STAT3. Deficiency in STAT3 greatly impaired the activation of RORγt expression and Th17 differentiation, suggesting that this induction is STAT3-dependent (87). Inversely, deficiency in suppressor of cytokine signaling 3 (SOCS3), a negative regulator of STAT3 activity, enhances Th17 differentiation (88). Whether STAT3 regulates RORγt expression directly by binding to the RORγt promoter needs to be established. Additional evidence for a role of RORγt in Th17differentiation came from studies showing that Thp cells isolated from RORγ null mice exhibited a marked reduction in their ability to undergo differentiation along the Th17 lineage (89). Conversely, exogenous expression of RORγt in Thp cells greatly increased the expression of IL-17 cytokines and IL23R in the absence of cytokines. Subsequent studies showed that, like RORγt, RORα is highly induced during Th17 cell differentiation in a STAT3-dependent manner (90).

Deficiency in RORα reduced IL-17 and IL23R, but not IL-17F or IL-22 expression, while exogenous expression of RORα in Thp cells or Jurkat cells enhanced IL-17, IL-17F, IL-22, and IL23R expression (90). These observations indicate that RORα also functions as a positive regulator of Th17 differentiation, suggesting a degree of functional redundancy between RORγt and RORα. This was consistent with findings showing that Th17 differentiation was completely impaired in Thp cells deficient in both RORα and RORγ (90). Thus, both RORα and RORγt are required for optimal differentiation of Thp cells into Th17 cells; however, RORγt appears to be the major player in this process because RORγt deficiency has a more pronounced effect on the expression of Th17 cytokines than loss of RORα. Whether RORα and RORγ have any unique roles in the regulation of gene expression during or after Th17 differentiation needs further study.

In addition to the induction of RORγt, IL-17, and IL-17F, differentiation along the Th17 lineage is associated with increased expression of IL-21 and IL23R (86).

IL-21 expression is not affected in RORγ-deficient mice, suggesting that this regulation occurs upstream of RORγ. In contrast to IL-21, the induction of IL23R is greatly reduced in RORγ-deficient mice, suggesting that its expression is regulated by RORγt.

1.4.10 Transcriptional regulation of IL-17 positive cell

A substantial amount of work concentrated on the signaling transcriptional events that occurring during Th 17 development (Figure 5).

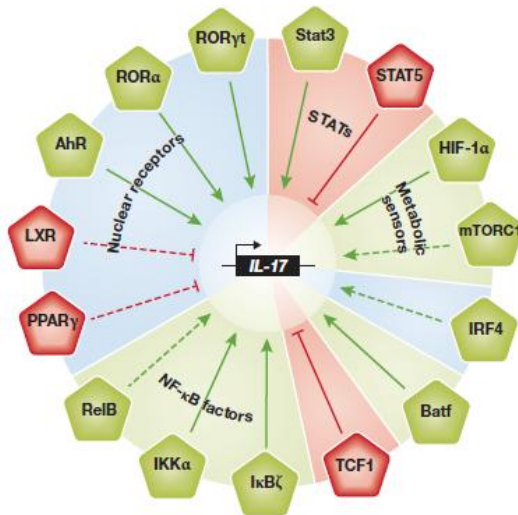

Figure 5-Transcriptional regulation of the IL-17 program.

Members of various transcription factor families are implicated in the regulation of *IL-17* transcription either directly (solid line) or indirectly (dashed line). Green indicates positive regulation and red negative regulation [110].

The transcription factors Rorγt, AhR and IκBζ are specifically induced during Th17-cell development and have key roles in their differentiation and function (91,92). STAT3, IRF4, Runx1 and Batf, on the other hand, influence Th17 development, but their expression is not restricted to Th17 cells (93,94).

As anticipated, the transcriptional modulators that target these key transcription factors can either enhance or impair the differentiation of Th17 cells.

The positive and negative regulators of the key transcription factor IRF4—ROCK2 and IRF4 binding protein, respectively were clearly shown to alter the activity of IRF4, thereby resulting in the spontaneous development of autoimmune disorders in mice with aberrantly activated ROCK2 and deficient in IRF4 binding protein (95).

The nuclear receptor LXR negatively regulates the IL-17 immune response by upregulating the transcription factor Srebp-1c, which interacts physically with AhR to interfere with its function in promoting Th17 development (96).

In addition, the nuclear receptor PPARγ was shown to negatively regulate Th17 differentiation by repressing the induction of Rorγt. Similarly to the differential role of STAT4 and STAT6 for Th1 and Th2 differentiation, respectively, STAT3 downstream from IL-6 signalling has a central role in the initiation of Th17 development. The components of the NF-κB signalling pathway regulate numerous immune responses, and their dysregulation is linked to inflammatory and autoimmune diseases, as well as cancer (97). Interestingly, several of these molecules regulate Rorγt and IL-17A expression. Thymic γδT cells express the surface lymphotoxin-β-receptor and, after its ligation, RelB mediates the induction of Rorγt in γδ T cells, but not αβ T cells, which is required for the differentiation of IL-17-producing γδ T cells and an innate IL-17 response to *Escherichia coli* infection (98). Besides NF-κB, kinase inhibitors for NF-κB signalling also play a role in Th17 differentiation. Although IKKα was originally identified as an inhibitor of NF-κB, IKKα can promote IL-17 transcription in Th17 cells by binding to the IL-17 locus in a NF-κB-independent way (99).

Furthermore, IκBζ is specifically induced during Th17 differentiation and, in cooperation with Rorγt, is essential for robust IL-17 transcription (91).

Several groups have demonstrated recently that the kinase mammalian target of rapamycin (mTOR) and the transcription factor HIF1-α, which are cellular metabolic sensors, control Th17 fate determination. The mTOR complex 1 (mTORC1), but not mTORC2, regulates the small GTPase Rheb signalling pathway, which promotes Th1 and Th17, but not Th2, differentiation (100).

HIF-1α is upregulated during Th17 differentiation, possibly in a STAT3-and mTOR-dependent manner, which in turn enhances Rorγt expression.

After this event, a molecular complex with STAT3, HIF-1α, Rorγt and P300 induces the robust expression of TH17-associated genes (101).

1.4.11 Suppression of ROR transcriptional activity by FOXP3

Treg and Th17 cells are reciprocally regulated, involving several positive and negative regulatory networks [83,84]. The balance between the expression of RORγt and FOXP3 plays a critical role in determining whether uncommitted CD4+ T helper cells differentiate into Treg or Th17 cells (Figure 6).

Regulation of FOXP3 expression by TGFβ and IL-6 plays a critical role in Treg and Th17 lineage determination [83,84]. In the presence of TCR engagement, TGFβ induces FOXP3 expression and Treg differentiation in murine Thp cells. Although TGFβ can moderately enhance the expression of RORα and RORγt, FOXP3 is induced at much higher levels, thereby shifting the ROR/FOXP3 balance towards FOXP3 and Treg differentiation [86,102]. Addition of IL-6 or IL-21 inhibits the induction of FOXP3 by TGFβ and activates RORα and RORγt expression, thereby shifting the ROR/FOXP3 balance in favor of Th17 differentiation. These observations indicate that FOXP3 represses Th17 differentiation by antagonizing RORγt function rather than inhibiting its expression [83]. This antagonism is mediated through a direct interaction of FOXP3 with RORα and RORγt.

1.4.12 Interaction with coregulatory proteins

For many receptors, binding of a ligand functions as a switch that induces a conformational change in the receptor that involves a repositioning of H12 (AF2) [83]. When RORs are in a transcriptionally-active conformation, H12 with H3 and H4 form a hydrophobic cleft and a charge clamp that involves the participation of a conserved Lys in H3 and a conserved Glu in H12 within RORs [68].

The clamp serves as an interaction interface for LXXLL motifs present in coactivators and related motifs in corepressors [95].

Receptor:coactivator complexes, through their histone acetylase activity, induce histone acetylation that results in decompaction of chromatin and increased transcription of target genes [94], while receptor association with corepressor complexes leads to histone deacetylation and subsequently compaction of chromatin and repression of gene expression [95]. RORs interact with corepressors, as well as coactivators, suggesting that they can function both as repressors and activators of gene transcription. Recently, repression of ROR-mediated transcriptional activation by the forkhead box transcription factor p3 (FOXP3) was shown to depend on a direct interaction between RORs and FOXP3 [103].

1.5 RORs and cancer

1.5.1 RORs and increased cancer susceptibility

A number of studies have provided evidence a role for RORs in cancer.

Mice deficient in the expression of RORγ exhibit a high incidence of thymic lymphomas that metastasize frequently to liver and spleen [104]. As a consequence, the lifespan of RORγ null mice is greatly reduced. The enhanced lymphoma formation may be related to changes in thymic homeostasis observed in RORγ KO mice [105]. Although the molecular mechanism underlying enhanced lymphoma formation has not yet been established, it may be related to the dysregulation of differentiation and proliferation in RORγ-/- thymocytes.

Interestingly, type B leukemogenic virus (TBLV), which causes T-cell lymphomas in mice, was found to be frequently integrated at the RORγ locus [106].

However, in contrast to RORγ null mice, RORγ expression correlated positively with lymphomas. Whether RORγ is implicated in human lymphomas requires further study. Another potential link between RORγ and cancer is emerging from studies showing increased expression of Th17-associated genes, including RORγ, IL-17, and IL-23, in gastric tumors, an increase in the population of circulating Th17 cells in gastric cancer patients [107], and a high incidence of Th17 cells at sites of ovarian cancer [108]. These elevated levels of proinflammatory cytokines may contribute to cancer pathogenesis. Alterations in RORγt expression or activity may cause changes in the Th17 cell population and production of proinflammatory cytokines, thereby affecting cancer progression positively or negatively.

1.5.2 Tumour-associated Th17 cells

Recently, TH17 cells have been investigated in patients with diverse cancer types, including ovarian cancer and prostate cancer.

Although not the predominant T cell subset within the tumour, Th17 cells are present in the tumour microenvironment. Th17 cells do not home to lymphoid tissues, but some of the above homing molecules might be involved in Th17 cell migrate to inflammatory tissues and tumours [111]. Th17 cells express other cytokines in addition to IL-17, and this might be functionally relevant in several physiological and pathological settings. Human tumour-infiltrating Th17 cells express negligible levels of the anti-inflammatory cytokine IL-10, but around 50–90% of Th17 cells produce high levels of effector cytokines such as IL-2, (GM-CSF), interferon-γ (IFNγ) and tumour necrosis factor (TNF) [111].

Therefore, tumour-associated TH17 cells exhibit an effector T cell cytokine profile similar to that of effector T cells that have been described in infectious diseases [112,113]. A similar cytokine profile has been observed in TH17 cells associated with distinct human tumour types, including carcinomas of the skin, intestine, pancreas, liver and ovaries [111]. These data indicate that TH17 cells might have a protective role in tumour immunopathology by promoting antitumour immunity.

1.5.3 Evidence for antitumour activity

The relationship between Th17 cells and tumour immunopathology is controversial [114]. However, there are several lines of evidence suggesting that Th17 cells can promote protective antitumour immune responses. Firstly, tumour-infiltrating Th17 cells express several effector cytokines, similar to that observed in patients with infectious diseases [112,113]. This suggests that tumour-associated Th17 cells might be functional effector T cells. Consistent with this possibility, Th17 cells are negatively correlated with the presence of T-Reg cells[111] and are positively correlated with effector immune cells, including IFNγ+ effector T cells, cytotoxic

CD8+ T cells and natural killer (NK) cells, in the same tumour microenvironment [111]. These observations are supported by data from both human and mouse tumours [111]. Transgenic T cells polarized to a Th17 cell phenotype following treatment with TGFβ and IL-6 were shown to induce tumour eradication in mice [115]. In addition, IL-17-deficient mice show accelerated tumour growth and lung metastasis in many tumour models, and forced expression of IL-17 in tumour cells was shown to suppress tumour progression [116]. In patients with prostate cancer, a significant inverse correlation is found between Th17 cell differentiation and tumour progression [117]. Treatment with specific antibody against cytotoxic T lymphocyte antigen 4 (CTLA4) [118] induces Th17 cells in patients with melanoma and the levels of IL-17 detected in tumour-associated ascites positively predicts patient survival. Taken together, these data provide strong evidence that Th17 cells can have protective roles in tumour immunity.

1.5.4 Pro-tumour role of Th17 cell-associated cytokines

Th17 cells infiltrate into tumor sites and draining lymph nodes in cancer patients [119]. The number of IL-17A-producing cells correlates with poor survival in patients with hepatocellular carcinoma, whereas the number was decreased in patients with advanced ovarian cancer, lung cancer, or non-Hodgkin's lymphoma. Thus, Th17 cells and IL-17A may play different roles depending on the tumor type and stage. Transplantation of IL-17A-overexpressing tumor cells into immunodeficient mice induced angiogenesis through induction of vascular endothelial growth factor (VEGF) expression, resulting in enhanced tumor growth [120]. T cellderived IL-17A also dramatically increases the release of angiogenic and chemoattractive IL-8 from tumor cells [121]. Delayed growth of subcutaneously transplanted B16 melanoma cells and MB49 bladder carcinoma cells was observed in IL17a$^{-/-}$ mice, whereas IFNγ$^{-/-}$ mice showed accelerated growth and augmented IL-17A production in the tumor [122].

In this case, IL-6 was expressed in response to IL-17A-activated Stat3 in the tumor cells, upregulating the expression of prosurvival and proangiogenic genes.
IL-17A is also involved in the development of colonic cancer in multiple intestinal neoplasia (Min) mice, a model of familial adenomatous polyposis [123]. Interestingly, enterotoxigenic *Bacterioides fragilis* (ETBF), an intestinal commensal bacteria, can accelerate colonic inflammation and tumor formation in Min mice [124]. In this model, ETBF selectively induced Th17 cell differentiation via Stat3 activation, and blocking IL-17A as well as IL-23R dramatically reduced tumor development indicating that a Stat3- and Th17 cell-dependent pathway contributed to the disease. It was shown that IL-17 is required for the development of MDSCs in tumor-bearing mice. A defect in IL-17R reduces the number of MDSCs in the blood, spleen, and tumors. This is further supported by data showing that the administration of exogenous IL-17 increases the number of MDSCs in wild-type tumor-bearing mice, whereas neutralization of IL-17 in wild-type tumor-bearing mice reduced the number of MDSCs [125].
TAM and MDSCs both generally recognized as dominant promoting forces, produce large amounts of the Th17 cell driving cytokines TGFbeta and IL-6 [126].
IL-23 is an IL-12 cytokine family member, which is produced by APCs and promotes the expansion and survival of Th17 cells. It has been reported that IL-23-deficient mice are resistant to chemically induced tumours [127].
This resistance is associated with decreased expression of matrix metalloproteinase 9 (MMP9) in the skin, a decrease in the expression of angiogenic markers and high levels of $CD8_+$T cell infiltration. Given the close relationship between IL-23 and Th 17 cells, it has been proposed that Th17 cells or IL-17 derived from Th17 cells can promote tumorigenesis in this model. However, this hypothesis remains to be tested and antitumour effects of IL-23 have been observed in several mouse tumour models [128].

1.6 Hematopoiesis

Hematopoiesis is a process of blood cell production and maturation in the bone marrow hematopoietic stem cells. The process begins with the hematopoieti stem cell. (HSCs). Because mature blood cells are predominantly short lived, stem cells are required throughout life to replenish multilineage progenitors and the precursors committed to individual hematopoietic lineages. HSCs reside as rare cells in the bone marrow in adult mammals and sit atop a hierarchy of progenitors (common myeloid progenitor (CMP), common lymphoid progenitor (CLP)) that become progressively restricted to several or single lineages. These progenitors yield blood precursors devoted to unilineage differentiation and production of mature blood cells, including red blood cells, megakaryocytes, myeloid cells (monocyte/macrophage and neutrophil), and lymphocytes. As with all other stem cells, HSCs are capable of self-renewal the production of additional HSCs and differentiation, specifically to all blood cell lineages. HSCs are defined operationally by their capacity to reconstitute the entire blood system of a recipient. Developmentally, there are two waves of hematopoiesis, so probably with two corresponding origins for HSCs [130]. Primitive hematopoiesis, distinguished from definitive hematopoiesis by large and nucleated erythrocytes and specifically expressed fetal hemoglobin isoforms, occurs as a transient wave preceding the advent of definitive hematopoiesis. Primitive hematopoiesis in mammals begins within the blood islands in yolk sac. Independently, the stem cells for definitive hematopoiesis originate within embryonic SP/AGM (splanchnopleur, aorta, gonads, and mesone-phros). Only these definitive hematopoietic stem/progenitor cells originating from AGM, but not primitive HSCs are able to repopulate the entire hematopoietic system in the lethally irradiated adult recipient mice [131].

Definitive HSCs expand in number in the AGM, and then migrate to and colonize fetal liver and spleen where they continue to differentiate into recognizable hematopoietic precursors. After birth, definitive hematopoiesis is primarily confined to bone marrow, and in some pathological conditions also to extramedullary sites such as spleen, liver, and occasionally lung and brain.

Hematolymphoid differentiation and maturation from HSCs to terminally differentiated cells involves loss of self-renewal capacity, transient acquisition of high proliferative potential, and subsequent restriction to a mature blood cell lineage, that, with the exception of memory T and B cells as well as some tissue macrophages and Langerhans cells, is postmitotic. The sequence of differentiation events (Figure 6) goes from HSCs to nonself-renewing multipotent progenitors (MPPs). MPPs, via intermediate populations debated in their specific details elsewhere, give rise to CMPs, and CLPs. The CLP and CMP are considered as primitive progenitor cells which then give rise to more-differentiated progenitors (also termed as committed precursor cells). Ultimately, these cells give rise to unilineage committed progenitors. Each step of the Hematopoietic progression that is survival, proliferation or differentiation is well supported by cytokines and growth factors.

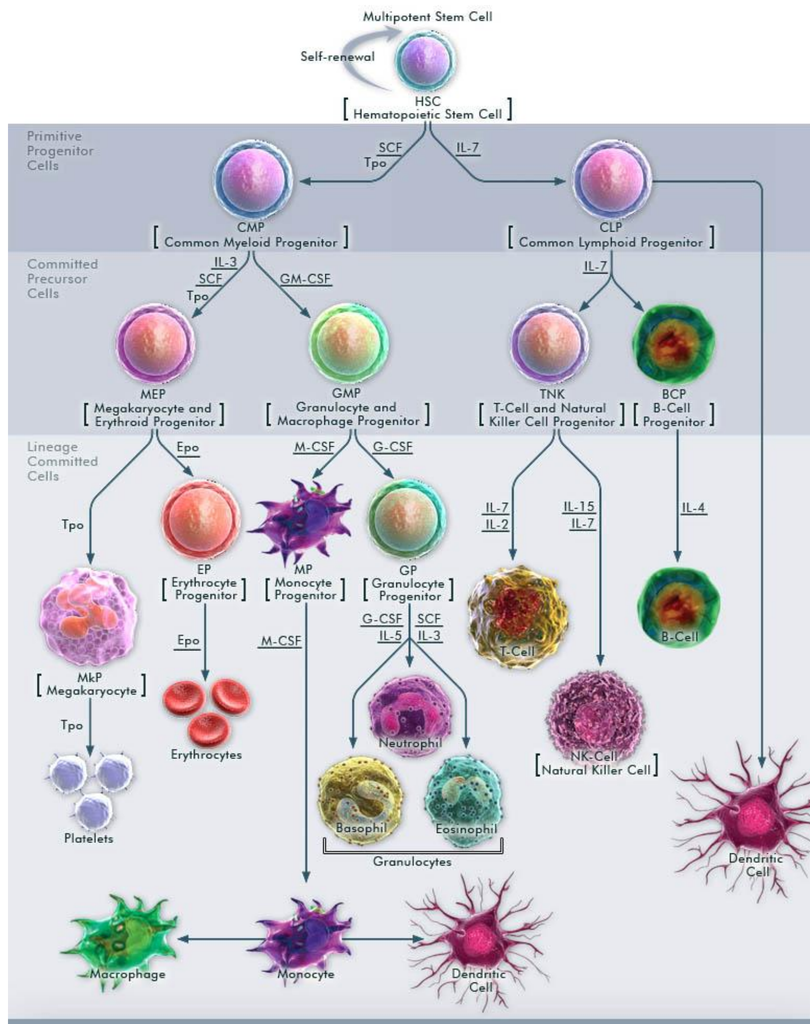

Figure 6- Hematopoiesis from multipotent stem cell

Hematopoiesis is a hierarchical progression of Multipotential HSCs that gradually lose one or more developmental options, becoming progenitor cells committed to a single lineage; these progenitors then mature into the corresponding types of peripheral-blood cells. Each step of the Hematopoietic progression that is survival, proliferation or differentiation is well supported by cytokines and growth factors (ex. GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), G-CSF (Granulocyte Colony Stimulating Factor), M-CSF(Macrophage Colony-Stimulating Factor)). [129]

1.6.1 Bone marrow stem cell niche

Key properties of stem cells such as their self-renewal and developmental capacity can be controlled in a nonautonomous manner by their cellular microenvironment. Bone marrow microenvironment is usually referred to as a stem cell niche. A niche is a group of stromal cells that allows a stem cell to maintain its identity [132].

The cells of a niche will prevent a previously specified cell from losing its stemness through loss of quiescence and potency or precocious differentiation.

Bone marrow stromal cells are known to provide microenvironmental support for hematopoietic stem cells through the secretion of growth factors and cytokines (Figure 6), but also through direct contact in cell–cell interactions and through production of extracellular matrix [133]. All the stromal cells in bone marrow, except macrophages, originate from pluripotent bone marrow mesenchymal stem cell (BM-MSCs) that exhibit ability to self-renew as well as to differentiate into cells of mesodermal lineage, including osteocytes, adipocytes, chondrocytes, myocytes, tenocytes, myocardiocytes, and hematopoietic supportive stroma [133].

Although the concept of niches is well accepted and experimentally proven in various invertebrate systems, mammalian stem cell niches remain poorly understood, as the precise location of the stem cells themselves often remains elusive. Several studies have attempted to localize HSCs in bone sections or by using confocal/two-photon intravital imaging based on three-color fluorescence microscopy [134]. Putative HSCs have been found near the endosteum lined by OBs (endosteal niche) or in association with sinusoidal endothelium (perivascular niche). Moreover, the location of some important cellular components of the niches may not be restricted to the endosteum or the perivascular niche area, but may be part of both environments, raising the possibility that both niches and the location of HSCs may not be as distinct as is currently assumed.

1.7 T cell in regulation of hematopoiesis

The involvement of the immune system in regulation of hematopoiesis was suggested during 1970s of the last century.

Different studies pointed to the cooperation between hematopoietic cells and T cells for the maintenance of optimal hematopoiesis in mice [135,136].

Further research has demonstrated that thymus-derived T lymphocytes can produce cytokines that have powerful effects on hematopoiesis.

Understanding how T cells regulate both steady-state hematopoiesis and induced hematopoiesis during an immune response is critically important for increasing our knowledge of both hematopoietic control and immune regulation.

The potential of the specific T helper cell subsets, Th1 and Th2 cells, to modulate the hematopoietic response in different ways, although not well characterized, has been demonstrated and reported [137]. However, defining the role of newly recognized subset of IL-17- secreting T cells, Th17 cells, as well as the regulatory T cells (T-regs), in regulation of hematopoiesis is still in its infancy.

Only recent data implied to the presence of T-regs in hematolymphoid compartments and demonstrated the ability of T-regs to modulate hematopoietic progenitor cells activity, suggesting that T-regs are not restricted in their regulatory actions within the adaptive and innate immune systems [138].

As for the IL-17, even the early studies pointed to this cytokine as an example of bridging between T-cell function and haematopoiesis, demonstrating that IL-17 induces production of various pro-inflammatory cytokines, but also those with haematopoietic effects, such as GM-CSF, G-CSF (granulocyte-colony-stimulating factor), and IL-6 [139]. As the understanding of its function improved, it was shown that IL-17 is at the heart of a regulatory circuit controlling neutrophil homeostasis, while other studies demonstrated that IL-17 also affects other cells of hematopoietic system, such as erythroid progenitors, as well as mesenchymal stem cells.

These findings focused the attention on molecular mechanisms underlying the effects of IL-17 on hematopoietic and mesenchymal stem cells in order to better understand the regulatory role of IL-17 in hematopoiesis, both during the normal, steadystate hematopoiesis and disturbed, altered hematopoietic responses.

1.7.1 Effects of IL-17 on hematopoietic cells

Initial evidence of IL-17's stimulatory effect on myeloid cells was obtained after in vitro experiments which demonstrated that IL-17 can induce the proliferation of CD34 + human stem cells, as well as their maturation into neutrophils, only when cocultured with fibroblasts [140]. This way it was suggested that IL-17 affects hematopoietic stem cells indirectly, through the induction of fibroblasts to release secondary cytokines with hematopoietic effects, including G-CSF and IL-6.

Further on, the in vivo expression of IL-17 in an experimental model of adenovirus-mediated gene transfer of the murine IL-17 cDNA induced a profound stimulation of both bone marrow and splenic granulopoiesis and led to expansion of myeloid hematopoietic stem and progenitor cells (high proliferative potential colonies—HPPC, colony-forming-unit–granulocyte-erythrocyte-megakaryocyte-monocyte—CFU-GEMM, and colony-forming-unit-granulocyte–macrophage—CFUGM) and neutrophilia, in part through release of secondary cytokines, such as G-CSF [141]. Data obtained in the same experimental model also showed that for optimal granulopoiesis, beside G-CSF release, IL-17-mediated effects require the presence or induction of the transmembrane form of stem cell factor [142] and confirmed that IL-17 stimulates the production and differentiation of granulocyte lineage cells by inducing secondary hematopoietic cytokines [143].

Data, obtained in a different experimental approach, demonstrated that even a single injection of IL-17 recombinant protein elicits a cascade of biological

changes in vivo, affecting the cells of granulocytic lineage, as well as the levels of cytokines released, primarily IL-6, in both murine bone marrow and spleen [144].

The most obvious in vivo response of granulocytic cells to IL-17 administration was observed at the level of bone marrow morphologically recognizable proliferative granulocytes and spleen metamyelocytes, indicating that more mature progenitors respond first to its action. The role of IL-17 in erythropoiesis has not been as extensively studied as its role in granulopoiesis.

Different studies demonstrated that hematopoietic effects of IL-17 were highly dependent on the microenvironment, since the effects on the erythroid compartments in mouse spleen were opposite to those observed in the bone marrow. The differences were attributed to different cytokine profiles induced by IL-17 related to the tissue microenvironment in which hematopoiesis occurs [144].

Beside the influence of IL-17 during steady-state hematopoiesis, its effects on hematopoietic cells during the state of disturbed or altered hematopoiesis were also analyzed. Tan W. et al. 2006 showed that following sublethal radiation-induced myelosuppression, in vivo overexpression of murine IL-17 substantially enhances granulopoietic restoration, characterized by increase in neutrophils, as well as both splenic and bone marrow progenitor cells [145]. Data obtained during the naturally acquired Syphacia obvelata infection in mice confirmed that the activity of IL-17 differs depending on physiological/pathological status of the organism.

It was demonstrated that this pinworm parasite induces significant hematopoietic changes during infection, characterized by increased myelopoiesis and erythropoiesis in infected animals. This stimulation of hematopoiesis was accompanied by altered sensitivity of the bone marrow myeloid and erythroid progenitors from infected mice to IL 17, as compared to non-infected controls, and the changes in reactivity were manifested both at the cellular and molecular level [146,147].

1.7.2 Effects of IL-17 on mesenchimal stem cell

The idea that T cells act through the bone marrow stromal cells to support hematopoiesis came with the reports that bone marrow grafts depleted in T cells lead to delayed or failed engraftment [148,149]. In the first reports, IL-17 was presented as a cytokine that achieves its effects primarily by acting on different stromal cells (fibroblasts, osteoblasts, chondrocytes, bone marrow stromal cells), stimulating them to secrete other soluble and membrane-bound factors, among which are IL-6, G-SCF, GM-CSF, SCF, and NO [150,151]. IL-17 also induces many genes in stromal cells, including those implicated in hematopoiesis, such as IL-6 gene and its transcriptional regulators (C/EPB family, IjBf), different CC and CXC chemokines, and apoptosis related proteins, such as FAS and Lipocalin 2 [152,153]. The particularly high levels of IL-17RA (IL-17 receptor A) expression on stromal cells, including bone marrow mesenchymal stem cells, are in line with this notion [154]. However, only recently, IL-17 was shown to act as potent growth factor for both murine and human BMMSCs, affecting their proliferation and differentiation potential. As regarding the influence of IL-17 on the MSCs differentiation, the reported results are diverse, and it appears that the effects of IL-17 are highly dependent on the specific microenvironment and/or specific host organism requirements.

1.7.3 The role of IL-17 in hematopoiesis

The IL-17 is emerging as critical player in inflammatory diseases and host defense responses to extracellular bacteria and fungi, acting largely by inducing neutrophil recruitment [155]. It is well established that IL-17 as a response cytokine released within the microenvironment of infections is required for mounting adequate immune responses in both innate and adaptive immunity.

Substantial data supported that the neutrophil homeostasis and trafficking to tissues are regulated by the IL-23/IL-17/G-CSF–cytokine-controlled loop.

Neutrophil turnover has been shown to play an important role in the homeostatic regulation of IL-17 and its control of granulopoiesis [156].
When neutrophil migration into tissues is blocked by adhesion molecule deficiency, macrophages and dendritic cells secrete excessive levels of IL-23, a key cytokine that drives Th17 development and IL-17 production [157], leading to increased G-CSF-dependent granulopoiesis. Neutrophil phagocytosis by macrophages and dendritic cells suppresses their production of IL-23, thus decreasing IL-17 synthesis and G-CSF-dependent granulopoiesis [156]. Therefore, IL-17 regulates granulopoiesis by its control of G-CSF expression, while circulating neutrophils act in a negative feedback loop to block excessive production of Th17 cells and IL-17. In Th17-mediated pathologic inflammation, the neutrophil response elicited by IL-17-dependent regulation plays a role in the initiation, but also in the maintenance of inflammation. Moreover, it was shown that IL-17 cooperates synergistically with various inflammatory cytokines, such as TNF-a, IL-1b, and interferon-c, augmenting the induction of pro-inflammatory responses from various target cells [157], thus placing this cytokine in the midst of a complex network that amplifies inflammation.

1.8 Steady state and emergency hematopoiesis

The hematolymphoid system is a paradigmatic, somatic stem cell–maintained organ with enormous cellular turnover, that is, the continuous de novo generation and subsequent death of cells. It is estimated that, in a 70-kg adult human, approximately 3×10^5 erythrocytes and 3×10^4 white blood cells are produced per second in steady-state (steady-state hematopoiesis), and it is documented that during specific demand, for example, on blood loss, infectious challenges and cancer cellular production can increase several-fold over baseline levels (demand-adapted hematopoiesis) (158).

Obviously, such high cellular throughput requires tight control, as hematopoietic failure (eg, aplasia) or excess (eg, leukemia) within a short time is not compatible with life. It is equally obvious that the control mechanism needs to involve "long-distance" regulatory feedback loops as the blood cell production sites in bone marrow (BM) are spatially disconnected from blood cell effector sites in the periphery.

1.8.2 Demand-adapted regulation of early hematopoiesis in infection and inflammation

The hematopoietic response to acute systemic bacterial infection, often referred to as emergency myelopoiesis, is a prime example for demand-adapted hematopoiesis. In contrast to less severe local infection not causing systemic symptoms and blood count alterations, emergency myelopoiesis is characterized by systemic signs such as blood leukocytosis, neutrophilia, the emergence of immature neutrophils (clinically called left-shift), and increased production of myelomonocytic cells in BM. Hematopoietic responses in acute viral infection as well as in chronic bacterial or viral infection are more variable, ranging from lymphopenia and neutropenia to lymphocytosis and neutrophilia.

Despite differences in kinetics and magnitude of the responses elicited by distinct pathogens, the crucial initial step is shared in any of these settings and involves sensing and recognition of a given pathogen. Specific antigen receptor–independent pathogen recognition in the innate branch of the immune system is accomplished through specialized pattern-recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) belong to the PRR family and recognize conserved microbial products derived from exogenous pathogens and possibly also some host-derived endogenous ligands [159,160]. Ligation of TLRs, via sophisticated downstream signaling cascades, induces cell- and context-dependent proliferation, differentiation, and migration, as well as

protein production and secretion, all processes geared to activate and orchestrate efficient immune response against insulting invaders [161].

The classic concept of induction of emergency myelopoiesis assumes that activation of PRRs on tissue-resident hematopoietic and nonhematopoietic cells at the site of pathogen entry triggers a cascade of signaling events, leading to release of immune cell attractants (eg, chemokines) and survival and maturation factors (eg, cytokines). Chemokines recruit immune cells such as granulocytes from the blood into the affected tissue, where they exert their effector function [162].

In parallel, cytokines with pleiotropic functions stimulate immune cells locally, and, on insufficient local control of the infection, increase to reach relevant systemic levels, ultimately enhancing production of myelomonocytic cells from myeloid committed precursors in the BM to replenish consumed peripheral cells [163]. In locally noncontrolled infection, a large number of soluble factors are rapidly increased in serum and are able to act at hematopoietic sites in BM. These factors include (1) conserved infectious agent products or "patterns" (eg, bacterial- or fungal-specific membrane products, or species-specific DNA and RNA motifs); (2) cytokines (eg, colony-stimulating factors, interleukins, interferons); and (3) mobility factors (eg, chemokines). The most prominent examples are myeloid differentiation enhancing cytokines such as granulocyte- CSF (G-CSF), M-CSF, and granulocyte-macrophage-CSF (GM-CSF), as well as early acting cytokines as IL-3, IL-6, and Flt3 ligand [164]. It has been demonstrated that in systemic infection those cytokines are released, reaching serum concentrations up to hundred-fold above steady-state levels in both mice and humans [165].

To react to any of these, responsive cells need to express the respective receptors. Different studies show that hematopoietic stem and progenitor cell (HSPC) expressed growth factor receptors, PRRs, and mobility receptors.

Some of these receptors have been used to identify and characterize the HSPCs themselves, such as the receptor for stem cell factor (c-Kit), stem cell antigen-1

(Sca-1), CD34, fetal liver kinase-2 (Flk2; also called Flt3), macrophage-colony–stimulating factor (M-CSF) receptor and the IL-7 receptor α (IL-7Rα) [166,167].
It is thus important to note that these receptor expression patterns might be altered during inflammatory challenges, and steady-state defined phenotypes of HSPCs might thus not apply in these situations. Altered expression of IL-7Rα, a receptor critical for lymphoid cell commitment, has been observed in experimental malaria infection [168]. Lineage marker negative (lin^-) c-KithiIL-7Rα+ cells, which do not exist in steady-state hematopoiesis and have similar myeloid and lymphoid potential to MMPs, are induced in response to infection via IFN-γ and contribute to host defense by dominant myeloid cell production [168].
Similarly, under pathologic conditions such as infection and cancer, some low-level myeloid colony-forming activity has been unexpectedly detected in $Gr\text{-}1^-CD11b^-$ cells that, in steady-state, represent both immature as well as fully differentiated granulocytes [169]. On the basis of their inhibitory effect on T-cell function, they operationally have been defined as myeloid-derived suppressor cells or activated immature myeloid cells, although their origin, developmental pathways, and existence of putative subsets remains to be determined [169].
In addition, it has been demonstrated by many studies that Sca-1, a glycosyl phosphatidylinositol-anchored protein, originally defined as a lymphocyte activation marker because of its up-regulation on T-cell activation [170] and now frequently used to define HSPCs [171], is up-regulated on systemic infectious or noninfectious inflammation [172]. Collectively, the expression of a variety of cytokine and PRRs on HSPCs suggests an active role on modulation of early hematopoiesis during systemic infection and inflammation.

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CHAPTER 2

Outline of the thesis

The hematolymphoid system is a paradigmatic, somatic stem cell–maintained organ with enormous cellular turnover, that is, the continuous de novo generation and subsequent death of cells. It is estimated that, in a 70-kg adult human, approximately 3×10^5 erythrocytes and 3×10^4 white blood cells are produced per second in steady-state ("steady-state hematopoiesis"), and it is documented that during specific demand, for example, on blood loss, infectious challenges and cancer, cellular production can increase several-fold over baseline levels ("emergency hematopoiesis"). While emergency myelopoiesis to infection or trauma is characterized by systemic signs such as blood leukocytosis, neutrophilia, the emergence of immature neutrophils, and increased production of myelomonocytic cells in bone marrow, chronic cancer inflammation-driven myelopoiesis converges in splenic accumulation of immature myeloid cells (MDSCs) and macrophages (TAMs) recruitment at the tumor site.

While the pro-tumor functions of MDSCs and TAMs are well characterized, a large gap remains in our understanding of the mechanisms that translate persistent inflammation into reactive emergency myelopoiesis.

Among these, the IL-17 axis is becoming of great interest, as in response to inflammation or infection it supports G-CSF mediated emergency granulopoiesis and promotes neutrophil apoptosis and macrophage phagocytosis of early apoptotic neutrophils, a crucial mechanism for IL-10 induction in macrophages and resolution of inflammation. It was shown that IL-17 is required for the development of MDSCs in tumor-bearing mice. A defect in IL-17R reduces the number of MDSCs in the blood, spleen, and tumors. This is further supported by data showing that the administration of exogenous IL-17 increases the number of MDSCs in wild-type tumor-bearing mice, whereas neutralization of IL-17 in wild-type tumor-bearing mice reduced the number of MDSCs.

Therefore, TAMs and MDSCs produce large amounts of the TH17 cell-driving cytokines TGFbeta and IL-6.
Based on this, we hypothesized that IL-17 and the retinoic acid orphan nuclear receptor gamma (RORγ) might be involved in mediating the fate and functional polarization of myeloid components that arise during emergency myelopoiesis.

We identified in tumor-bearers mice subsets of myeloid-derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs) that express RORC1, strikingly in response to G-CSF- and GM-CSF-driven "emergency" hematopoiesis.
To evaluate the functional relevance of RORC-expressing myeloid cells to tumor progression, we studied tumor models in chimeric mice (Wt/RORC$^{-/-}$), obtained by transplanting bone marrow of donor RORC-deficient mice in lethally irradiated C57BL/6 Wt recipient mice. This model was useful to evaluate the role of RORC/IL-17A axis in MDSCs expansion, TAMs differentiation and cells distribution in spleen and tumors. We also characterized the role of RORC in the control of MDSCs survival and suppressive capacity. As polarized inflammation plays a key role in cancer development, we analysed the expression of prototypical M1 and M2 cytokine genes in TAMs and MDSCs.

To evaluate the role of the RORC/IL-17A signature in the control of myeloid precursor differentiation and maturation first we determined RORC and IL-17A protein expression in early myeloid progenitors and immature myeloid cells from both bone marrow and spleen. Then, we analysed the role of RORC in progenitor commitment, screening bone marrow and spleen for common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs) and megacaryocyte/erythroid progenitors (MEPs).

To shed light on the molecular signalling networks of RORC-driven emergency granulo–myelopoiesis, we evaluated the expression of positive and negative transcriptional regulators in BM and spleen from tumor-bearing mice.
We focused on cEBPbeta, known as a major positive regulator of G-CSF- and GM-CSF- driven emergency granulo-myelopoiesis, and IRF8, known as regulator that mediates rapid cell-fate switching of the common GMP to compensate for increased needs of components of the mononuclear-phagocyte system (MPS) to persistent inflammation.

Tissue resident macrophage population plays a key role in maintaining tissue neutrophil homeostasis in the steady state and in settings of acute inflammation. However, regulation of neutrophil homeostasis by tissue macrophages in cancer related inflammation has not been demonstrated to date. To evaluate the role of this cross-talk on MPS system homeostasis and tumor progression, we analized myeloid cell accumulation and distribution in tumor chimeric mice after macrophages depletion.

The present study clarifies the role of innate IL-17 and RORC immune responses in cancer-related inflammation. We provide the first evidence of a critical role for RORC expression in the transcriptional control and development of MDSCs subsets and TAMs. Further, our findings infer that RORC-driven emergency granulo-myelopoiesis is a tightly controlled and programmed response to an inflammatory tumor environment and is critical for maintaining tissue homeostasis.

CHAPTER 3

New pathways of protumor “emergency“ granulo/monocytopoiesis

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Abstract

Inflammation promotes G-CSF (Granulocyte colony-stimulating factor) and GM-CSF (Granulocyte-macrophage colony-stimulating factor) mediated "emergency" myelopoiesis to ensure supply of immune cells to increased demand. Cancer-driven myelopoiesis stimulates expansion of protumor myeloid-populations, including myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). We identified novel subsets of retinoic acid orphan nuclear receptor gamma (RORC1) expressing MDSCs and TAMs in human and mouse tumor bearers. RORC1 orchestrated myelopoiesis by suppressing negative (SOCS3, Bcl3) and promoting positive (C/EBPβ) regulators of granulopoiesis, while enhancing expression of the monopoiesis-inducing transcription factor IRF8. RORC1 supported protumor innate responses by protecting MDSCs from apoptosis, mediating M2 polarization of TAMs and limiting tumor infiltration by effector neutrophils.

Accordingly, ablation of RORC1 in the hematopoietic compartment prevented cancer-driven myelopoiesis, event that correlated with inhibition of tumor growth and metastasis. Our findings identify RORC1 as a key regulator of myeloid lineage commitment and function in cancer.

Introduction

Immunologic stress, such as infection and cancer, modifies the magnitude and composition of the hematopoietic output, a feature of immunoregulation defined as "emergency" hematopoiesis, to guaranty proper supply and function of immune cells to increased demand [1]. Tumors can reprogram myeloid cell differentiation and commitment to create permissive conditions needed for disease progression [2]. However, the molecular pathways guiding cancer-driven "emergency" myelopoiesis remain largely unknown. G-CSF and GM-CSF were reported to drive "emergency" myelopoiesis by securing supply of neutrophils and macrophages from bone marrow and extra-medullary hematopoietic stem cell niches (HSCs) [1, 3]. Newer studies reveal that monocytic (M-) and granulocytic (PMN-) MDSCs and TAMs, the two major myeloid populations associated with cancer development [2], differentiate from a common myeloid progenitor (CMP) into functionally altered myeloid cells [4]. Macrophage colony-stimulating factor (M-CSF), promotes macrophage differentiation from marrow precursors cells and is required for differentiation and expansion of tissue macrophages involved in tissue homeostasis [5] and tumor progression [6]. It is becoming evident that reciprocal regulation of macrophage versus neutrophil/granulocyte differentiation might critically control tissue homeostasis. Depletion of tissue macrophages lead to exacerbated G-CSF-mediated granulopoiesis/neutrophilia [7, 8] and studies support the role of tissue macrophages as regulators of HSCs niche homeostasis [9, 10]. Thus, a better understanding of the molecular networks that dictate polymorphonuclear versus mononuclear lineage fates as well as their functional programming during "emergency" myelopoiesis is mandatory to clarify the mechanisms controlling tissue homeostasis

Investigation of signaling pathways controlling hematopoiesis revealed that G-CSF-induced "emergency" granulopoiesis is mediated through the transcription factors c-EBPβ [11] and STAT3 [12], whereas M-CSF supports monocyte differentiation through activation of the transcription factors PU.1 and IRF8 [13]. Of relevance, in stress conditions IL-17A promotes G-CSF- and stem cell factor-mediated neutrophilia/granulopoiesis [14]. Despite IL-17 expression in tumor bearing men and mice has been so far greatly restricted to the adaptive arm of immunity [15], TAMs and MDSCs produce large amounts of the Th17 cell driving cytokines TGFβ and IL-6 [16], suggesting that adaptive and innate immunity share IL-17-related molecular signaling pathways. Indeed, IL-17 expressing cells with macrophage morphology have been described in patients with breast cancer [17] and IL-17-induced recruitment of MDSCs in gastric cancer [18].

Despite the reported prevalence of IL-17 expressing cells in tumor bearers, its role in cancer remains controversial, Th17 cells have been reported to promote [19] or inhibit [20] cancer development. Whereas the Th17 immune response is controlled by the orphan nuclear receptor RORC full length protein (RORC1) and the RORC-t splice variant (RORC2) [21], the signaling pathways that drive functional differentiation of IL-17^{+} innate immune cells have been poorly investigated to date. In subjects with arthritis mast cells express a dual IL-17A/RORC finger print in response to TLR4 ligands [22] 1 and a population of RORγt-expressing neutrophils that express IL-17 in response to IL-6 and IL-23 was recently identified in a model of fungal infection [23]. These findings suggest a major role for IL-17 and related signaling cytokines and transcription factors in myeloid lineage commitment and function in inflammatory diseases, including cancer.

The present study identifies RORC1 as a major player in cancer-driven myelopoiesis. In particular, we demonstrate that RORC dampens the supply of anti-tumor effector neutrophils and macrophages by re-directing myeloid lineage commitment to expansion of "M2"-like pro-tumor MDSCs and TAMs subsets.

Materials and Methods

Ethics Statement

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); 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. Humanitas Clinical and Research Center Institutional Regulations and Policies providing internal authorization for persons conducting animal experiments. Mice have been monitored daily and euthanized when displaying excessive discomfort.

Animals

C57BL/6 mice were purchased from Charles River (Calco, Italy).

Homozygous RORC mutant mice (B6.129P2(Cg)-$Rorc^{tm1Litt}$/J) [32] were kindly donated by Prof. Dr. Dan Littman (New York University) and Prof. Dr. Sanjiv Luther (University of Lausanne). The presence of the RORC mutant transgene has been checked by PCR on tail DNA as previously described (Dan Littman Lab homepage:http://www.med.nyu.edu/skirballlab/littmanlab//genotyping_protocols/ Rorgko.pdf). For additional information about the homozygous RORC mutant mice strain see Sun et al. [32]. RORC mutant mice were bred and maintained at the Humanitas Clinical and Research Center (Rozzano, Italy).

Male BALB/NeuT mice were described elsewhere [25,26], and were mated with C57BL/6 females to obtain the F1 CxB6NeuT strain.

The presence of the HER2/neu transgene has been checked by PCR on tail DNA as previously described [26]. The CxB6NeuT strain was bred and maintained at the Fondazione IRCCS Istituto Nazionale Tumori (Milan, Italy). IL-17Ako mice were kindly donated by Prof. Dr. Burkhard Becher (University of Zuerich, CH) and Prof. Dr. Y. Iwakura (University of Tokyo).

Bone marrow Transplantation (BMT)

Eight-week-old male mice were lethally γ-irradiated with two dose of 475 cGy and BMT performed as previously described [27]. RORC deficient ($RORC^{-/-}$) CD45.2 $(Ly5.2)^{+}$ and $RORC^{+/+}$ BM ($RORC^{+/+}$) CD45.2 $(Ly5.2)^{+}$ was transferred to Wt male C57BL/6 or Wt male CxB6NeuT CD45.1 $(Ly5.1)^{+}$. In addition, IL-$17A^{-/-}$ BM and IL-17A expressing BM, both $(Ly5.2)^{+}$, were transferred to $RORC^{+/+}$ male CxB6NeuT CD45.1 $(Ly5.1)^{+}$. BMT engraftment was checked by staining with Percp-conjugated CD45.1 antibody and PE-conjugated CD45.2 antibody (BD Biosciences) and subsequent FACs analysis. At 10-12 weeks after BMT, 10^{5} MN/MCA1 tumor cells were injected i.m. in the left hind limb of chimeric mice. The tumor reached usually palpable size at day 14 post tumor cell injection and tumor size was monitored three times a week until the day of sacrifice. Experimental fibrosarcomas were induced by a single subcutaneous dose injection of methylcholanthrene in corn oil (200 ug) at week 10-12 after BMT. 12 weeks post methylcholanthrene injection 40-50 % of the mice developed a fibrosarcoma. Tumor growth was monitored 3 times a week starting from week 8 post methylcholanthrene injection. Breast carcinoma development was monitored 3 times a week, 6-8 weeks after BMT of $RORC^{-/-}$ and $RORC^{+/+}$ BM to CxB6NeuT mice.

Cell Culture and Reagents

TAMs were obtained 3-4 weeks after tumor cell injection and isolated and identified as previously described [28]. Thioglycollate-elicidited peritoneal exudate cells (PEC) were isolated from healthy or tumor bearing mice as previously described [28]. PECs and TAMs were incubated in RPMI 1640 containing 10% FCS, 2 mmol/L glutamine and 100 units/mL penicillin-streptomycin.

BM MDSCs were generated from Bone marrow (BM) cells isolated from naïve Wt or RORC$^{-/-}$ mice. BM cells were cultured in the presence of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) (40 ng/mL), granulocyte colony-stimulating factor (G-CSF) (40 ng/mL) and interleukin-6 (IL-6) (20 ng/mL) for up to 5-6 days as previously described [24]. Spleen-derived MDSCs of Wt/Wt and Wt/RORC$^{-/-}$ mice were isolated by depletion of CD19 and CD11c positive populations followed by sequential positive selection with biotinylated Ly6G^{+} (PMN-MDSCs) and CD11b^{+}Ly6C (M-MDSCs) beads according to the manufacturer's instructions (Miltenyi Biotech). The purity of both BM-MDSCs and spleen-derived M-MDSC (CD11b^{+}Ly6C^{+}Ly6G^{low}),
PMN-MDSCs (CD11b^{+}Ly6G^{+}Ly6C^{low}) populations was >90%, as determined by flow cytometry, and the viability as determined by AnxV- binding (Immunostep) was >95% for Wt/Wt MDSCs subsets, whereas in contrast MDSCs isolated form Wt/Rorc$^{-/-}$ animals showed a decreased viability (as stated in the text).

BM-MDSCs were cultured in RPMI 1640 containing 5% FCS, 2 mmol/L glutamine, 100 units/mL penicillin-streptomycin, 10 mM Hepes and
20 uM beta-mercaptoethanol and M-MDSCs and PMN-MDSCs subsets were cultured in DMEM containing 5% FCS, 2 mmol/L glutamine, 100 units/mL penicillin-streptomycin, 10 mM Hepes and 20 uM beta-mercaptoethanol.

For the different *in vitro* treatments (as stated in the text), we used the following concentrations: 100 ng/mL LPS (LPS from *Salmonella* Abortus, Equi S-form; Alexis), 200 U/mL IFN-g (Peprotech) , LPS (100 ng/mL) + IFN-γ (200 U/mL), GM-CSF (40 ng/mL) + G-CSF (40 ng/mL) (Peprotech), IL-1β(40 ng/mL) (Peprotech) or TSNT obtained from MN/MCA1 harvested from Wt tumor bearers (diluted 1:1 in culture media).

Lineage cell separation from BM

Untouched Isolation of lineage–negative cells (depleted from mature hematopoietic cells such as T cells, B cells, monocytes/macrophages, granulocytes and erythrocytes) from BM harvested from naïve Wt or $RORC^{-/-}$ mice was performed by using the murine lineage cell separation kit from Miltenyi (130-090-858) as indicated by the manufacturers protocol.

Separated lineage–negative progenitor cells were cultured in the presence of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) (40 ng/mL) (Peprotech), granulocyte colony-stimulating factor (G-CSF) (40 ng/mL) (Peprotech), IL-1β (20 ng/mL) (Peprotech) or culture media alone for up to 7-10 days. The arisement and prevalence of $ckit^{+}$ macrophage/monocyte and granulocyte progenitors was analysed by FACS analysis using the following surface antibodies: CD117 (ckit)- , CD11b-. Ly6C-, Ly6G-, Gr1-, F4/80-.

In vivo treatments

Wt/Wt and Wt/$RORC^{-/-}$ tumor free and MN/MCA1-bearing chimeric mice were treated with the following drugs starting from day 14 post tumor cell injection when the tumor became palpable: RORγ Antagonist (SR1555 Cayman Chemical) and RORα/γ Agonist (SR1078, Calbiochem) were administered at the dose of 10 mg/kg ip four times a week for 2 weeks, control mice received PBS injections;

CSFR1 antagonist (Roche) or isotype Ab were administered at an initial dose of 60 mg/kg followed by two doses of 30 mg/kg two times a week for a total of 2 weeks; Anti-GCSF MAB 414 (R&D Systems), anti- GM-CSF 415NA (R&D Systems) were administered 10 mg/mouse ip daily for a total of 2 weeks; control mice received PBS injections.

Patients

10 patients (40 yr old) with T2 or T3 CRC were enrolled in the study after signing Cancer Research Center Humanitas IRB-approved consent.

Patients did not receive radiation or chemotherapy before sample collection. Peripheral blood was collected at the time of surgery from all patients.

All blood samples were analyzed within 3 hrs after collection by FACS-analysis (see section Flow Cytometry).

Myxed leukocyte reaction (MLR)

M-MDSCs ($CD11b^+Ly6C^+Ly6G^{low}$) populations were isolated from MN/MCA1-bearing Wt/Wt or Wt/$RORC^{-/-}$ chimeric mice by Miltenyi bead separation as described above and stimulated with IFN-γ (200 U/mL) or kept in culture media alone for 72 hrs. Supernatants were harvested at 24, 48 and 72 hrs of *in vitro* stimulation for NO determination. Splenocytes were mechanically harvested from the spleens of naïve Wt OT-1 mice and added to M-MDSCs post 72 hrs of *in vitro* stimulation with IFN-γ. Briefly, M-MDSCs were mixed with a constant number of OT-1 splenic cells at a 1:1 to 1:16 ratio in a 96-well plate ($2x10^5$ OT-1 splenocytes/well). Cells were incubated in DMEM containing 5% FCS, 2 mmol/L glutamine, 100 units/mL penicillin-streptomycin, 10 mM Hepes and 20 uM beta-mercaptoethanol and pulsed with ovalbumin peptide ($OVA_{257–264}$) (250 μg/mL).

As stimulation controls OT-1 splenocytes alone were pulsed with OVA peptide (250 µg/mL), ConA (5 µg/mL) or kept in culture media. Post 48 hrs of *in vitro* stimulation with OVA peptide, cells were pulsed with [^{3}H]-thymidine (1 µCi/well) for 16 to 18 hours and read on a β-plate reader (Perkin Elmer).
Percentage of suppression was calculated as follows: 100 − [(CPM of OT-1 splenocytes + MDSCs)/(CPM of OT1 splenocytes) × 100].

Flow cytometry

Fluorescence activated cell sorting (FACS) analysis was performed on mouse blood, spleen, BM and MN/MCA1 or isolated TAMs, MDSCs subsets and *in vitro* generated BM-MDSCs. Surface immune phenotyping was performed using anti-mouse CD45-PerCP, CD11b-PE, FITC or -APC, Gr1-PE or-APC, F4/80-PE or -APC, CD115 (CSFR1)-PE and C5aR-PE, all from BD Biosciences (San Diego, CA). Ly6G-FITC and Ly6C-FITC or –PE were from Miltenyi Biotech (Teterow, Germany). FCII/IIIR –PE or –APC (CD16/CD32) were from Bio Legend (San Diego, SA). Lineage markers (PE-CD3, -CD4, -CD8, -Mac-1, -Gr-1, -Ter119, and -B220) were from (BD) and antibodies for hematopoietic progenitor phenotyping (Sca-1, c-Kit (CD117), IL7Rα, CD34 and CD16/32) were purchased from (BD). Post surface staining cells were treated with Fix/Perm buffer to stabilize the cell membrane and afterwards permeabilized with Perm buffer, both components of the eBioscience Foxp3-staining buffer set (cat. 00-5523).
Post pemeabilization cells were stained with anti-mouse/human RORC-APC, anti-mouse IL-17A-APC or anti-mouse TNFa-Alexa648, all from eBioscience (San Diego, CA). Anti-mouse IFNγ-PE was from BD Bioscience (San Diego, CA). CEBP/beta un- conjugated was from Millipore (Billerica, Massachusetts) and a secondary goat anti rabbit Alexa Fluor® 488 conjugated antibody was used (Invitrogen, Molecular Probes, Carlsbad, CA).

IRF8 un-conjugated antibody was from (BD) and a secondary goat anti rabbit Alexa Fluor® 488 conjugated antibody was used (Invitrogen, Molecular Probes, Carlsbad, CA).

Human blood samples were surface stained using anti-human CD14-PE, HLADR-Pacific Blue and CD15-FITC, all from BD Bioscience (San Diego, CA). Intracellular staining was performed using the eBioscience Foxp3-staining buffer (cat. 00-5523) and anti-mouse/human RORC-APC antibody from eBioscience and anti-human IL-17A-PE antibody from BioLegend (San Diego, CA) .

Real-Time PCR

Total RNA was reverse-transcribed by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) as previously described [29] , amplified using Fast Syber Green Master Mix (Applied Biosystems), and detected by the 7900HT Fast Real-Time System (Applied Biosystems). The sequences of gene-specific primers are available upon request. Data were processed using SDS2.2.2 software (Applied Biosystems). Results were normalized to the expression of the housekeeping gene β-actin and then expressed as fold up-regulation with respect to the control cell population.

Enzyme linked immunosorbent assay (ELISA)

MN/MCA1 Supernatants were tested in sandwich ELISA (R&D Systems).

Tumor cell-free supernatants were tested for the indicated cytokines/growth factors: Murine TNFα, GM-CSF, G-CSF, M-CSF, IL-1b, IL-17A and VEGFA (Duoset Elisa kit, R&D).

Determination of nitric oxide (NO) production

NO was measured by Griess reaction as the amount of NO^{-2} produced, using a nitrate/nitrite assay kit (Griess Reagent System, Promega).

Confocal Microscopy Analysis

Cells were seeded on Poly-L-Lysine (Sigma-Aldrich) coated sterile rounded glasses at $2x10^5$ cells/ml in medium and fixed with 4% PFA for 10 minutes at room temperature. Cell permeabilization was obtained after 1 hour incubation with PBS 0.1% Triton-X100 (Sigma-Aldrich) plus 5% normal goat serum (Dako Cytomation, Carpinteria, CA USA) and 2% BSA, (Amersham Biosciences, Piscataway Township, NJ USA). Cells were then incubated with rat anti-mouse F4/80 monoclonal antibody (2 g/ml; IgG2b, MCA497G AbD Serotec) or anti-mouse CD115 monoclonal antibody (2g/mL, IgG2a, BD Bioscience) and with hamster anti-mouse CD31 monoclonal antibody (2 g/ml; IgG, MAB 1398Z, Millipore) in PBS 0.05% Tween 20 for 1 hour at room temperature. Irrelevant mouse IgG and IgG2b (2 g/ml) were used as control. A secondary goat anti hamster IgG Alexa® Fluor 488 conjugated (2 g/ml) or goat anti rat AlexaFluor® 647 conjugated (2 g/ml) were used (Invitrogen, Molecular Probes, Carlsbad, CA USA).

In each step cells were extensively washed with 0.05% Tween 20 in PBS (pH 7.4). For DNA detection DAPI (Invitrogen, Molecular Probes) was used.

Samples were mounted with FluorPreserve Reagent (Calbiochem San Diego, CA USA) and analyzed with an Olympus Fluoview FV1000 laser scanning confocal microscope operating with lasers with 405, 488, 594 and 647 nm excitations.

The resulting fluorescence emission was collected using a 460-to-490 nm (for DAPI), 500-to-550 nm (for Alexa® Fluor 488), 570-680 nm (for Alexa® Fluor 594) and 620-750 nm (for Alexa® Fluor 647) band-pass filters.

Samples were imaged with an oil immersion objective (40×1.30 NA Plan-Apochromat; Olympus).

Statistics

Statistical significance was determined by one-tailed Student's *t* test with *n* 3.

**P* 0.05; ***P* 0.01; ****P* 0.001.

Results

Divergent RORC/IL-17A fingerprint in tumor-associated myeloid cells

IL-17A supports G-CSF-driven "emergency" myelopoiesis [30] and the cytokine expressing myeloid cells have been reported in inflammation [23,24 , 23].

To clarify the role of IL-17A^{+} innate immune cells in tumor progression, we screened the myeloid compartment of fibrosarcoma (MN/MCA1) bearing C57BL/6 mice for IL-17A expression. A tumor volume of 1-1,5 cm^{3} and few lung metastasis (<5) at day 23 post tumor cell injection, was defined as early stage disease (ED), whereas tumors larger than 2 cm^{3} and a high number of lung metastasis (>15) (day 25-28) was defined as advanced stage disease (AD) (Figure S1). While in AD few blood and spleen CD45^{+}CD3^{+} T cells expressed IL-17A, CD45^{+}CD3^{neg} cells expressed significant levels of IL-17 (Figure 1A). Few CD45^{+}CD3^{neg} cells expressed IL-17 in NT and ED, suggesting IL-17 as a novel regulating feature of advanced cancer inflammation. FACS-analysis of the CD45^{+}CD3^{neg} cell pool confirmed that splenic Gr1^{+}CD11b^{+} MDSCs represent the major myeloid population in AD bearing mice (Figure 1B). Analysing the composition of the splenic myeloid compartment, we observed a dramatic increase of polymorphonuclear CD11b^{+}Gr1^{+}Ly6G^{hi}Ly6C^{low} (PMN-MDSCs) and a milder, but significant, increase of m monocytic CD11b^{+}Gr1^{+}Ly6C^{hi}Ly6G^{low} (M-MDSCs) and CD11b^{+}F4/80^{+} macrophages (Figure 1B).

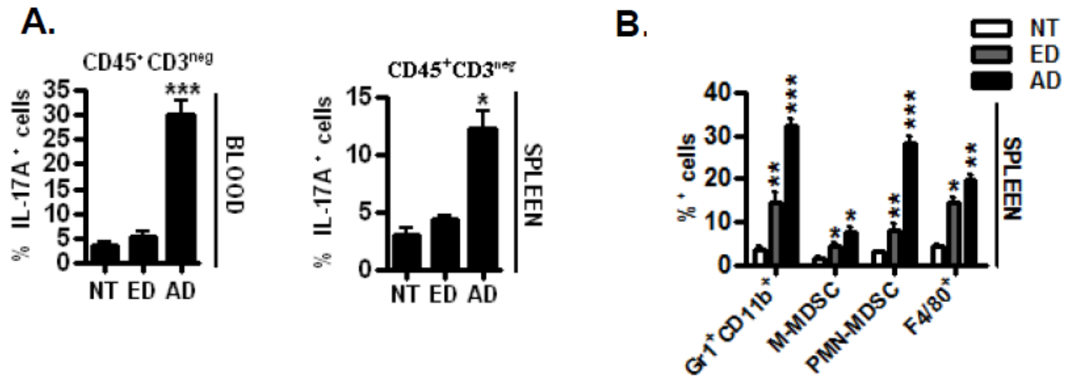

Figure 1- A) IL-17A is expressed by different cell subsets in tumor microenvironment.

The represented data are mean percentages ± SD of $CD45^+IL\text{-}17\ A^+$ cells in fresh blood and spleen from tumor free (NT) or MN/MCA1-bearing wt mice. Tumor bearing animals were divided into 2 groups as stated in the text: animals with early (ED) and advanced tumor disease (AD).

B) $Gr1^+CD11b^+$ are the major myeloid subset in the spleen from tumor bearing mice.

The represented data are mean percentages ± SD of $Gr1^+CD11b^+$, $CD11b^+Ly6C^+$ (M-MDSCs) $CD11b^+Ly6G^+$ (PMN-MDSCs) and $CD11b^+F4/80^+$ cells in the spleen from tumor free or MN/MCA1-bearing wt animals. Mean ± SD of 6 independent experiments are shown.

To determine the myeloid growth factor/s that drive “emergency” granulo-monocytopoiesis in cancer, MN/MCA1 bearing mice were treated with neutralizing antibodies to G-CSF,GM-CSF or the M-CSF receptor (M-CSFR) CSFR1[5].

As shown (Figure 2A)**,** inhibition of G-CSF and GM-CSF limited the accumulation of splenic PMN-MDSC with no effect on M-MDSCs. While the anti-CSFR1 antibody decreased the number of $F4/80^+$ spleen macrophages, surprisingly the number of splenic PMN-MDSCs dramatically increased. Further, the anti-G-CSF antibody significantly increased the $CD11b^+F4/80^+$ spleen macrophages.

Confirming the relevance of myelopoiesis to cancer inflammation *in vivo*, neutralization of either GM-, G-CSF or M-CSFR resulted in inhibition of tumor growth (Figure 2B) and metastasis (Figure 2C).

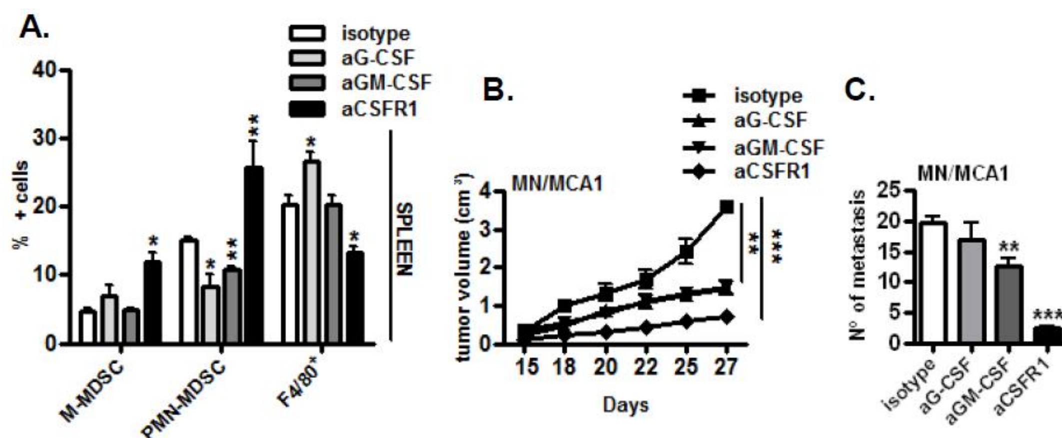

Figure 2- Colony growth factors are required for both tumor-driven "emergency" myelopoiesis and tumor progression. **A)**: The represented data are mean percentages ± SD of M-MDSCs, PMN-MDSCs, F4/80^{+} and F4/80^{+} cells in the spleen of MN/MCA1-bearing animals with AD, measured within the CD45^{+} gate. Tumor bearing mice were treated with antagonist antibody to G-CSF, GM-CSF, M-CSFR (CSF1R) or isotype control antibody, starting from day 14 post tumor cell injection when tumors became palpable. Treatment regimens are stated in the text. **B)**: Tumor growth in Wt C57BL/6 mice grafted with MN/MCA1 cells and treated with antagonist antibodies to G-CSF, GM-CSF, M-CSFR (CSF1R). **C)**: Average number of lung metastases at the day of sacrifice. Mean ± SD of 1 representative experiment with n=6 mice/experimental group are shown.

Our findings strongly suggest a reciprocal antagonic regulation of the polymorphonuclear and monocytic lineages in CSFs driven "emergency" myelopoiesis in cancer.

Next, we evaluated the expression of IL-17A in cancer-associated myeloid subsets. IL-17A was selectively expressed by PMN-MDSCs in blood and spleen and significantly increased in AD (Figure 3A). In contrast, IL-17A was poorly or not expressed by M-MDSC, CD11b^{+}F4/80^{+} macrophages or CD11b^{neg}CD117^{+} mast cells (data not shown).

Despite PMN-MDSCs from AD tumor bearing mice expressed endogenous IL-17A, these cells failed to release IL-17 in response to degranulating signals as CD16/32 cross-linking antibody and C5a (data not shown) [31].

RORγ (RORC1) and its splice variant RORγt (RORC2) are master regulator for IL-17A gene transcription in Th17 cells[32], innate lymphocytes[33], γδ T [34] and NKT cells [35]. Hence, we tested by FACS-analysis whereas IL-17 expression in myeloid cells was associated with RORC expression. In accordance, the majority of blood and spleen PMN-MDSCs from AD mice expressed RORC, while its expression was restricted to a minor subset of IL-17A^{neg} M-MDSCs (Figure 3B). Noteworthy, RORC was highly expressed by splenic IL-17A^{neg}CD11b^{+}F4/80^{+} macrophages, in particular in AD (Figure 3C). Of relevance, RORC was expressed by the majority of F4/80^{+} TAM (≥90%), and thyoglicollate-elicited peritoneal macrophages (PEC) from both, tumor free (NT-PEC) and tumor bearing mice (T-PEC) (Figure 3D).

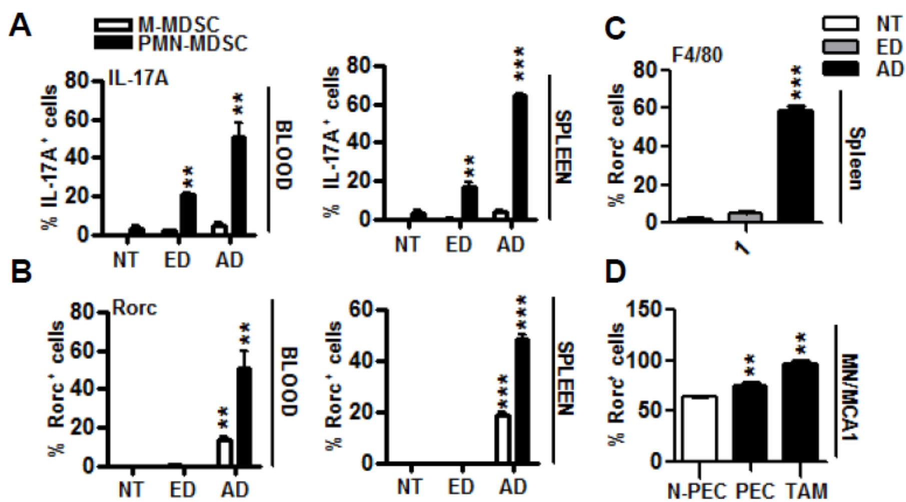

Figure 3- The IL-17A/RORC signature is expressed by myeloid subsets that arise during cancer-driven "emergency" myelopoiesis. The represented data are mean percentages ± SD of: PMN-MDSCs IL-17A^{+} and M-MDSCs IL-17^{+} (**A,B**), F4/80^{+}RORC^{+} cells (**C**), either detected in fresh blood or spleens. Mean ± SD of 6 independent experiments in tumor free or MN/MCA1

bearing Wt C57BL/6 (ED and AD) mice are shown. **D)**: FACS-analysis for RORC expression in PECs (from NT and MN/MCA1 bearing mice) and TAMs isolated from MN/MCA1 are shown**.**

Few M-MDSCs and PMN-MDSCs were detected at the tumor site, both of which expressed RORC in the absence of IL-17A (data not shown).

We report for the first time RORC expression unlinked from IL-17A by the monocytic lineage that emerges from tumor-associated myelopoiesis.

The expression of RORC or IL-17A by MDSCs in human cancer patients has not been reported to date. To fill this gap, blood samples obtained from healthy donors (HD) (n=10) or patients with advanced colorectal cancer (CRC) (stage II/III) (n=10) were analysed. Noteworthy, $RORC^{+}$ blood M-MDSC ($CD14^{+}HLA\text{-}DRA^{neg}$) and PMN-MDSC ($CD15^{+}HLA\text{-}DRA^{neg}$) increased significantly in CRC patients (Figure S2A). Similar to mice (Figure 3B), RORC expression dominated in the PMN-MDSC subset (Figure S2B). In contrast, IL-17A was neither detected in human blood M-MDSCs nor PMN-MDSCs (data not shown).

These results together with our findings in mice indicate that RORC expression by myeloid subsets constitutes a hallmark of pro-tumor “emergency” myelopoiesis. ELISA of tumor supernatants (TSN) harvested from AD mice revealed the presence of growth factors (G-CSF, GM-CSF, M-CSF, VEGF) and cytokines (IL-1β, TNFα) implicated in differentiation of myeloid lineages (Figure S3).

To identify factor/s that might condition myeloid cells to a RORC/IL-17 finger print in cancer, BM-derived MDSC (BM-MDSCs) or PEC from tumor free Wt mice were cultured for 48 hrs with either combination of GM-CSF and G-CSF or the MN/MCA1 supernatant. Both treatments induced significant IL-17A expression in BM-derived PMN-MDSCs, as opposed to minor expression in M-MDSCs (Figure S4A). BM-derived M-MDSC and PMN-MDSCs expressed basal RORC levels (Figure S4B), plausibly induced by GM-CSF and G-CSF used for their *in vitro* generation [11].

Basal RORC expression was significantly increased by combination of GM-CSF/G-CSF in BM-derived PMN- and M-MDSCs, while the TSN had a milder effect on RORC induction (Figure S4B).
Confocal microcopy demonstrated that RORC expression and nuclear translocation was induced in naive PEC challenged for 72 hrs with either GM-CSF/G-CSF, IL-1β or LPS (Figure S4C). In contrast, IFNγ failed to induce RORC expression and nuclear translocation.
Finally, we determined the mRNA levels for RORC1 and the RORC splice variant Rorγt (RORC2) [32,36] in PEC stimulated for 72 hrs with LPS and in thymocytes[31] from healthy Wt mice (Figure S4D). Noteworthy, RORC1 mRNA levels in LPS-stimulated PEC equaled RORC1 mRNA expression in Wt thymocytes, while RORC2 was not expressed by PEC. In summary, these data indicate that in cancer the monocyte-macrophage lineage expresses a RORC1 fingerprint unlinked from IL-17A, whereas the granulocyte/neutrophil lineage expresses a dual RORC/IL-17A signature.

RORC regulates MDSCs differentiation and survival

To evaluate the *in vivo* relevance of RORC expressing myeloid cells in cancer, we generated BM chimeras transplanting donor RORC-deficient BM cells into lethally irradiated C57BL/6 Wt recipients mice (RORC$^{-/-}$>Wt), to be compared to Wt>Wt mice. Tumor growth (Figure 4A) and metastasis (Figure 4B) were significantly reduced in RORC$^{-/-}$ >Wt in comparison to Wt>Wt chimeras. The effect of RORC deficiency in bone marrow cells on tumor progression was tested in two additional tumor models. RORC$^{-/-}$ BM was transplanted into HER2/neuT (NeuT) transgenic mice, a model of spontaneous mammary carcinoma [38] and into mice that were subsequently exposed to methylcholanthrene-induced carcinogenesis (fibrosarcoma) [39]. Consistently, tumor growth in both tumor models was significantly inhibited in RORC$^{-/-}$>Wt chimeras (Figure 4C,D).

FACS-analysis confirmed the IL-17/RORC expression pattern observed in the MN/MCA1 model (Figure 1). Briefly, in both additional tumor models IL-17A expression was restricted to PMN-MDSC, whereas M-MDSC, PMN-MDSC and $CD11b^{+}F480^{hi}$ macrophages expressed RORC (data not shown).

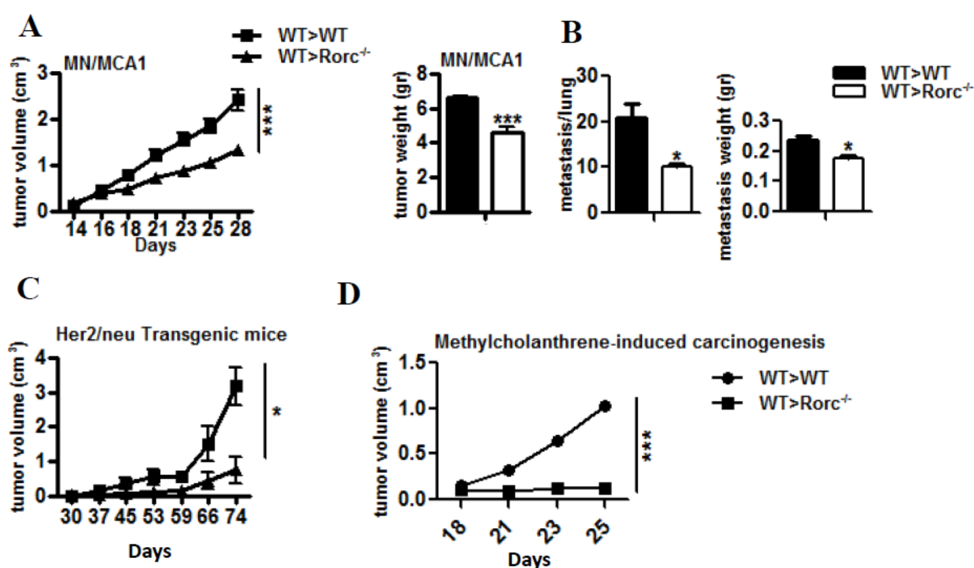

Figure 4- Tumor progression is reduced in RORC-deficient tumor-bearers mice.

A) Tumor (MN/MCA1) growth kinetic from day 14 post tumor cell injection to day 28 is shown. The Histogram bars show final tumor weights at the day of sacrifice. Mean tumor volumes (cm^3) and weights (gr) +/- SD in 18 Wt>Wt and $RORC^{-/-}$>Wt chimeric mice are shown. **B)** The number of macroscopic lung metastasis and weight (gr) of metastasized lungs is shown. Mean counts and weights +/- SD in 12 MN/MCA1 bearing Wt>Wt and Wt>RORC at the day of sacrifice are shown. **C) RORC is required for spontaneous- and chemically-induced carcinogenesis**. Breast tumor growth (Her2/neu-driven breast cancer) in CxB6NeuT chimeric mice, transplanted with Wt or RORC deficient BM. Mean tumor volumes (cm^3) +/- SD in 6 Wt>Wt and $RORC^{-/-}$>Wt chimeric mice are shown. **D**) Tumor growth (methylcolanthrene-induced) in Wt/Wt and $RORC^{-/-}$>Wt chimeric mice. Mean tumor volumes (cm^3) +/- SD in 6 Wt>Wt and $RORC^{-/-}$>Wt chimeric mice are shown.

Next, we evaluated the role of RORC in "steady state" versus cancer-driven "emergency" myelopoiesis. $RORC^{-/-}$>Wt tumor bearers showed a significant reduction of splenic M-MDSC and PMN-MDSC in comparison to Wt>Wt tumor bearers (Figure 5B). Surprisingly, PMN-MDSC and M-MDSC were significantly increased in the spleen from tumor free (Figure 5A) $RORC^{-/-}$>Wt versus Wt>Wt chimeras (Figure 5B). Of relevance, PMN-MDSC infiltration was significantly increased in tumors of $RORC^{-/-}$>Wt chimeras (Figure 5C).

While the number of F4/80^{+} macrophages in spleen did not change in RORC deficient tumor bearers (Figure 5E), we observed a significant decrease of CD11b^{+}Ly6C^{low}F4/80^{+} macrophages paralleled by an increase of PMN-MDSCs at the tumor site (Figure 5F). Further, the increase of splenic MDSC subsets in $RORC^{-/-}$>Wt tumor free mice was paralleled by a significant increase of F4/80^{+} macrophages (Figure 5D).

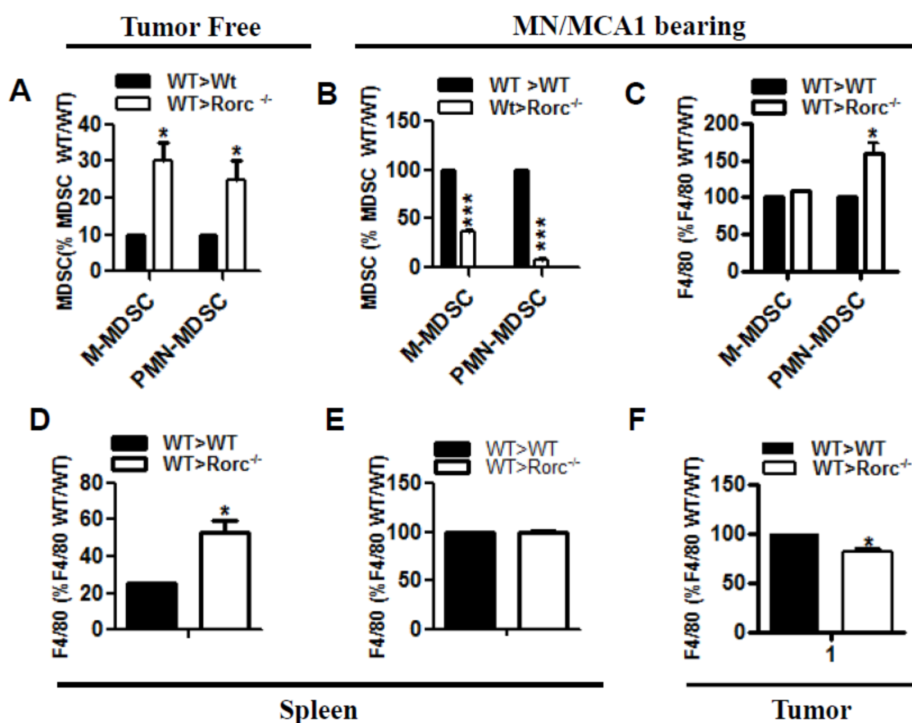

Figure 5- Prevalence of myeloid subsets in spleen and MN/MCA1 of Wt>Wt and RORC$^{-/-}$>Wt chimeric mice. Represented data are mean percentages ± SD of PMN-MDSCs, M-MDSCs, F4/80^{+} subsets, in the spleen from tumor free chimeric mice (**A,D**). Mean percentages ± SD of cell subsets in the spleen of tumor-bearing chimeric mice (**B,E**) and in primary tumor (MN/MCA1) (**C,F**).
Mean ± SD of 6 independent experiments in tumor free and MN/MCA1- bearing (AD) Wt>Wt and RORC$^{-/-}$>Wt chimeric mice, with 6 mice per experimental group (n=6).

These results indicate that RORC promotes expansion of splenic MDSCs and TAMs selectively in tumor driven "emergency" myelopoiesis, while in contrast RORC might limit tissue infiltration by effector myeloid cells in "steady state" mice as well as at the primary site of inflammation.
Hence, we tested whether RORC might control MDSCs survival [40].
BM-derived MDSCs from RORC-deficient mice displayed a higher percentage of AnxV^{+} binding cells in comparison to Wt BM-MDSCs (Figure S5A).
Next, to clarify the role of RORC in MDSCs survival to inflammatory challenge, BM-derived Wt and RORC-deficient MDSCs were cultured for 48 hrs with MN/MCA1 supernatant or with combination of GM-CSF/G-CSF. Both treatments reduced the number of AnxV^{+} binding Wt MDSCs, whereas no protection from apoptosis was observed in RORC-deficient MDSCs (Figure S5B).
Neutrophil activation and maturation is paralleled by up-regulation of FCγII and III receptors (CD32 or CD16, respectively) [41] and the complement C5a receptor (C5aR) [42]. We observed that spleen PMN-MDSCs isolated from Wt>Wt tumor bearing mice expressed lower levels of CD16/CD32 and C5aR in comparison to spleen PMN-MDSCs isolated from RORC$^{-/-}$>Wt tumor bearers (Figure S6B). Further, Wt splenic PMN-MDSC expressed higher RORC levels when compared to mature thioglycollate elicited neutrophils (N-PEC), while IL-17A expression remained unchanged (Figure S6A) This pattern of receptor expression suggests that RORC might suppress neutrophil maturation favoring accumulation of immature PMN-MDSCs .

In the attempt to pharmacologically validate our observations, tumor free or MN/MCA1 bearing Wt mice were treated with the RORC agonist SR1078. Despite no major differences were observed in primary tumor growth (data not shown), SR1078 increased lung metastases (Figure 6A) and splenic M-MDSCs and PMN-MDSCs (Figure 6B). Despite CD11b^{+}F4/80^{+} macrophages were not modified in spleen and tumor (data not shown), SR1078 significantly increased RORC expression in splenic macrophages (Figure 6D,E).

Noteworthy, SR1078 did not affect steady state myelopoiesis in tumor free mice (Figure 6F). As expected, SR1078 increased RORC expression in M-MDSCs and PMN-MDSCs (Figure 6C) while IL-17A expression was selectively induced in PMN-MDSCs (Figure 6D). These data further confirm that RORC nuclear receptor is a novel positive regulator of extramedullary myelopoiesis in cancer.

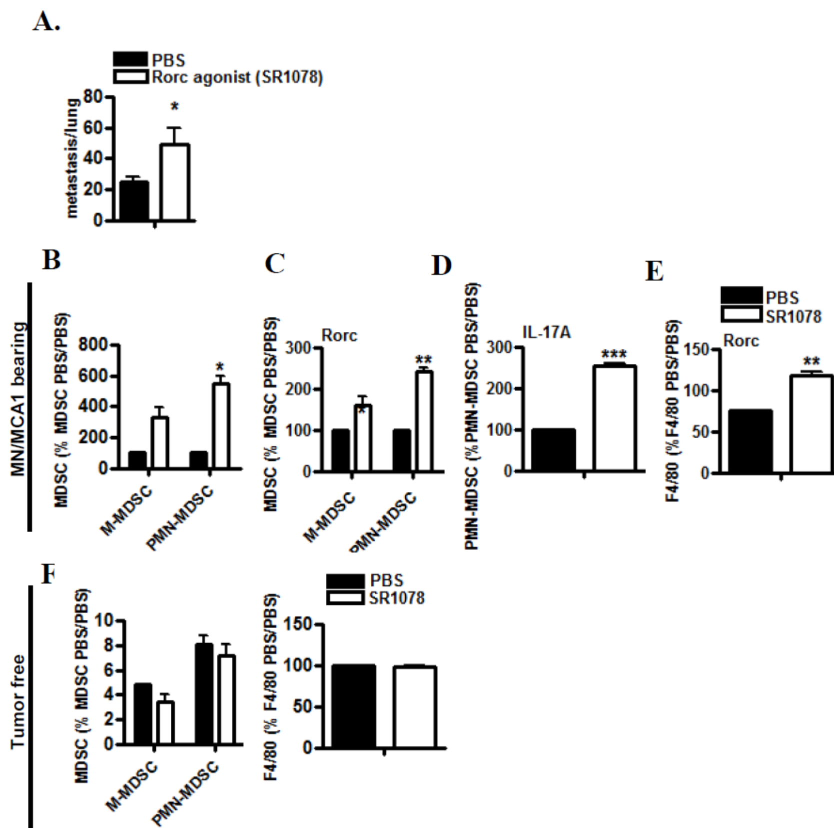

Figure 6- RORC agonist (SR1078) affects tumor development- A) The number of macroscopic lung metastasis in mice treated with either PBS or the RORC agonist (SR1078). Mean ± SD of 6 animals/group (n=6) are shown.

RORC agonist (SR1078) induces a RORC/IL-17A phenotype in splenic myeloid subsets.
The represented data are mean percentages ± SD of PMN-MDSCs, M-MDSCs, $CD11b^{+}F4/80^{+}$ and subsets in the spleen of tumor-bearer (**B**) and tumor-free (**F**) mice treated with PBS or RORC agonist (SR1078).

RORC agonist (SR1078) induces a RORC/IL-17A phenotype in tumor-associated myeloid subsets. The represented data are mean percentages ± SD of $RORC^{+}$ MDSCs subsets (**C-left**) or $F4/80^{+}$ (**E**) and $IL\text{-}17^{+}$ PMN-MDSCs (**D**) in the spleen of tumor bearer mice treated with PBS or RORC agonist (SR1078).

RORC tunes type I innate and adaptive immunity

The lack of RORC in the hematopoietic compartment resulted in decreased number of TAMs (Fig. 2C)., suggesting that RORC might be implicated in terminal differentiation of macrophages. To confirm this assumption, TAMs from Wt>Wt and $RORC^{-/-}$ >Wt tumor bearing chimeras were screened for CD115 expression, a marker of terminally differentiated macrophages [43]. As shown, the number of $CD11b^{+}F4/80^{+}CD115^{+}$ macrophages decreased in spleen (Figure 7A) and tumor (Figure 7B) of $RORC^{-/-}$>Wt chimeras . In agreement, we observed by confocal microscopy (Figure7C) fewer $CD115^{+}F480^{+}$ macrophages in tumor tissues of $RORC^{-/-}$>Wt chimeras. We examined the suppressive capacity of M-MDSC in response to IFN-γ [4]. However, no major differences were observed in
Wt vs $RORC^{-/-}$ cells, in terms of nitric oxide production and suppressive activity (data not shown).

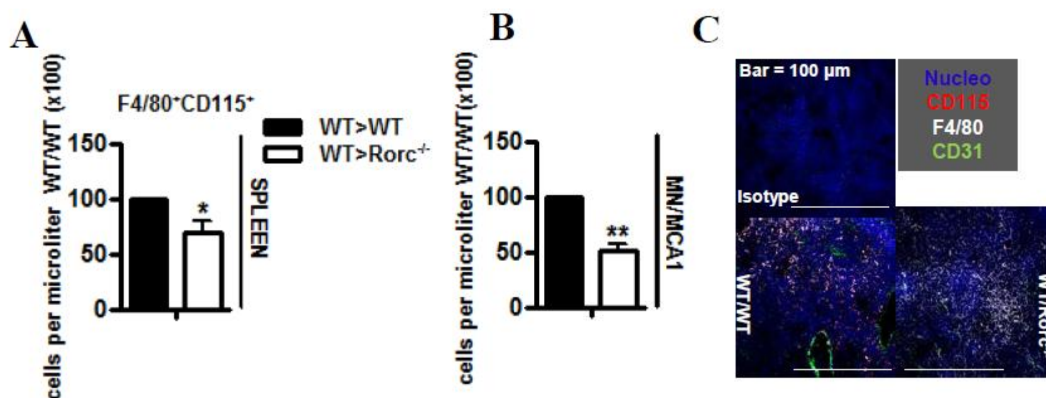

Figure 7- RORC drives the expansion of F4/80^{+}CD115^{+}TAM.

The histogram bars show the the number of CD115-expressing F4/80^{+} in spleen (**A**) and primary tumor (MN/MCA1) (**B**) from Wt>Wt and RORC$^{-/-}$>Wt chimeric mice. **C**) Expression of CD115 in F4/80^{+} cells in MN/MCA1 obtained from Wt>Wt and RORC$^{-/-}$>Wt chimeric mice.
Tumor tissues were stained with anti-CD115 antibody (red), anti- F4/80 antibody (white) and anti-CD31 antibody (green). Irrelevant mouse IgG was used as control. Characteristics of the nuclei were revealed by addition of DAPI (blue). Bar represents 100 μm**.**

As macrophage polarization plays a key role in cancer progression[44], we analysed by RT-PCR the expression of prototypical M1 and M2 genes in TAMs from tumor bearing Wt>Wt and RORC$^{-/-}$ >Wt chimeras. TAMs were treated with 100 ng LPS (M/L) or 200U/ml IFN-γ for 4 hrs to induce M1 polarized response or exposed to LPS for 20hrs (L/M) to induce M2-polarization, as previously described [45]. In addition, spleen PMN-MDSCs were stimulated 4 hrs *in vitro* with IFN-γ, LPS or combination of IFN-γ plus LPS. RORC-deficient TAMs displayed enhanced expression of M1 (IL-12p40, TNFα, IL-1β) and decreased expression of M2 (IL-10, TGFβ, Ym1) genes under M/L and L/M conditions, in comparison to their Wt counterparts (Figure S7A**).** In analogy, RORC-deficient PMN-MDSCs displayed increased TNFα and IL-1βmRNA levels (Figure S7B) and increased secretion of IL-1β (Figure S7C) in response to LPS/IFN-γ.

Thus, RORC acts as negative regulator of M1 and promoter of M2 cytokine genes in TAM and spleen PMN-MDSCs. This observation was further supported by the increased levels of pro-inflammatory cytokines (TNFα, IL-1β) and growth factors (G -CSF, GM-CSF, VEGF) determined in MN/MCA1 supernatants from RORC$^{-/-}$>Wt (Figure S8A) correlating with marked splenomegalia observed in RORC$^{-/-}$ >Wt MN/MCA1 (Figure S8B) and Her2/neu (Figure S8C) bearing mice. Supporting the inflammatory phenotype in RORC$^{-/-}$>Wt mice, we found increased CD4^{+}IFN-γ^{+} cells in tumor free (Figure S9A) and tumor bearing (Figure S9B) RORC$^{-/-}$>Wt mice, while the total CD45^{+}CD4^{+} cells decreased as described previously [46]. In contrast, tumor infiltrating CD4^{+}IFN-γ^{+} and total CD45^{+}CD4^{+} T cells increased in RORC$^{-/-}$>Wt chimeras (Figure S9C). Further, F4/80^{+}TNFα^{+} macrophages increased in spleen (Figure S9D,E) and tumor (Figure S9G) from RORC$^{-/-}$>Wt mice. Supporting the inhibitory role of RORC in activation of type I antitumor immunity, CD4^{+}Foxp3^{+} T regulatory cells (Treg) significantly decreased in the spleen from RORC$^{-/-}$ >Wt mice (Figure S9F).

The spleen is a major reservoir of RORC^{high}/C/EBPβ^{high} myeloid precursors

The stem cell factor receptor/c-Kit, expressed on Thy-1^{low}Sca-1^{+}lineage-negative (Lin^{-}) hematopoietic stem cells (HSCs), contributes to the differentiation of HSCs progenitors [47] and is down-modulated in response to growth factors [48]. Therefore, we next evaluated RORC and IL-17A protein expression in early myeloid progenitors (c-kit^{+}) from BM and immature myeloid cells (c-kit^{low}), from spleen of MN/MCA1 bearing Wt mice (Figure 8A) shows the gating strategy used to identify granulocyte progenitors (population A: c-kit^{low}F480^{neg}Ly6C^{neg}Ly6G^{high} and population B: c-kit^{high}F480^{+}Ly6C^{neg}Ly6G^{+}) and monocyte/macrophage progenitors (population C: c-kit^{low}F480^{+}Ly6G^{low}Ly6C^{+} and population D: c-kit^{low}F480^{+}Ly6G^{low}Ly6C^{low}).

Reflecting the RORC/IL-17A expression pattern observed in mature myeloid cells (Figure 1), dual expression of RORC and IL-17A was restricted to immature granulocyte precursors (population B) in BM (Figure 8B, left), while BM cells from the monocyte/macrophage lineage did not express IL-17A and expressed low levels of c-kit. Of relevance, RORC and C/EBPβ were selectively co-expressed by immature BM granulocyte progenitors (population B), evidencing that RORC supports cancer driven medullar granulopoiesis but not monocytopoiesis.

In contrast, in the spleen of WT tumor bearers (Figure 8B, right) RORC and C/EBPβ were highly co-expressed in monocyte/macrophage (population C+D) and granulocyte progenitors (population A+B), suggesting that RORC is the major driver of extra-medullar granulo-monocytopoies in cancer inflammation.

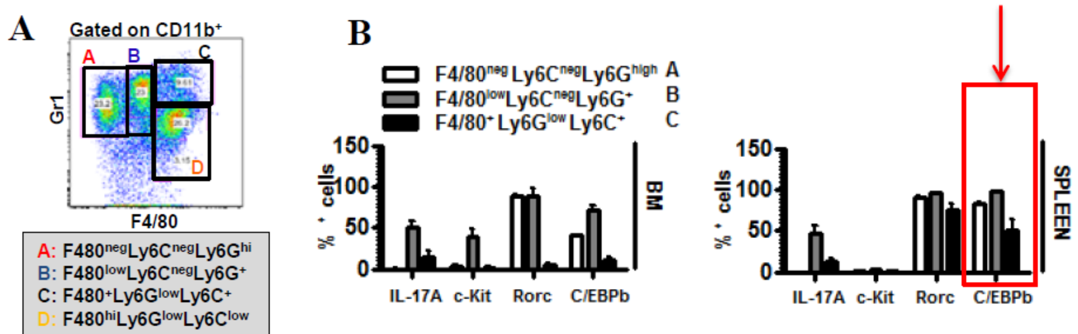

Figure 8- RORC/IL-17 signature in early myeloid progenitors (c-kit^{+}) and immature myeloid cells (c-kit^{low}) in spleen and bone marrow from MN/MCA1 bearing Wt mice.

A) The gating strategy used for identifying F4/80^{neg}C^{neg}G^{high} granulocyte (A), F4/80^{+}C^{neg}G^{+} monocyte (B), F4/80^{+}G^{low}C^{+} macrophage/monocyte (C) and F4/80^{+}G^{low}C^{low} progenitor (D) subsets is shown . **B**) The represented data are mean percentages ± SD of IL-17A, RORC, c-kit and C/EBPβ expressing cells in the (A), (B) and (C) progenitor subsets, in BM (left) and spleen (right) from MN/MCA1 bearing Wt mice. Mean ± SD of 3 independent experiments is shown.

RORC controls critical regulators of myelopoiesis

Because RORC is required for cancer-driven myelopoiesis, we analyzed by FACS the commitment of common hematopoietic progenitors in tumor bearing Wt>Wt and RORC$^{-/-}$>Wt chimeras. While in the spleen few LSK (lin)$^{-}$c-kit^{+}Sca-1^{+}) were measured (data not shown), LSK cells were significantly increased in the BM from RORC$^{-/-}$>Wt chimeras (Figure 9A). Next, spleen and BM were screened for the presence of common myeloid progenitors (CMP), granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroid progenitors (MEP).

CMPs were significantly enhanced in BM of RORC$^{-/-}$>Wt chimeras, while, in accordance, a decrease in GMP was observed (Figure 9B). These findings reveal a blockage in differentiation of early hematopoietic progenitors in RORC$^{-/-}$>Wt chimeras. To evaluate the impact of this blockage on the arisement of myeloid progenitor populations . BM and spleen from MN/MCA1 bearing Wt>Wt or RORC$^{-/-}$>Wt chimeras were screened for granulocyte (population A+B) and monocyte/macrophage precursors (population C+D). In both, BM and spleen from tumor bearing RORC$^{-/-}$>Wt chimeras, a significant decrease in granulocyte (population A+B) and monocyte/macrophage precursors (population C)

(Figure 9C) was measured, while we observed a significant increase in macrophage precursors (population D) (Figure 9D). These findings suggest that RORC is needed for the expansion of immature M-MDSCs and PMN-MDSCs from CMP/GMP, while RORC might be required for differentiation and terminal differentiation of macrophage progenitors.

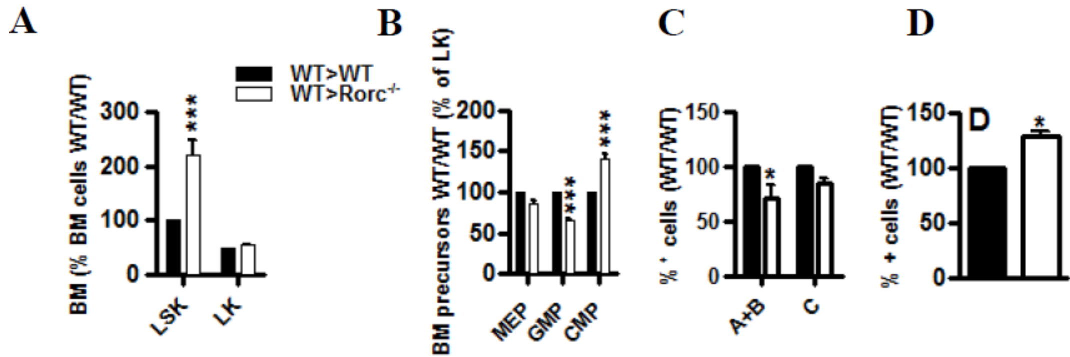

Figure 9- Prevalence of bona fide hematopoietic stem cells and myeloid progenitors in BM of Wt/Wt and Wt/RORC-/- MN/MCA1 tumor bearers.

A) LSK and LK populations in BM from RORC expressing (filled bars) and RORC deficient (open bars) MN/MCA1 bearing chimeric mice. BM cells and splenocytes were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, and Ter-119), IL-7R, c-Kit, and Sca-1 antibodies. Bar graphs show the percentages ± SD of LSK and LK in BM and spleen. **B**) CMP, GMP, and MEP populations in BM RORC expressing (filled bars) and RORC deficient (open bars) MN/MCA1 bearing chimeric mice. BM cells and splenocytes were stained with anti-lineage cocktail (Mac-1, Gr-1, B220, CD4, CD8, Ter-119, and IL7R), c-Kit, Sca-1, CD34, and FcγR antibodies. The percentages indicate CMP, GMP, and MEP in the gated LK population. Mean ± SD of 3 independent experiments is shown.

Increased number of macrophage precursors and reduced numbers of granulocyte and monocyte precursors in BM cells from MN/MCA1 bearing Wt/RORC-/- chimeric mice.

Bar graphs show the percentages ± SD of granulocyte -A+B- and monocyte/macrophages -C- precursors BM cells (**C**) and macrophage precursors -D- (**D**) from MN/MCA1 bearing Wt/Wt (filled bars) and Wt/RORC-/- (open bars) chimeric mice. Mean ± SD of 3 independent experiments is shown.

To shed light on the mechanisms of RORC-driven emergency myelopoiesis, we evaluated positive and negative transcriptional regulators in BM and spleen from Wt>Wt and $RORC^{-/-}$>Wt tumor bearing chimeras. C/EBPβ CCAAT/enhancer binding protein (C/EBPβ) is a major positive regulator of G-CSF and GM-CSF driven "emergency" myelopoiesis[49], while c/EBPβ appears as a major regulator of "steady state" granulopoiesis[50]. PU.1 and C/EBPβ contribute both to "emergency" granulo-monocytopoiesis [51,52].

We observed decreased mRNA expression of PU.1, C/EBPβ and C/EBPβ in spleen (Figure S10A, right) and BM (Figure S10A, left) of $RORC^{-/-}$>Wt tumor bearers, paralleled by decreased C/EBPβ protein levels in splenic –granulocyte (population B) and monocyte/macrophage (population C) precursors (Figure S10B).

Importantly, we observed in BM and spleen (Figure S10C) from $RORC^{-/-}$>Wt MN/MCA1 tumor bearers increased mRNA levels of the suppressor of cytokine signaling-3 (Socs3) and the transcriptional co-regulator B cell leukemia/lymphoma 3 (Bcl3), which have been both reported as potent inhibitors of G-CSF-driven "emergency" granulopoiesis [53,54]. Further, in agreement with the IFN-γ-induced inhibition of G-CSF-driven neutrophilia[55], IFN-γ induced a strong increase of Socs3 and Bcl3 mRNA in splenic PMN-MDSCs from $RORC^{-/-}$ tumor bearers (Figure S10D). Finally, we observed a decreased number of IRF8 expressing $CD11b^{+}F4/80^{+}$ macrophages in spleen and tumor tissue from $RORC^{-/-}$ >Wt (Figure S10E), evidencing that RORC is required for the modulation of transcriptional regulators that mediate terminal maturation of macrophages and dictate the fate of myeloid commitment from the polymorphonuclear to the monocyte/macrophage lineage [13]. Next, $(lin)^{-}$c-kit^{+}Sca-1^{-} progenitor cells isolated by magnetic bead separation from "steady state" Wt or $RORC^{-/-}$ bone marrows, were challenged with IL-1β, G-CSF or GM-CSF. The arisement of myeloid progenitor subsets to CSFs was evaluated by FACS-analysis.

The gating strategy used is shown in (Figure S11A). RORC deficient
$(lin)^{-}c\text{-}kit^{+}Sca\text{-}1^{-}$ progenitor cells challenged with IL-1β, G-CSF or GM-CSF failed to differentiate to immature granulocyte precursors (population B), monocyte/macrophage precursors (population C+D), while RORC deficiency had no effect on the expansion of more mature granulocyte subsets (population A) (Figure S11B**).** Our results show that a decreased number of macrophages in tumors or in steady state mice leads to increased tissue neutrophils (Fig. 2C), suggesting that tissue macrophages provide a negative feedback for neutrophil accumulation in tissues.

To prove this assumption, MN/MCA1 bearing Wt or $Rorc^{-/-}$ mice were treated with an anti-CSFR1 antibody. The CSFR1 produced significant depletion of the $CD11b^{+}F4/80^{+}$ macrophages in tumor of Wt (Figure 9A) and RORC deficient mice (data not shown). The antibody preferentially depleted the $CD115^{+}$ macrophage population, which co-expressed high RORC levels (Figure 9A). The depletion of tissue macrophages was paralleled by the inhibition of immature $RORC^{+}$ MDSC subsets, while promoting a strong tumor infiltration by $RORC^{neg}Ly6G^{high}$ neutrophils (Figure 9B). Tumor infiltration by $RORC^{neg}Ly6G^{high}$ neutrophils of macrophage depleted RORC defcicent tumors was increased in comparison to Wt macrophage depleted tumors (Figure 9B) Neutrophils in tumors from macrophage depleted mice expressed significantly higher levels of C5a and CD16/32 in comparison to mice treated with the isotype antibody (Figure 9C). This phenotype suggests that tumor tissue macrophages favor infiltration by immature MDSC populations in disadvantage of mature neutrophils, contributing to a protumoral microenvironment. Further, treatment with anti-CSFR1 resulted in reduced number of monocyte/macrophage precursors (population C+D), while increasing granulocyte progenitors (population A+B) (Fig.5D). Supporting the role for reciprocal negative regulation of monocytes/macrophages and granulocytes in cancer inflammation mediated tissue homeostasis , treatment with anti-GCSF

antibody increased the number of splenic F4/80$^+$TNFα$^+$ M1-like macrophages, paralleled by elevation of CD4$^+$IFNγ$^+$ Th1 cells (Figure 9E).

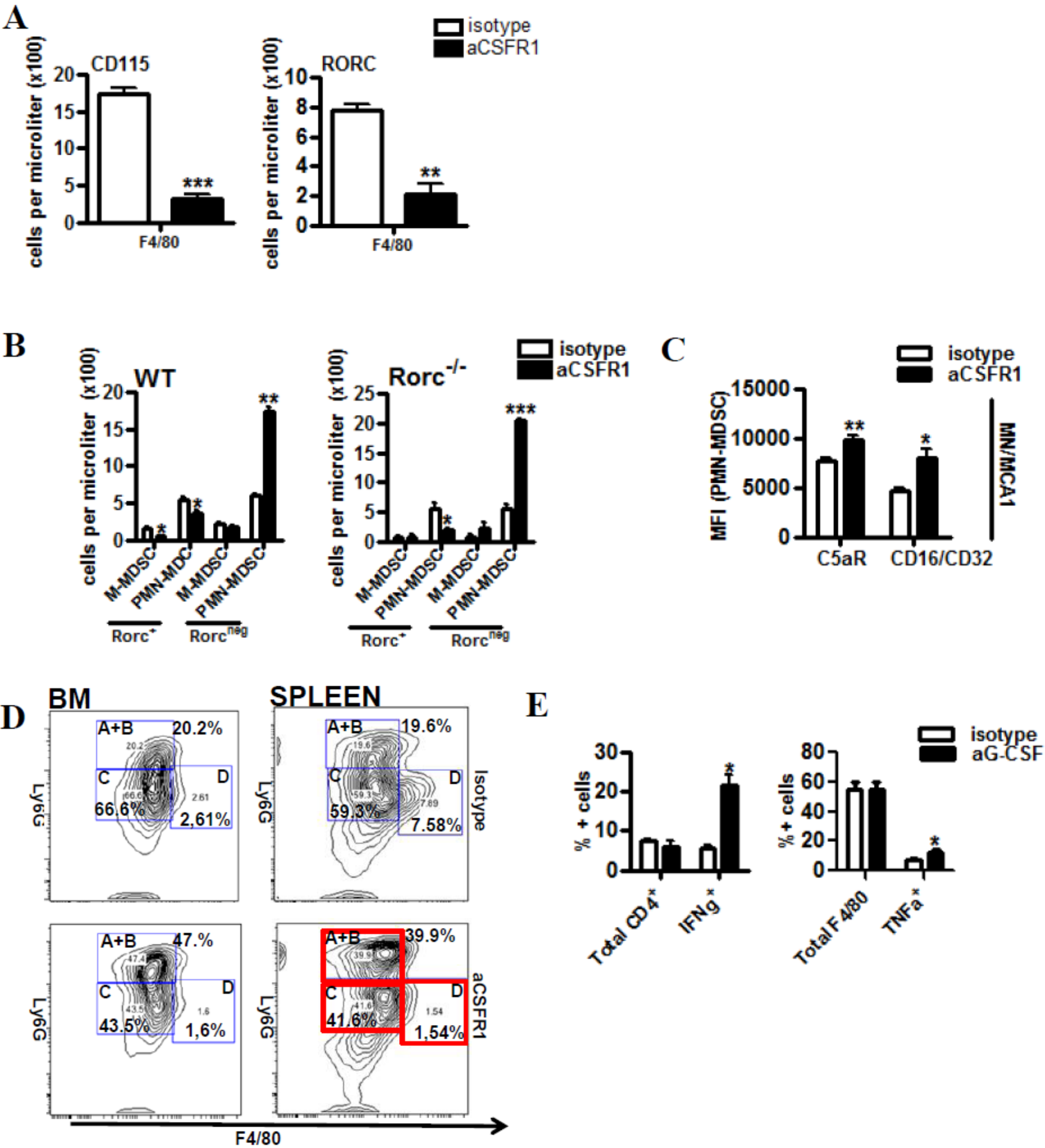

Figure 9- Depletion of F4/80$^+$CD115$^+$RORC^{high} tissue resident macrophages during cancer development using an anti-CSFR1 antibody.

A) The represented data are mean numbers ± SD of CD115$^+$ (left) and RORC$^+$ (right) expressing CD11b$^+$F4/80$^+$ in spleen (upper row) from Wt/Wt chimeric mice treated with isotype IGg (open bar) or anti-CSFR1 antibody (filled bar). Mean ± SD of 6 mice/experimental group (n=6) is shown.

B) Prevalence of myeloid subsets in MN/MCA1 from anti-CSFR1 antibody treated mice. The represented data are mean percentages ± SD RORC$^+$ or RORC^{neg} CD11b$^+$Ly6C$^+$ and CD11b$^+$Ly6G$^+$ subsets in MN/MCA1 from Wt/Wt and Wt/RORC$^{-/-}$ chimeric mice treated with isotype IGg (open bar) or ant-CSFR1 antibody (filled bar). Mean ± SD of 6 mice/experimental group (n=6) is shown.

C) Mature neutrophils accumulate in tumor tissues from macrophage–depleted mice. The represented data are mean percentages ± SD of CD16/CD32 and C5aR expressing CD11b$^+$Ly6G$^+$ cells in MN/MCA1 from Wt/Wt and Wt/RORC$^{-/-}$ chimeric mice treated with isotype IGg (open bar) or anti-CSFR1 antibody (filled bar). Mean ± SD of 6 mice/experimental group (n=6) is shown.

D) Markedly increased number of granulocyte progenitors and depletion of macrophage/monocyte precursors in BM cells and splenocytes from MN/MCA1 bearing mice. Representative FACS DOT PLOTS for F4/80^{neg}C^{neg}G^{high} granulocyte, F480$^+$C^{neg}G$^+$ monocyte and F480$^+$G^{low}C^{low} macrophage progenitor subsets in BM cells and splenocytes from MN/MCA1 bearing Wt/Wt mice treated with IGg isotype antibody and anti-CSFR1 antibody are shown.

E) Adaptive and innate type I inflammatory cells markedly increase in tumors from mice treated with G-CSF antagonist. Bar graphs show mean percentages ± SD of CD4$^+$ and CD4$^+$IFNγ$^+$ and F4/80$^+$TNFα$^+$ subsets in MN/MCA1 from Wt/Wt mice treated with PBS (open bars) or anti-G-CSF antibody (filled bars). Mean ± SD of 6 mice/experimental group (n=6) is shown.

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Supplemental data

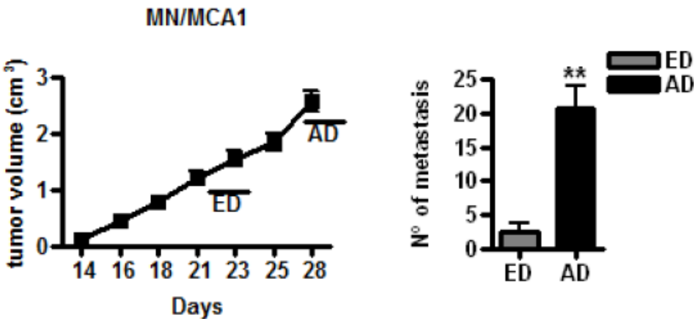


Figure S1- Criteria for classification of tumor bearing animals with early (ED) and advanced disease (AD). **A)**: Kinetic of tumor (MN/MCA1) growth from day 14 post tumor cell injection up to day 28 is shown. Mean tumor volumes (cm^3) +/- SD in 30 Wt C57BL/6 mice are shown.

B): The number of macroscopic lung metastasis is shown. Mean counts +/- SD in 30 Wt C57BL/6 mice are shown.

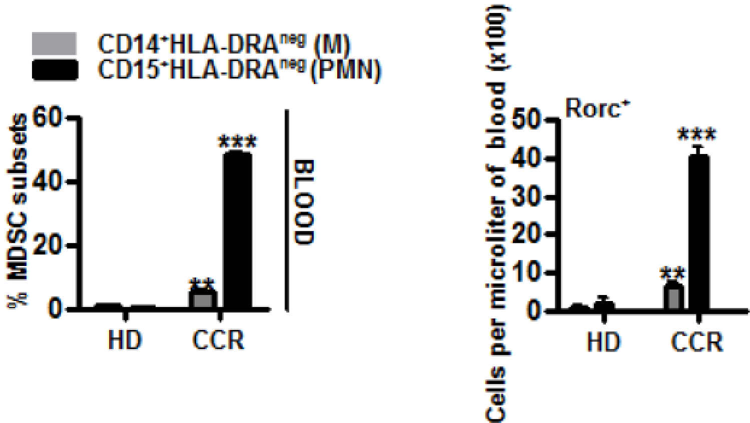


Figure S2- Peripheral blood cells from CRC patients were analyzed for RORC$^+$ and IL-17A$^+$ MDSCs subsets by routine flow cytometry analysis. Peripheral blood samples from healthy donors (HD) (n=10) and CRC patients (n=10) were analyzed by FACS analysis for the presence of M-MDSCs (CD14$^+$HLA-DR^{neg}) and PMN-MDSCs (CD15$^+$HLA-DR^{neg}) (**A, left**). RORC (APC-A log) (**A, right**) expression was determined in M-MDSCs and PMN-MDSCs by intracellular staining followed by FACS analysis. 10 milliliters of blood were collected from HDs or CRC patients under Institutional Review Board approved studies . 50 000 live leukocyte events were acquired for each sample.

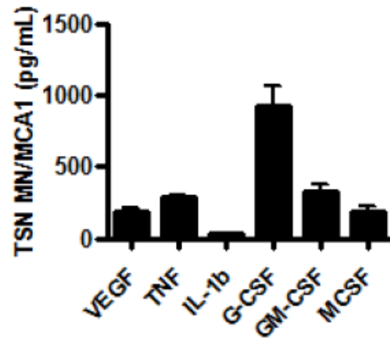
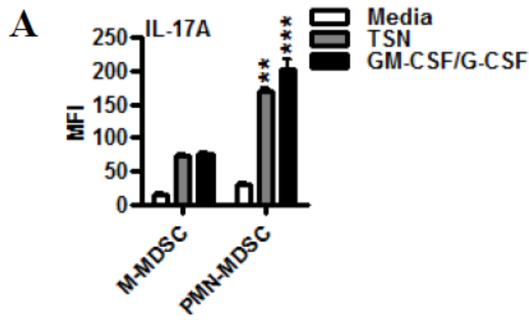


Figure S3- Levels of selected cytokines and growth factors in MN/MCA1 supernatants.
Protein expression (pg/mL) for VEGF, GM-CSF, G-CSF, M-CSF, IL-1β and TNFα were measured in MN/MCA1 supernatants by ELISA. Mean expression levels (pg/mL) +/- SD in 3 MN/MCA1 supernatants harvested from tumor bearing Wt C57BL/6 mice are shown.

A



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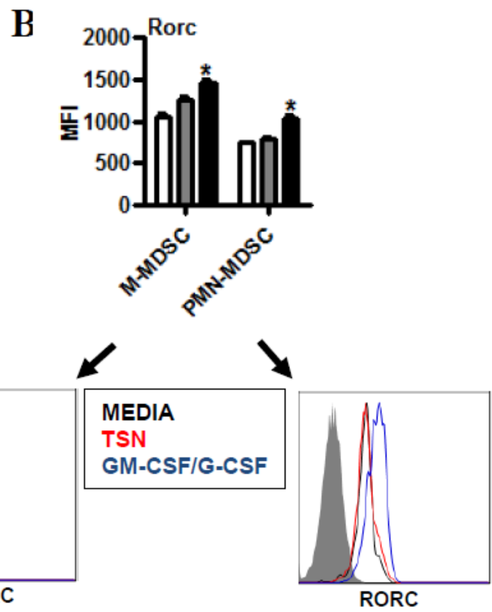

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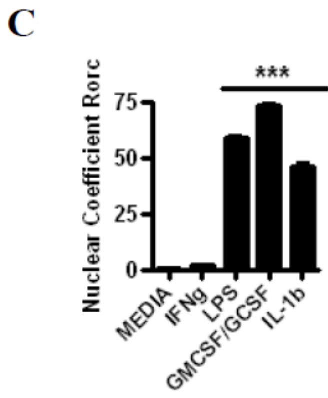

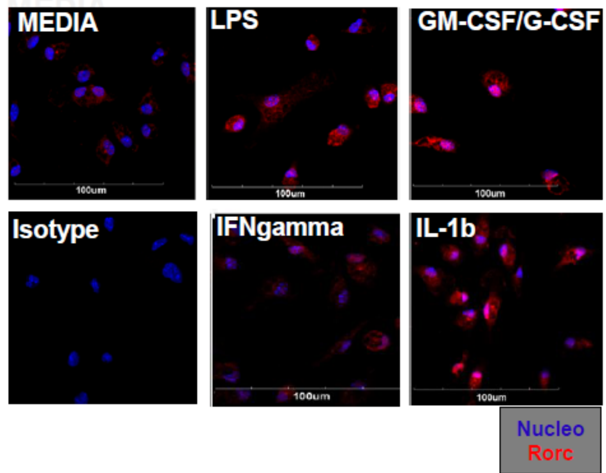

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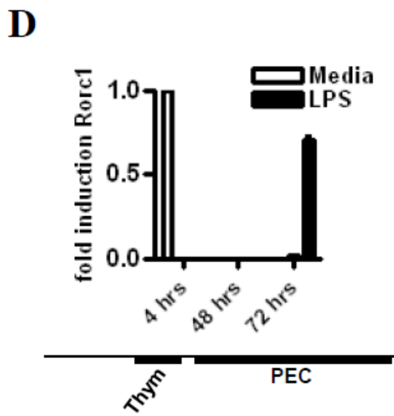


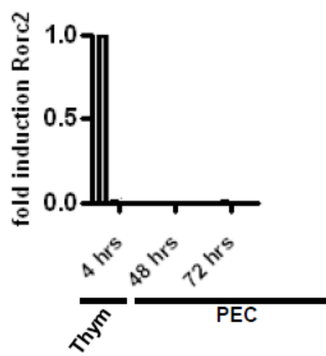


Figure S4- *In vitro* induction of IL-17A and RORC expression in BM-MDSCs.
BM-derived MDSCs (BM-MDSCs) were stimulated in vitro for 48 hours and then analyzed for IL-17A (APC-A log) (**A**) and RORC (APC-A log) (**B**) expression levels, within the $CD11b^+Ly6C^+$ and $CD11b^+Ly6G^+$ gates. Mean fluorescence intensity (MFI) +/- SD in M-MDSCs and PMN-MDSCs for IL-17A and RORC are shown in BM-MDSCs obtained from 3 representative experiments. **C) Expression and nuclear translocation of RORC** in PEC harvested from naïve Wt C57BL/6 mice following activation with LPS or cytokines/colony growth factors. PEC were treated for 72 hrs with LPS, GM-CSF+G-CSF, IL-1β, IFNγ or maintained in RPMI 1640 alone (MEDIA). Post 72 hrs of *in vitro* stimulation, PEC were fixed with PFA, permeabilized in PBS 0.1% Triton-X100 and stained with anti-RORC antibody (red). Irrelevant mouse IgG was used as control. Nuclei were revealed by addition of DAPI (blue). The thickness of the optical slices was 1 µm. Bar represents 100µm**.** Left: Histograms showing the fluorescence intensity (Nuclear Coefficient) of nuclear RORC. **D) RORC1 and RORC2 mRNA expression levels in PEC and thymocytes.** PEC harvested from naïve Wt C57BL/6 mice were stimulated *in vitro* with LPS for 24, 48 and 72 hrs. mRNA expression levels for RORC1 (black bar) (left histogram) and RORC2 (black bar) (right histogram) were compared to constitutive expression levels observed in unstimulated thymocytes, obtained from naïve Wt C57BL/6 mice (open bar).

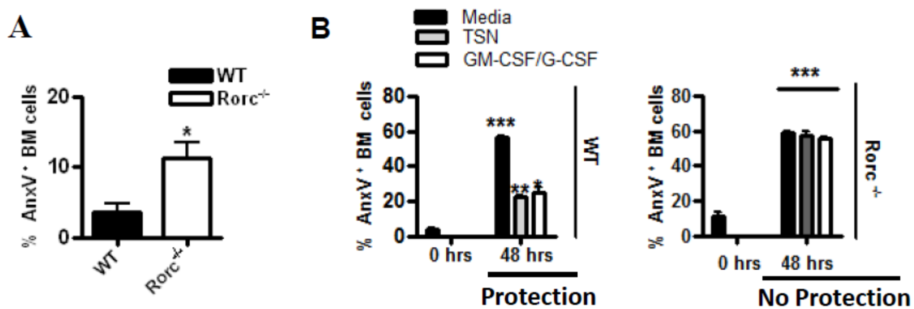


Figure S5- A) RORC promotes survival of MDSCs. BM derived MDSCs generated *in vitro* from RORC expressing and RORC deficient BM were analyzed by FACS analysis for AnnexinV binding. Mean ± SD of 3 independent experiments is shown.

B) Tumor-derived factors and GM-/G-CSF promote MDSCs survival. FACS analysis of Annexin V binding to Wt (left) and $RORC^{-/-}$ (right) BM-derived MDSCs treated with tumor supernatants (TSNT) or with combination of GM-CSF/G-CSF. AnnexinV binding was determined in freshly generated MDSCs (0 hrs) and after 48 hrs of *in vitro* culture, as indicated. Mean ± SD of 3 independent experiments is shown.

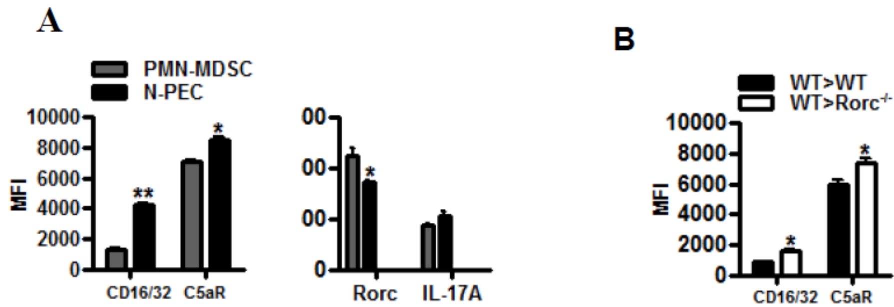
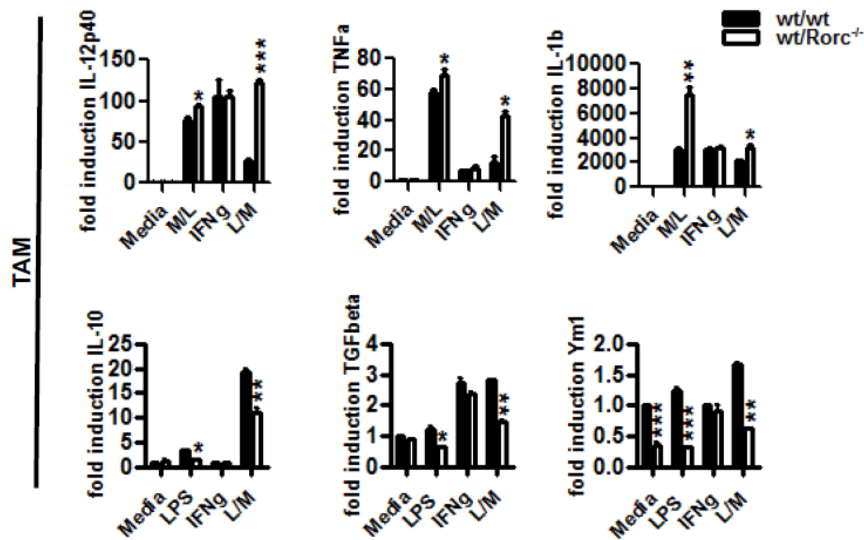


Figure S6- RORC impairs neutrophil maturation and promotes accumulation of immature myeloid cells. Left: The histogram bars show the *means* (± *SD*) of the relative *mean* fluorescence intensity (MFI) of FCII/IIIR (CD16/CD32), C5aR (**A**) RORC and IL-17A (**B**) in PMN-MDSCs harvested from the spleen of MN/MCA1 bearing Wt mice and in peripheral elicited cells (PEC) from tumor free Wt animals. **C**) The histogram bars show the mean percentages ± *SD of* FCII/IIIR^{+} (CD16/CD32) and C5aR^{+} PMN-MDSCs separated from the spleen of MN/MCA1 bearing Wt>Wt and Rorc$^{-/-}$ >Wt chimeric mice. Data from six independent experiments performed are shown.

A



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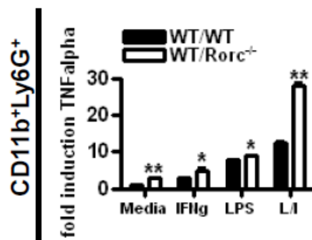

fold induction IL-1beta

Media IFNg LPS L/I

C

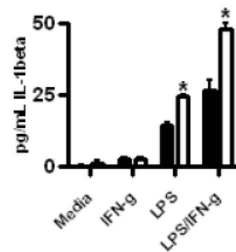

Figure S7- RORC promotes M2 skewing of TAM and inhibits M1 cytokine expression in PMN-MDSCs. A) Total RNA from control (Media), 4 hrs activated (with IFNγ or LPS) and 20 hrs LPS-treated (L/M) TAM, were harvested from MN/MCA1 from Wt/Wt (filled bar) and Wt/RORC$^{-/-}$ (open bar) tumor bearing chimeric mice and analyzed by RT-PCR for the expression of representative M2 (IL-10, TGFβ, Ym1) and M1 (IL-12p40, TNFα, IL-1β) genes. **B)** Total RNA from control (Media) and activated (IFNγ, LPS or LPS+IFN-γ) PMN-MDSCs, obtained from the spleen of MN/MCA1 bearing Wt>Wt (filled bar) and RORC$^{-/-}$>Wt (open bar) chimeric mice, were analyzed by RT-PCR for the mRNA expression of TNFα and IL-1β representative M1 genes.

C) Secretion of IL-1beta (pg/ml) by PMN-MDSCs was measured by ELISA. Results are given as fold increase over the mRNA level expressed by untreated cells (Media) and show the mean ± SD from triplicate values.

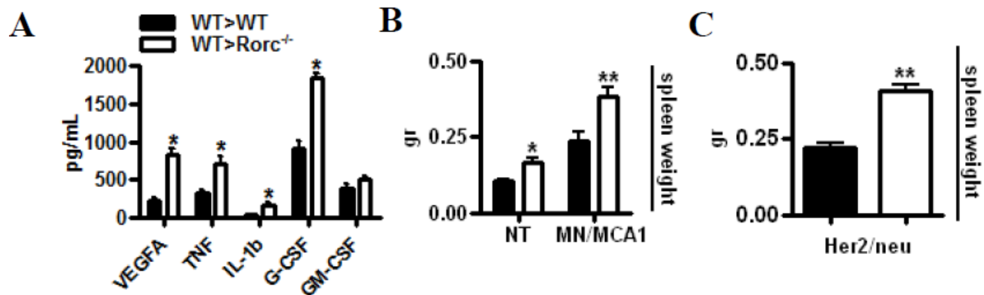

Figure S8- A) Lack of RORC in the bone marrow of chimeric Wt/RORC$^{-/-}$ tumor bearers results in **increased levels of pro- inflammatory cytokines** (**A**) (TNFα, IL-1β) and **growth factors** (VEGFA, G-CSF, GM-CSF) into the tumor microenvironment, associated with **splenomegalia** (**B**). **A**): ELISA, results show the average of 3 independent experiments ± SD.

B): Mean spleen weights (gr) +/- SD was calculated in 10 W>/Wt (NT) and RORC$^{-/-}$>Wt MN/MCA1 bearing chimeric mice and 6 Wt>Wt and RORC$^{-/-}$>Wt Her2/neu chimeric mice.

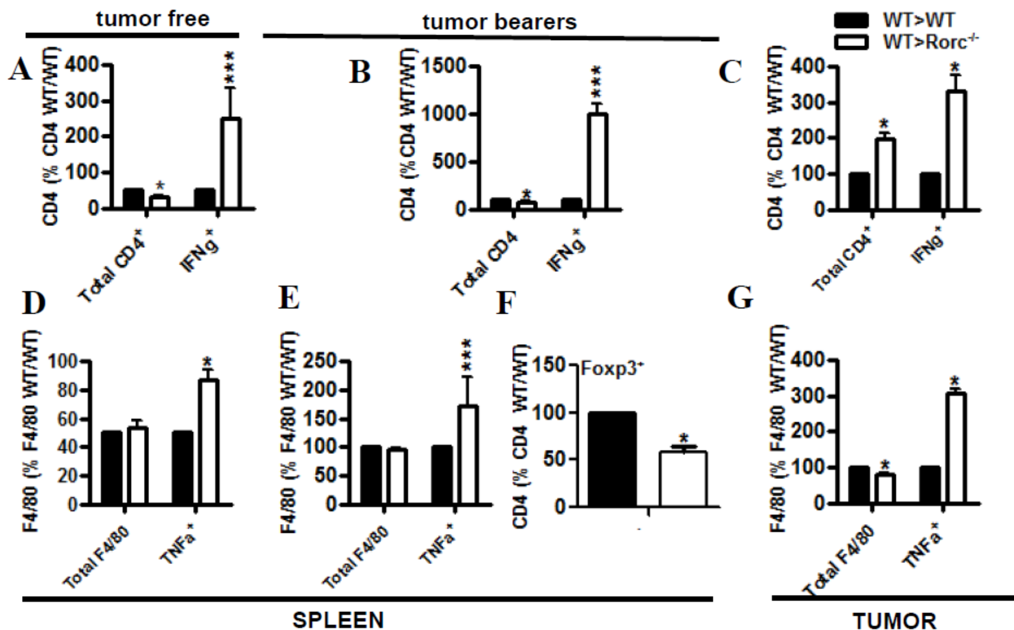

Figure S9- RORC keeps type I innate and adaptive effector responses in tumor bearers in check. The represented data are mean percentages ± SD of CD4^{+}, CD4^{+}IFNγ^{+} and CD4^{+}Foxp3^{+} within the CD45^{+} gate and F4/80^{+} and F4/80^{+}TNFα^{+} subsets within the CD11b^{+}CD45^{+} gate, in spleen from tumor free (**A,D**) and spleen (**B,E,F**) and tumor (**C,G**) from MN/MCA1 tumor bearing Wt>Wt (filled bars) and RORC$^{-/-}$>Wt (open bars) chimeric mice. Mean ± SD of 6 independent experiments is shown.

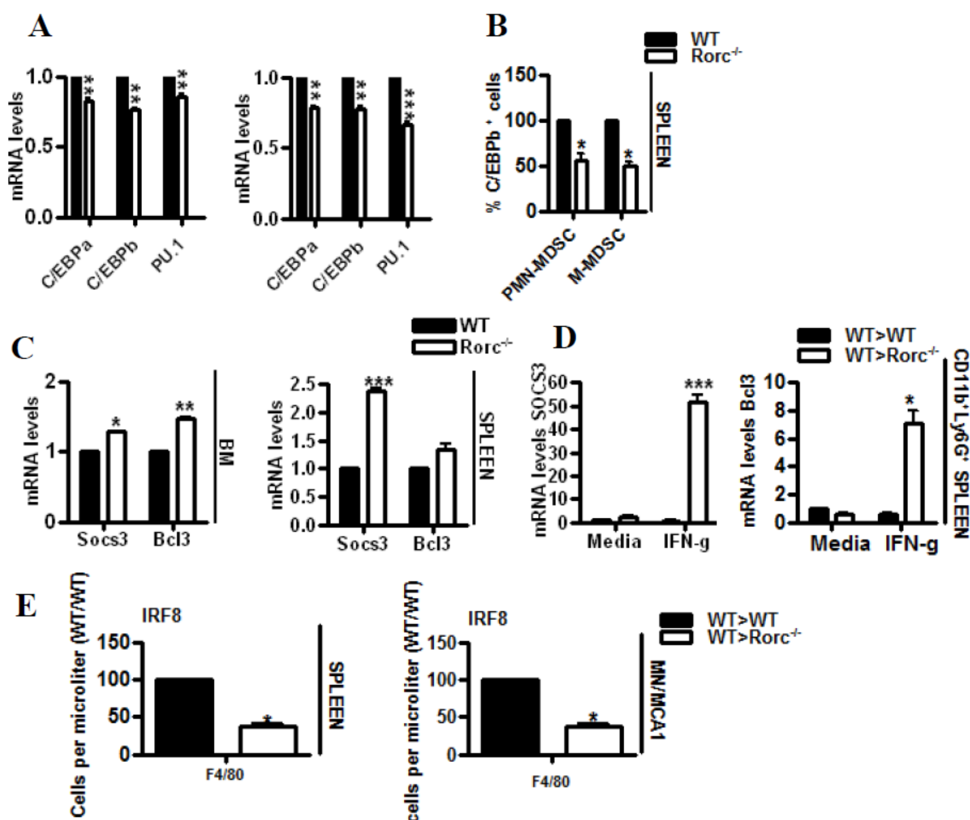

Figure S10- RORC expression is required for C/EBPβ expression by splenic myeloid precursors. A) Total RNA from BM cells (**left**) and splenocytes (**right**) harvested from Wt>Wt (filled bar) and RORC$^{-/-}$>Wt (open bar) MN/MCA1 bearing chimeric mice were analyzed by RT-PCR for the expression of key regulatory transcription factors (C/EBPβ, C/EBPβ and Pu.1) of "emergency" granulo-myelopoiesis. Results are given as fold increase over the mRNA level expressed by Wt/Wt cells and are representative of at least 3 different experiments; shown are mean ± SD from triplicate values. **B)** Mean percentages ± SD of C/EBPβ protein expressing M-MDSCs and PMN-MDSCs in ckit^{low} splenocytes measured by FACS analysis are shown. Mean ± SD of 3 independent experiments is shown.

RORC functions as negative regulator of SOCS3 and Bcl3 expression in BM cells and splenocytes. C) Total RNA from BM cells (left) and splenocytes (right) harvested from MN/MCA1 bearing Wt>Wt (filled bar) and RORC$^{-/-}$>Wt (open bar) chimeric mice were analyzed by RT-PCR for the expression of negative transcriptional regulators (SOCS3, and Bcl3) of "emergency" granulo-myelopoiesis. **D**) IFNγ-induced expression of SOCS3 and Bcl3 in spleen PMN-MDSC from Wt/Wt (filled bar) and Wt/RORC$^{-/-}$ (open bar) chimeric mice.

Results are given as fold increase over the mRNA level expressed by Wt>Wt cells and are representative of at least 3 different experiments; shown are mean ± SD from triplicate values.

E) RORC is required for IRF8 expression by macrophages. **E**) Mean percentages ± SD of IRF8 expressing CD11b^{+}F4/80^{hi} and CD11b^{+}F4/80^{low} subsets in spleen and MN/MCA1 from Wt>Wt (filled bar) and RORC$^{-/-}$>Wt (open bar) chimeric mice measured by FACS analysis are shown.

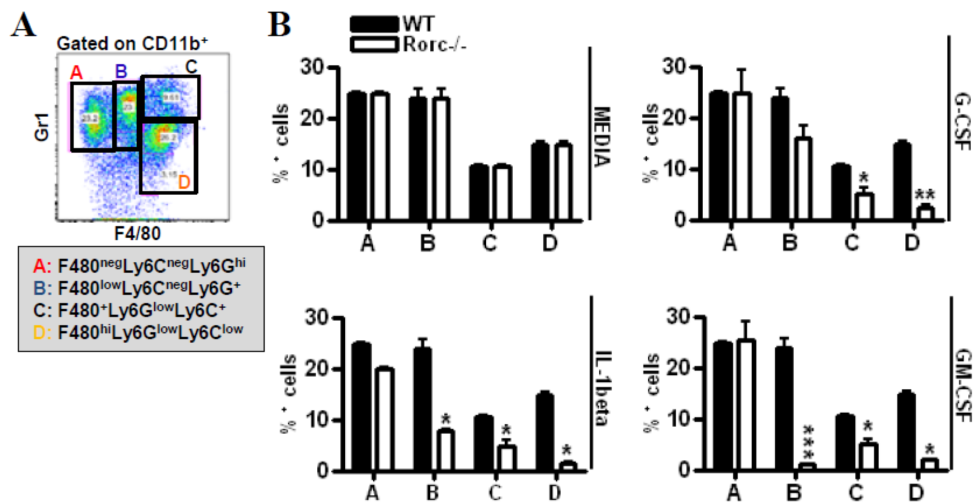

Figure S11- RORC is required for the commitment of granulocyte and monocyte/macrophage progenitors to master growth factors of "emergency" granulo-myelopoiesis.

A): The gating strategy used for identifying (A) F480^{neg}C^{neg}G^{high} granulocyte, (B) F480^{+}C^{neg}G^{+} monocyte, (C) F480^{+}G^{low}C^{+} macrophage/monocyte and (D) F480^{+}G^{low}C^{low} progenitor subsets is shown. **B**) (lin)$^{-}$c-kit^{+}Sca-1^{-} progenitor cells were isolated by magnetic bead separation from "steady state" Wt (filled bar) or RORC$^{-/-}$ (open bar) bone marrows and challenged with IL-1β, G-CSF or GM-CSF *in vitro* (*"*Methods*")*. Next, the distribution of granulocyte and monocyte/macrophage subsets was evaluated by FACS analysis. The represented data are mean percentages ± SD of (A), (B) and (C) progenitor subsets. Mean ± SD of 3 independent experiments is shown.

CHAPTER 4

Discussion

Circulating G-CSF and GM-CSF levels rise dramatically during inflammation and infection, eliciting rapid mobilization of mature neutrophils and monocytes from bone marrow and extra-medullary progenitor tissue reserves (i.e. spleen), that outstrip the capacity of steady-state myelolopoiesis and rely on distinct programs of accelerated "emergency" myelopoiesis [1-3]. While "emergency" myelopoiesis to infection or trauma increases rapidly the inflammatory neutrophil and monocyte/macrophage pools [1], chronic cancer inflammation-driven myelopoiesis converges in splenic accumulation of immature myeloid cells (MDSCs) [4,5] and macrophages recruitment at the tumor site (TAMs)[6,7]. While the pro-tumor functions of MDSCs and TAMs are well characterized[5,6], a large gap remains in our understanding of the mechanisms that translate persistent inflammation into reactive "emergency" myelopoiesis. IL-17 is becoming of great interest, as in response to inflammation or infection it supports G-CSF mediated "emergency" granulopoiesis [8-11] and promotes neutrophil apoptosis [12,13] and macrophage phagocytosis of early apoptotic neutrophil (ANs), a crucial mechanism for IL-10 induction in macrophages and resolution of inflammation [13]. Based on this, we hypothesized that IL-17 and the related transcription factor RORC might be involved in mediating the fate and functional polarization of myeloid components that arise during "emergency" myelopoiesis. In line with this hypothesis,

Zhang et al. showed that IL-17 enhanced the IFNγ-induced M1 polarization of macrophages, through increased STAT-1 phosphorylation, while it suppressed their IL-4–mediated M2 conversion via STAT-6 inhibition[14]. In addition, IL-10 and IL-10R-deficient macrophages stimulated with LPS produced large amounts of IL-17, whereas addition of exogenous IL-10 to IL-10 deficient macrophages abolished IL-17 secretion [15]. Recent data suggest an unexpected role for IL-17 in orchestrating resolution of innate inflammation, by promoting M2 differentiation and ensuring efficient clearance of apoptotic neutrophils [16].

Importantly, IL-17-mediated M2 polarization requires the presence of IL-10 [13], whose presence in the microenvironment is probably required to convert IL-17 from a pro-inflammatory to a regulatory molecule [17]. Consistent with this view, the activation of liver X receptors (LXRs) by apoptotic neutrophils, suppressed the IL-23/IL-17 cytokine cascade [18]. We demonstrate now that "emergency" myeloid hematopoiesis is mediated by the orphan retinoic nuclear receptor RORC (RORγ), and characterize novel subsets of IL-17^{+}RORC^{+} PMN-MDSCs, IL-17^{neg}RORC^{high} M-MDSCs and TAMs that arise strikingly in response to signals that drive "emergency" myeloid hematopoiesis (GM-CSF, G-CSF, IL-1β, TLR4 ligands). Our results demonstrate that RORC has a crucial role in expansion of pro-tumor myeloid populations, MDSCs and TAMs. In addition, we report that RORC acts as a potent inhibitor of M1-inflammation in neutrophils and macrophages, promoting expression of M2 genes in TAMs. We also show that RORC is required for the commitment of myeloid progenitors to granulocyte and monocyte/macrophage lineages by suppressing and promoting respectively the expression of negative (SOCS3, Bcl3) and positive (cEBPβ) transcriptional regulators of "emergency" granulo-myelopoiesis. In addition, RORC mediates macrophage-neutrophil plasticity through induction of the cell fate switching transcription factor IRF8, leading to terminal differentiation in F4/80^{low} tissue macrophages. In agreement, a recent study by Waight et al. demonstrated that during cancer-associated myelopoiesis G-CSF and GM-CSF-inhibited IRF8 to promote expansion of granulocytic/neutrophil-like MDSC [19].

RORC blocks terminal maturation and activation of monocytes and *granulocytes* and enhances M-MDSCs and PMN-MDSCs survival and expansion with decreased expression of the Fcγ receptor and M1 cytokines. Our data suggest that a crosstalk between neutrophils and monocytes/macrophages is crucial in mediating tissue homeostasis to persistent cancer inflammation.

Our results point toward RORC as a critical transcriptional regulator in mediating the fate of myeloid lineages and myeloid plasticity to inflammatory stress signals. This assumption is also supported by the fact that the transcriptome of macrophages and granulocytes is overwhelming similar [20].
Despite IL-17A has no direct role in mediating and programming “emergency” granulo-myelopoiesis and tissue homeostasis in processes of cancer inflammation, we propose IL-17A as novel indicator for immature myeloid responses in subjects with cancer. This hypothesis is supported by the observed drop of IL-17 expression in PMN-MDSCs located into the tumor microenvironment.
Lochner et al. have described a subset of $RORC^{+}Foxp3^{+}IL\text{-}10^{+}IL\text{-}17^{-}$ regulatory T cells that co-exist with $RORC^{+}IL\text{-}17^{+}IL\text{-}10^{neg}$ pro-inflammatory T cells [21]. Thus, potentially antagonistic pro-inflammatory IL-17–producing and regulatory $RORC^{high}$ immune cells uncoupled from IL-17 production coexist and are tightly controlled, suggesting that a perturbed equilibrium in $RORC/IL17/IL\text{-}10^{+}$ immune cells might lead to decreased immunoreactivity or, in contrast, to pathological inflammation. By depleting the accumulation of $RORC^{high}F4/80^{high}CD115^{+}$ TAMs, with antagonist antibody to CSFR1, we demonstrate that this population selectively suppresses the recruitment of mature ($CD16/CD32^{high}$) $RORC^{neg}$ neutrophils, favoring expansion of $RORC^{+}$ immature ($CD16/CD32^{low}$) PMN-MDSCs. Taken together, our results strongly suggest that high RORC expression act as pro-resolving mediator of myeloid inflammation, at the cellular and tissue level, by stopping recruitment of effector neutrophils to tissues. However, the pathways that sense inflammatory stress with consequent adjustment of the cross-talk monocytes/macrophages with neutrophils remain largely unknown.
Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) reside in specialized bone marrow (BM) niches and their mobilization from the BM to peripheral tissues is regulated by the sympathetic nervous system (SNS), as well as by hematopoietic growth factors that initiate “emergency” granulo-myelopoiesis as G-CSF [22].

Recent studies suggest that MSCs control the egression of HSCs from the bone marrow. In addition, Chow et al. have recently demonstrated that BM macrophages contribute to the retention of HSCs in the BM by acting on MSCs and suppressing myeloid cell mobilization in response to G-CSF [23]. These findings, together with our observation that M2-macrophages selectively suppress the migration of effector neutrophils to tissues, while promoting local expansion of immature myeloid cells, suggest that BM-derived tissue macrophages are key regulators of polarization and recruitment of myeloid progenitors and subsets to tissues, as well as by mediating mesenchymal stem cell spreading to tissues. Thus, antagonists to RORC might hold the potential to augment stem cell mobilization in cancer patients [24] and to block tumor progression by inhibiting expansion and accumulation of immature myeloid cells. The selective expression of nuclear receptors by myeloid cells to inflammatory stress signals, becomes interesting as several members of the nuclear receptor superfamily, including GR, VDR, liver X receptor (LXR), PPARs, and the retinoic acid orphan nuclear receptor family members RORα and RORC (RORgamma) regulate inflammation [25-26] and plausibly HSC niche homeostasis. In addition, RORC appears as a major regulator of tissue homeostasis being critical mediator of adipogenesis [27], insulin sensitivity [27] and the body clock [28]. RORC agonists might also provide novel myeloid-based pharmacological approaches in promyelocytic leukaemia (APL) and acute myelogenous leukaemia (AML), both characterized by a dramatic increase of immature myeloid progenitors. Indeed, All-trans retinoic acid (ATRA), a ligand for retinoic orphan nuclear receptors beta and gamma [29], induces differentiation and apoptosis of immature leukemic myeloid progenitors [30].

In summary, we provide the first evidence of a critical role for RORC expression in the transcriptional control and development of MDSC subsets and TAMs.

We demonstrate that in response to inflammatory stress monocytes/macrophages and neutrophils are controlled by RORC and their cross-talk is required to control the prevalence of effector neutrophils in tissues.

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CHAPTER 5

Effects of the chemotherapeutic drug Topotecan on anti-tumor immunity

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Unpublished results

Abstract

Recent studies report that some chemotherapeutic agents enhance antitumor immune responses through their capacity to eliminate immunosuppressive cells or trigger immunogenic cell death of tumor cells.

Here we demonstrated that the chemotherapeutic drug Topotecan (TPT) has an effect on the immunogenicity of dendritic cells (DCs) and the immunosuppressive activity of myeloid derived suppressor cells (MDSCs).

On both mouse and human DCs, TPT treatment significantly increases antigen processing.

TPT also up-regulates DC production of pro inflammatory cytokines (IL-1β,IL-12) and increases DC-mediated T cell-activation *in vitro* resulting in a skewing of T cell polarization towards type 1 (Th1).

Moreover, TPT partially blocks the suppressive capacity of mouse MDSCs *in vitro* in terms of nitric oxide production. Therefore MDSCs result less suppressive when tested in an antigen-specific co-culture assay *in vitro*.

Our preliminary results indicate that TPT, already approved for clinical treatment of selected cancer conditions, can affect the biology of myeloid cells.

Introduction

Immune responses against cancer are an important factor determining tumor evolution [1,2]. In many experimental and human cancers, T cell immune responses are involved in tumor growth control and restrain tumor progression.

By contrast, the role of cytotoxic agents on immune responses remains largely unresolved. Chemotherapy has long been suspected to induce systemic immunesuppression [3]. However, recent studies report that some chemotherapeutic agents can modify the tumor microenvironment to promote an effective anti-tumor immune response [4]. Chemotherapy can modulate the tumor microenvironment by inducing apoptosis in tumor cells themselves, thus potentially augmenting antigen processing and presentation through cross-priming [5]. Chemotherapy may also augment antitumor immunity by altering the tumor microenvironment to favor a more productive tumor-specific immune response [5].

Hypoxia is defined as the reduction of oxygen levels in organs, tissues or cells [6]. This decrease of oxygen tension is characteristic of infected tissues, wounds, rheumatic joints, and areas of tumors but also of normal spleen and joints [7]. Tumor cell oxygen deficiency is a key environmental stressor associated with resistance to radiation therapy and chemotherapy, selection of more invasive and metastatic clones and poor patience prognosis [8]. The hypoxic environment attracts infiltrating immune cells that move against oxygen gradients and respond to demanding conditions by switching to anaerobic metabolism to maintain their energy requirements. Several lines of evidence suggest that oxygen deprivation causes opposite effects on the innate and adaptive immunity in terms of cell survival and expression of immune functions [9]. Tumor-associated macrophages (TAMs), one of the major players in the connection between inflammation and cancer promoting disease progression, accumulate in tumor hypoxic areas and respond to the levels of hypoxia with a transcription program in which mitogenic, proinvasive, proangiogenic and prometastatic genes are up-regulated[10].

Also, hypoxia dissociates the inflammatory and tissue repair functions of dendritic cells (DCs) from their capacity to act as sentinels for adaptive immunity[11].

It has been demonstrated that hypoxia inhibits the expression of several differentiation and maturation markers in response to lipopolysaccharide (LPS), as well as DC stimulatory capacity for T-cell functions (11). These events are paralleled by impaired up-regulation of the chemokine receptor CCR7, an otherwise necessary event for the homing of mature DCs to lymph-nodes. In contrast, hypoxia strongly up-regulates the production of proinflammatory cytokines, particularly TNFα and IL-1β. Responses to hypoxia are mostly orchestrated by activation of the hypoxia-inducible factor (HIF) family of transcription factors[12].

The significance of HIF contribution to tumor growth is well documented. Significant association between HIF-1α over-expression and patient mortality has been shown in cancers of the brain, breast, cervix, oropharynx, ovary and uterus **[8].** A number of HIF-inducible genes, such as VEGF, mediate key steps in tumorigenesis. These features make HIF-1 an attractive target for therapy [13].

The majority of HIF-1 inhibitors (small molecules) identified so far are the results of either cell-based high throughput screening or empirical discoveries.

These compounds target upstream (inhibitors of HIF-1α protein translation, inhibitors of oncogenic pathways) or downstream (inhibitors of HIF DNA binding activity or HIF transcriptional activity) the transcription factor [12].

Beside its direct role in cancer cells, HIF has a crucial role in the cells of the tumor microenvironment. Up-regulation of HIF-1α and HIF-2α in myeloid cells exposed to hypoxia leads to increased transcription of many genes that regulate cell proliferation, metabolism, angiogenesis and immune-suppression [14,15].

The study of HIF inhibitors activity on cancer infiltrating cells is one line of research that still needs to be pursued. At present, whether and at which extent HIF inhibitors affect myeloid cells present in the hypoxic areas of solid tumors is unknown.

The effect of HIF inhibitors on infiltrating cells may indirectly contribute to tumor responsiveness to the therapy.

Topotecan (Hycamtin®) is a semisynthetic derivative of camptothecin [16].

This drug reversibly binds to and stabilizes the Topoisomerase 1 (Top 1) enzyme on the DNA, which in the presence of DNA replication, causes DNA damage and cytotoxicity. In a cell-based high throughput screening of the National Cancer Institute "Diversity Set" library, Topotecan was identified as a potent inhibitor of hypoxia inducible factor 1α (HIF-1α) activity[16].

The primary indication of topotecan is second-line therapy against advanced ovarian carcinoma. It was also granted FDA approval as a therapeutic option for recurrent SCLC (small cell lung cancer). Different studies show that this chemotherapeutic agent may induce cancer cell apoptosis not only by increasing the proapoptotic signal but also by decreasing the antiapoptotic signal, such as the PI(3)K-Akt survival pathway [17].

In this project we will characterize the immunomodulating properties of TPT whit a special focus on two myeloid population involved in tumor development: Dendritc Cells (DCs) and Myeloid-derived Suppressor Cells (MDSCs).

Here we show that the chemotherapeutic drug Topotecan (TPT) has an effect on the immunogenicity of dendritic cells (DCs) and the immunosuppressive activity of myeloid derived suppressor cells (MDSCs). It promotes antigen processing by DCs and the skewing of T cell polarization towards type 1 (Th1) known to be involved in antitumor immunity. Topotecan also reduces the suppressive activity of MDSCs reducing nitrite production.

Materials and methods

Reagents

Cell culture media, glutamine, antibiotics, and aseptically collected fetal bovine serum (FBS) were from Lonza. β-mercaptoethanol was from Sigma Aldrich. Cytokines were from PeproTech. All reagents contained <0.125 endotoxin units/ml, as checked by the *Limulus* amebocyte assay (Microbiological Associates, Walkersville, MD). LPS from *Salmonella Abortus Equi* S-form and Topotecan were obtained from Alexis.

Mice

Unless otherwise specified, C57BL/6J mice were used. OT-I mice were from Jackson Laboratories. Mice were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996 edition), under protocols approved by the institute Animal Care and Use Committee. Mice were monitored daily and euthanized if displaying excessive discomfort. The study was designed in compliance with principles set out in the Italian Governing Law, EU directives and guidelines.

Dendritic cells culture

Human DCs were differentiated from peripheral blood monocytes of healthy donors (18). Highly enriched blood monocytes were obtained from buffy coats (through the courtesy of the Ospedale di Desio, Milan, Italy) by Ficoll and Percoll (Amersham Biosciences) gradients as previously described (19). Monocytes were incubated for 6 days in complete RPMI medium (RPMI1640 medium, 10% FBS, 2mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin) supplemented with 50 ng/ml GM-CSF and 20 ng/ml IL-4. At day 6, immature DCs (>90% $CD1a^+$) were collected, resuspended at 106 cells/ml and stimulated as indicated in the text.
Mouse DCs were generated from bone marrow (BM) precursor cells (20).

BM cells, obtained from mouse femurs and tibias, were resuspended in complete IMDM medium (IMDM medium, 10% FBS, 2mM glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, 50 μM β-mercaptoethanol) supplemented with 30% of conditioned medium from GM-CSF-expressing NIH-3T3 cells (21), and seeded in Suspension culture dishes (Corning). The medium was replaced at days 3 and 7. At day 9, immature DCs (>90% $CD11c^+$) were collected, resuspended at 106 cells/ml and stimulated as indicated in the text. Hypoxia treatment was performed by placing cells in a InVivo2 400 hypoxic workstation (Ruskinn-Biotrace, Bridgend, United Kingdom) maintained at 1% O_2 and 37°C, or in a modular incubator chamber (Billups-Rothemberg, Del Mar, CA) flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2 and placed at 37°C in a conventional cell culture incubator (11).

Myeloid-derived suppressor cells culture

Mouse bone-marrow-derived MDSCs were obtained by incubation of bone marrow cells in complete RPMI medium supplemented with mIL6, mGM-CSF, and mG-CSF (40 ng/ml each) (22). At day 4, cells (>90% $CD11b^+$ $Gr1^+$) were collected and stimulated as indicated in the text. Splenic MDSCs ($Ly6C^+$ $CD11b^+$ cells) were purified from the spleen of tumor-bearing mice by magnetic separation (Miltenyi Biotec). MN/MCA1 fibrosarcoma cells (23) were inoculated intramuscularly in the leg and tumor growth was monitored with a caliper 3 times a week.

When tumors reached 3cm3 of volume, mice were euthanized, and spleens were collected and smashed to obtain a single cell suspension. MDSCs were first enriched by consequent serial negative selections with CD19, CD11c and Ly6G microbeads according to manifacturer,. Finally, remaining cells were positively selected with $CD11b^+$ microbeads. all these cells were aloso Ly6C positive and the purity, evaluated by flow cytometry, exceeded 90%.

Flow cytometry

Cell were incubated with fluorescence-labeled antibodies and analyzed with a FACS Canto I flow cytometer (Becton Dickinson) using the BD FACSDiva software. Anti-hCD1a, anti-hCD80, anti-hCD83, anti-hCD86, anti HLA-DR were from BD pharmingen; anti-mCD11c, anti-mCD86, anti-CD40, anti-I-A/I-E (MHC II) were from e-Bioscience; anti-mCD11b was from Biolegend; anti-mLy6C was from Miltenyi Biotec.

Mixed lymphocyte reaction (MLR)

DCs were plated at different concentrations in 96-well round-bottom microtest plates, in complete RPMI medium and matured in the absence or the presence of TPT for 24h. Each group was performed in triplicate. Cells were washed and 1 x 105 lymphocytes from peripheral blood of a different individual (for human DCs) or 1 x 105 splenocytes of a BALBc mouse (for mouse DCs) were added.

Co-cultures were incubated for 3 (mouse cells) or 5 days (human cells).

An aliquot of supernatant was collected to test IFN production, then cells were pulsed with 1 Ci/well [^{3}H] thymidine (Amersham). After 16 hours, cells were harvested using a Cell Harvester (Tomtech), and radioactivity was measured in a Liquid Scintillation Counter (Trilux MicroBeta, Wallac).

Macropinocytosis assay

DCs, matured in the absence or the presence of TPT, were incubated in complete medium with fluoroscein isothiocyanate-conjugate 70kDa dextran (2 mg/ml; Sigma Aldrich) at 37°C or at 0°C (negative control) for 1h in the dark, washed and analyzed by flow cytometry. Background fluorescence measured when the samples were incubated at 0°C was subtracted to the values of fluorescence measured when cells were incubated at 37°C.

Antigen-processing assay

DCs, matured in the absence or the presence of TPT, were incubated in complete medium with DQ-ovalbumin (DQ-OVA, 0.2 mg/mL; Molecular Probes) for 1h in the dark at 16°C to allow DQ-OVA internalization but not its processing.

DCs were then extensively washed and resuspended in complete medium and seeded in triplicates in 96-well plates incubated at 37°C or in ice (negative control). At the indicated time points, cells were transferred on ice to block protein processing. Fluorescence was analyzed by flow cytometry and results expressed as Mean Fluorescence Intensity (M.F.I.).

Nitrite production

Nitric oxide released in the MDSCs culture supernatant was evaluated by measuring nitrite accumulation at different time points using the Griess Reagent System (Promega) according manufacturer's protocol.

Real time PCR

Total RNA was extracted with TRIzol (Invitrogen) and reverse transcription of 1 μg of RNA was performed using the cDNA Archive kit (Applied Biosystems, AB). Real-time PCR was performed using the Fast Syber Green PCR Master Mix (AB) and detected by the 7900HT Fast Real-Time System (AB). Specific primers: miNOS Fw: gccaccaacaatggcaaca; mINOS Rev: cgtaccggatgagctgtgaatt; -actin FW: cccaaggccaaccgcgagaagat; -actin Rev: gtcccggccagccaggtccag.

Results were normalized to the expression of the housekeeping gene and expressed as fold up-regulation, with respect to the control cell population.

Suppression assay

MDSCs were plated at different concentrations in 96 well plates, in complete RPMI medium. Cells were stimulated with IFNγ (200U/ml), in the absence or the presence of TPT, for 72h. MDSCs were washed and 2 x 105 splenocytes from OT-I mice were added for additional 72h in the presence of 250 μg/ml ovalbumin (OVA) protein (Sigma). An aliquot of supernatant was collected to test NO production, then cells were pulsed with [^{3}H] thymidine (1 Ci/well, Amersham). After 16 hours, cells were harvested using a Cell Harvester (Tomtech), and radioactivity was measured in a Liquid Scintillation Counter (Trilux MicroBeta, Wallac).

Statistical analysis

Statistical significance was determined by Student's t. *$P < 0.05$; **$P < 0.01$; ***$p < 0.001$.

Results

Human DC treated with topotecan are more immunogenic.

Previous work from our laboratory demonstrated that hypoxia interferes with the maturation of DCs (11). We wanted to investigate if, by inhibiting the Hypoxia-Inducible factor (HIF), the master regulator of cellular responses to hypoxia, we could revert the effects of hypoxia on human monocyte-derived dendritic cells (MDDCs). We performed preliminary experiments with two drugs that have been reported to inhibit HIF: topotecan (TPT) and echinomycin (ECH) (24).

ECH was toxic on DCs at the tested concentrations (5-20 nM, data not shown), therefore its use was abandoned. On the contrary, MDDCs well tolerated the treatment with TPT, up to 1 M (data not shown).

We selected 500 nM as working concentration for DC treatment.

First, we analyzed the phenotype of MDDCs immature (CTRL) and matured with lipopolisaccharide (LPS) in the absence or in the presence of TPT in normoxic (20% O^2) and hypoxic (1% O^2) conditions. TPT did not significantly modify the expression of CD80, CD86, CD83, and HLA-DR, neither in normoxia or in hypoxia (Figure 1 A). As expected, when MDDCs were cultured in hypoxic conditions, markers expression was lower than in normoxia (12).

We measured the release of IL-1β and, as expected, MDDCs released greater amounts of this cytokine when matured in hypoxia (Figure 1B). Surprisingly, the treatment with TPT not only did not inhibit IL-1β production, as hypothesized, but promoted LPS-induced IL-1 release, both in normoxia and in hypoxia.

The effect was reproducible in all cell preparations tested, with a 2-fold increase in the TPT –treated DCs compared to control mature DCs.

Next, we evaluated the ability of DCs to capture, process and present the antigen to T cells. The treatment with TPT did not alter antigen uptake by macropinocytosis, as assessed in a FITC-dextran internalization assay (Figure 1C).

MDDCs matured in hypoxia showed an increase, albeit not significant, in their capacity to internalize FITC-dextran particles. This is in line with the notion that DCs matured in an hypoxic environment retain some characteristics of immature cells (11). When we measured the antigen-processing ability of DCs by using self-quenched dye conjugate ovalbumin (DQ-OVA), that exhibits green fluorescence upon proteolytic degradation, we found that the treatment with TPT enabled MDDCs to process the antigen more efficiently (Figure 1D).

Finally, we tested the capability of DCs to activate T cells in a Mixed-leukocyte Reaction (MLR). MDDCs were matured with LPS in the absence or the presence of TPT, washed, and co-cultured with allogeneic lymphocytes.

MDDCs matured with LPS promoted lymphocyte proliferation, independently from the treatment with TPT (Figure 1 E). However, T cells activated by co-culture with TPT-treated MDDCs produced higher amount of IFN, suggesting that TPT can induce a skewing of T cell polarization towards type 1 (Th1).

TPT-treatment consistently showed an effect both in normoxia and in hypoxia, although the statistical significance was reached only when DCs were matured in normoxia (Figure 1F; normoxia $p=0.027$, hypoxia $p=0.066$). These results indicate that TPT enhances the ability of human DCs to release the pro-inflammatory cytokine IL-1β and, more importantly, to process the antigen and to promote IFNγ production by T cells. Possibly, this activity of TPT is independent from HIF-inhibition, as suggested by the fact that the regulatory effect of TPT on DCs is present also in normoxic conditions.

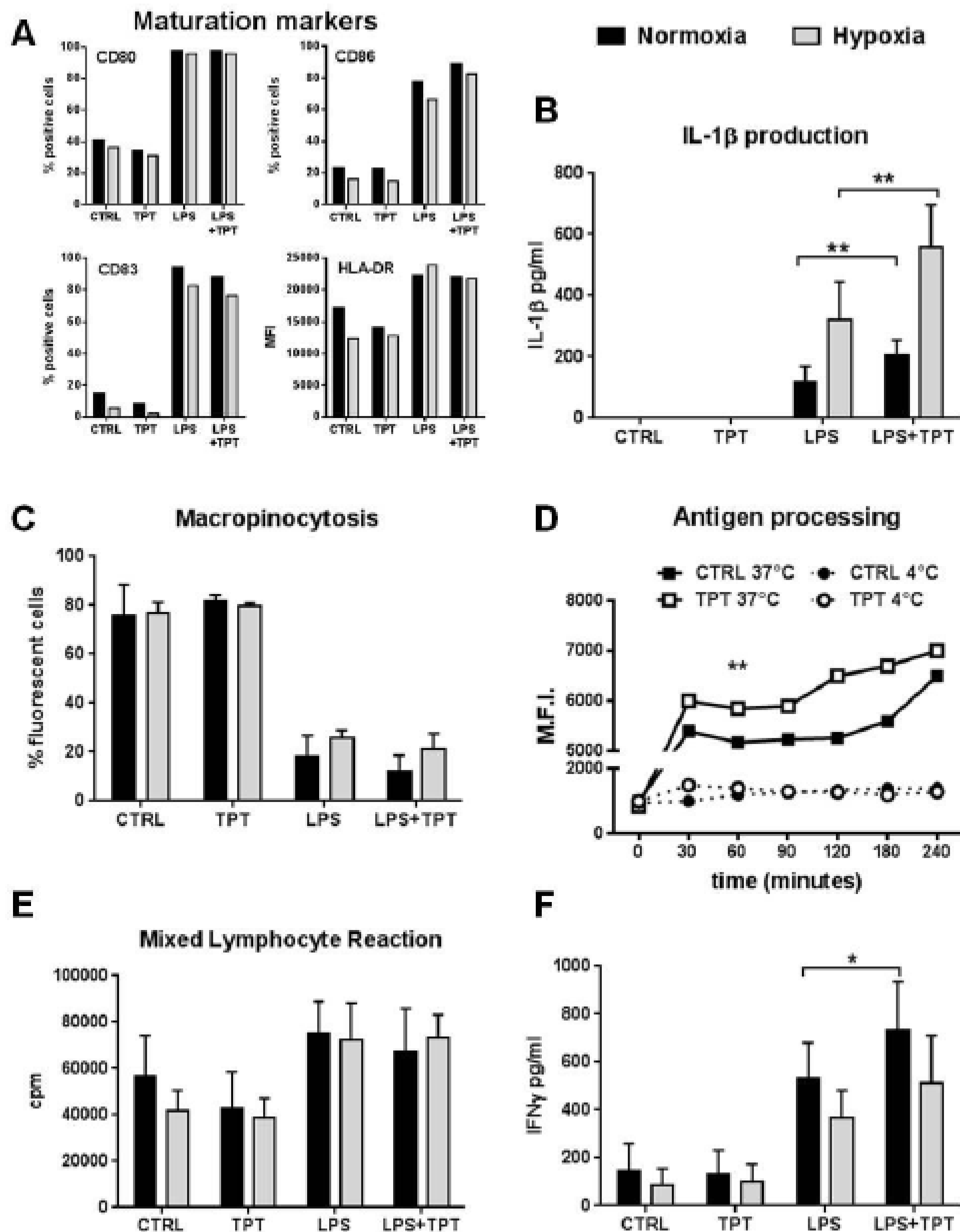

Figure 1- Effects of TPT on the maturation of human DCs. Human monocyte-derived DCs (MDDCs) were matured with LPS (100 ng/ml) in the absence or in the presence of topotecan (500 nM) in normoxic (20% O^2) or hypoxic (1% O^2) conditions for 24h. A) Flow cytometry analysis of maturation markers expression; CD80, CD86, and CD83 expression was measured as percentage of positive cells, HLA-DR expression was evaluated as Mean Fluorescence Intensity (MFI);
data from 1 out of 4 independent experiments with similar results are shown.

B) Measurement of IL-1β production by ELISA. The graph shows mean values SD (n=7, **p<0.01). C) Macropinocytosis assay: flow cytometry analysis of the uptake of FITC-labelled dextran particles (measured as green fluorescence cell positivity). The graph shows mean values SD (n=3). D) Antigen processing assay: flow cytometry analysis of DQ-OVA processing (measured as MFI of green fluorescence-positive cells). Data from 1 out of 3 independent experiments with similar results are shown (experiments were performed in triplicate; ** P<0.01). E and F) Mixed Lymphocyte Reaction (MLR): MDDCs were matured in the absence or in the presence of TPT, in normoxia or hypoxia, washed and co-cultured with allogeneic T cells (ratio 1:30) for 5 days.
The proliferation of T cells was evaluated by incorporation of [^{3}H]-thymidine in the last 16h of culture (E); the release of IFNγ in cell supernatant was measured by ELISA (F). Graphs show the mean values SD of 3 independent experiments performed in triplicate (n=3; * p<0.05).

Mouse DCs treated with TPT have a higher immunostimulant activity.

In the next series of experiments, we tested TPT on mouse bone-marrow derived DCs (BMDCs). As human DCs responses to TPT treatment did not seem to be influenced by oxygen levels (see above), we decided to continue our study using normoxic conditions only. Incidentally, preliminary experiments did not reveal significant differences between results obtained in normoxic or in hypoxic conditions in terms of phenotype and ability to stimulate T cells to proliferate and produce IFNγ (data not shown). The positive effect of TPT on IL-1β production by mature DCs was present both in normoxia and hypoxia, although the levels of
IL-1β released in hypoxia were higher (data not shown). Results obtained with mouse DCs corresponded to those obtained with human DCs. TPT did not significantly modified the expression of maturation markers (CD86, CD40, MHC class II), but enhanced IL-1β production by mature BMDCs (Figure 2A).
TPT-treated BMDCs showed an increased capacity to process the antigen compared to control cells (Figure 2B). As observed for human cells, BMDCs matured in the presence or in the absence of TPT induced allogeneic lymphocyte proliferation at a similar degree (Figure 2C).

Nonetheless, lymphocytes activated by BMDCs treated with TPT produced more IFNγ (Figure 2D). Thus, TPT has a potentiating effect on the immunogenicity both of mouse and of human DCs.

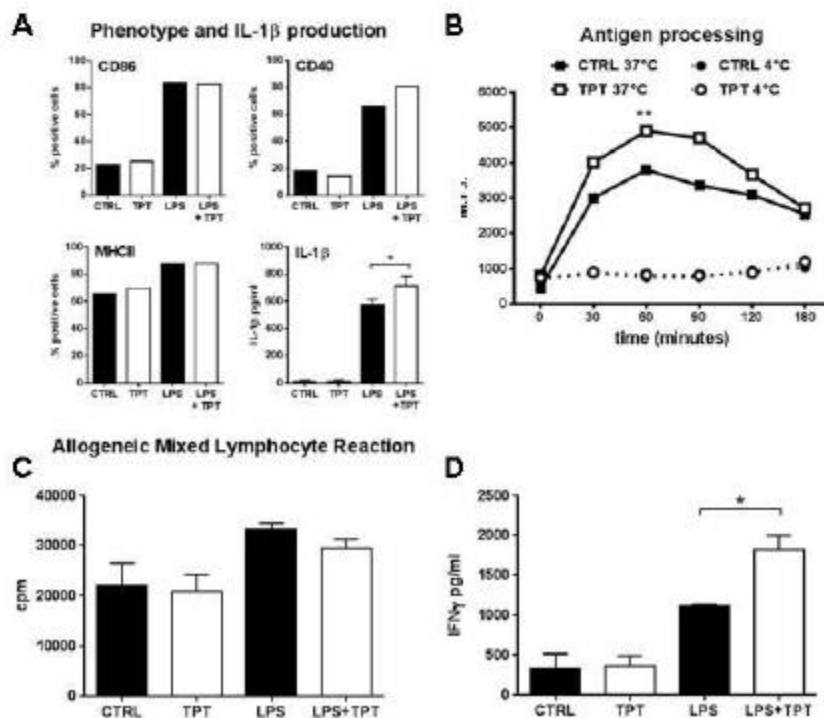

Figure 2- Effects of TPT on the maturation of mouse DCs. Mouse bone marrow-derived DCs (BMDCs) were matured with LPS (100 ng/ml) in the absence or in the presence of topotecan (500 nM) for 24h. A) Flow cytometry analysis of maturation markers expression and measurement of IL-1β production by ELISA. For the phenotype, data from 1 out of 3 independent experiments with similar results are shown. For IL-1 release, the graph shows mean values SD (n=3, *p<0.05). B) Antigen processing assay: flow cytometry analysis of DQ-OVA processing (measured as MFI of green fluorescence-positive cells). Data from 1 out of 3 independent experiments with similar results are shown (experiments were performed in triplicate; ** P<0.01). C and D) Mixed Lymphocyte Reaction (MLR): BMDCs were matured in the absence or in the presence of TPT, in normoxia or hypoxia, washed and co-cultured with allogeneic T cells (ratio 1:27) for 3 days. The proliferation of T cells was evaluated by incorporation of [^{3}H]-thymidine in the last 18h of culture (C); the release of IFNγ in cell supernatant was measured by ELISA (D). Data from 1 out of 2 independent experiments with similar results are shown (experiments were performed in triplicate; ** P<0.01).

Mouse MDSCs treated with TPT have a lower immunosuppressive activity.

We investigated the effect of TPT on another population of myeloid cells, whose role is relevant in anti-tumor immunity, namely myeloid-derived suppressor cells (MDSCs). One of the mechanisms by which MDSCs exert immunosuppressive activity is through the release of nitric oxide (NO). We observed that bone marrow-derived MDSCs (BMMDSCs) activated in the presence of TPT expressed lower levels of the enzyme inducible nitric oxide synthase (iNOS) (Figure 3A), and that the production of NO when MDSCs were stimulated with IFNγ was significantly reduced (Figure 3B). Importantly, splenic MDSCs purified from tumor-bearing mice treated with TPT *in vitro* were less suppressive compared to control MDSCs, when tested in an antigen-specific co-culture assay (Figure 3C and D).

These data suggest that TPT acts also on leukocytes that have a crucial role in impairing anti-tumor immunity, contrasting their immunosuppressive activity.

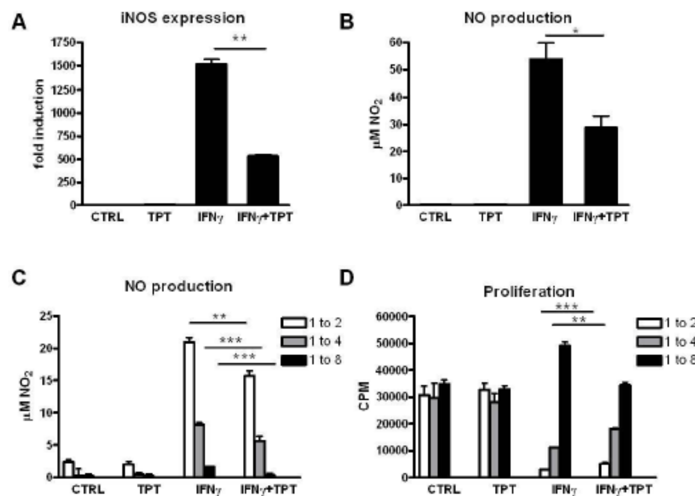

Figure 3- Effects of TPT on the activity of MDSCs. A-B) Mouse bone marrow-derived MDSCs were stimulated with IFNγ (200 U/ml) in the absence or in the presence of topotecan (500 nM) for 24h (A) or 48h (B). A) iNOS mRNA levels were assessed by real-time PCR.

Data from 1 out of 2 independent experiments with similar results are shown (experiments were performed in triplicate; ** P<0.01). B) NO production was measured using the Griess Reagent System. Data from 1 out of 2 independent experiments with similar results are shown (experiments were performed in triplicate; * P<0.05). C-D) Splenic Ly6C+ MDSCs were purified from tumor-bearing mice and cultured with IFNγ (200 U/ml) in the absence or in the presence of topotecan (500 nM) for 72h. Splenocytes from OT-I mice were then added for additional 72h in the presence of 250 g/ml OVA (ratio MDSCs: splenocytes: 1:2, 1:4, 1.8). C) NO production was measured using the Griess Reagent System. Data from 1 out of 2 independent experiments with similar results are shown (experiments were performed in triplicate; *** P<0.001). D) The proliferation of splenocytes was evaluated by incorporation of [^{3}H]-thymidine in the last 16h of culture. Data from 1 out of 2 independent experiments with similar results are shown (experiments were performed in triplicate; ** P<0.01, *** p<0.001).

Discussion

Chemotherapy is the treatment of choice for various tumors [5].

However, complete remission requires a sustained antitumor immune response in order to efficiently eradicate tumor cells that have survived chemotherapy.

Accumulating evidence indicates that the innate and adaptive immunity make a crucial contribution to the antitumor effects of conventional chemotherapy.

The immune system is elicited in two ways by conventional therapies.

Some therapeutic programs can elicit specific cellular responses that render tumor cell death immunogenic. Other drugs may have side effects that stimulate the immune system through transient lymphodepletion, by the subversion of immunosuppressive mechanisms or through direct or indirect stimulatory effects on immune effectors. Exemples of chemotherapeutic drugs that act on the immune system are gemcitabine and 5-fluorouracil.

Gemcitabine is a sintethic pyrimidine nucleoside analogue that is efficient in the treatment of pancreatic, breast and lung cancers. It inhibits B-cell proliferation and antibody production in response to tumor antigens, a phenomenon that may skew antitumor humoral immunity towards beneficial T-cell responses.

Moreover, Gemcitabine reduces the frequency of MDSCs. Gemcitabine-induced apoptosis of established tumours may enhance the DC-dependent cross-presentation of tumour antigens to T cells [25].

5-Fluorouracil (5FU) is a fluoropyrimidine that is commonly used against breast cancer and gastrointestinal malignancies. In vitro, 5FU induces the expression of heat shock proteins (HSPs) in tumour cells and facilitates antigen uptake by DCs and subsequent cross-presentation of tumour antigens [26]. Vincent et al. showed that 5FU showed a stronger efficacy over gemcitabine to deplete MDSCs and selectively induced MDSCs apoptotic cell death in vitro and in vivo [27].

The elimination of MDSCs by 5FU increased IFNγ production by tumor-specific CD8(+) T cells infiltrating the tumor and promoted T cell-dependent antitumor responses in vivo.

In this project we have characterized the effect of TPT on the activity of two myeloid populations: Dendritic Cells (DCs) and Myeloid-derived Suppressor Cells (MDSCs). First we analyzed the effect of TPT on human DCs.

TPT did not significantly modify markers expression neither in normoxia or in hypoxia. Surprisingly, the treatment with TPT not only did not inhibit IL-1β production, as hypothesized, but promoted LPS-induced IL-1 release, both in normoxia and in hypoxia. The treatment with TPT did not alter antigen uptake by macropinocytosis, as assessed in a FITC-dextran internalization assay.

When we measured the antigen-processing ability of DCs, we found that the treatment with TPT enabled DCs to process the antigen more efficiently.

Finally, we tested the capability of DCs to activate T cells in a Mixed-leukocyte Reaction (MLR). T cells activated by co-culture with TPT-treated DCs produced higher amount of IFNγ, suggesting that TPT can induce a skewing of T cell polarization towards type 1 (Th1). Results obtained with mouse DCs corresponded to those obtained with human DCs. These results indicate that TPT enhances the ability of DCs to release the pro-inflammatory cytokine IL-1 and, more importantly, to process the antigen and to promote IFNγ production by T cells.

Thus, TPT has a potentiating effect on the immunogenicity both of mouse and of human DCs. In addition, we tested the effect of TPT on MDSCs. We observed that TPT treated bone marrow-derived MDSCs (BMMDSCs) expressed lower levels of the enzyme inducible nitric oxide synthase (iNOS). Thus, splenic MDSCs resulted less suppressive when tested in an antigen-specific co-culture assay because of the reduced production of NO.

These data suggest that TPT acts also on leukocytes that have a crucial role in impairing anti-tumor immunity, contrasting their immunosuppressive activity.

Our studies demonstrated that TPT could augment antitumor immunity by altering the tumor microenvironment to favor a more productive tumor-specific immune response.

Further studies are needed to investigate the effect of Topotecan.

TPT seems to be more active in normoxic condition than in hypoxia.

This evidence suggest an hypoxia-indipendent, but probably HIF-dependent effect of TPT on DCs. In this view we decided to evaluate the involvement of other target such as NF-kB, whose activity is strictly associated with HIF. In fact, basal NF-kB activity appears to be required for HIF-1α mRNA expression and protein accumulation under hypoxia [28]. In this regard, it is interesting to point out that many cancers chemotherapeutics (e.g. etoposide, cisplatin, paclitaxel and vinblastine) are known to activate NF-kB [29,30].

As previously described, the chemotherapeutic drug TPT promotes an increased release of IL-1β protein by DCs. On the basis of this finding, we will investigate if this induction is due to a transcriptional effect or to the activation of the inflammasome-caspase 1 pathway. It was shown that the NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer [31]. Mice lacking the inflammasome adaptor protein PYCARD (ASC) and caspase-1 demonstrate increased disease outcome, morbidity, histopathology, and polyp formation. The increased tumor burden is correlated with attenuated levels of IL-1β and IL-18 at the tumor site. In this regard, we will evaluate if TPT can affect the relationship between tumor growth and inflammasome activation in mice.

We will use NLRP3 ko mice as control in this experiment.

We will test caspase 1 activation in different myeloid population (DCs, MDSCs, TAMs) localized in the tumor microenvironment.

To further evaluate the role of TPT in DC-mediated T cell activation, we will perform *in vitro* experiments of antigen specific T cell activation.

We will use antigen-specific transgenic T cells and coculture them with Topotecan-treated DCs pulsed with the same antigen. T cell activation will be evaluated in terms of proliferation and secretion of IFNγ.

We will then test the capability of treated DCs to stimulate T cells *in vivo*. Topotecan-treated or untreated DCs will be loaded with antigen and injected in the footpad of the animal. The draining lymph node will be recovered, cells will be restimulated with antigen peptide and IFNγ production will be measured.

This point will be useful to evaluate the possibility to design a DC-based vaccination protocol. Tumor vaccines offer the potential for preventing cancer in those at high risk for disease development, preventing relapse in those diagnosed with early cancer, and treating advanced disease. The ability of vaccines to induce a response robust enough to mediate tumor rejection is limited by the extent of disease burden, the suppressive effect of the local tumor microenvironment, and multiple layers of systemic immune tolerance established to keep the immune response turned off. Chemotherapy can be used with tumor vaccines in unexpected ways, breaking down these barriers and unleashing the full potential of the antitumor immune response [32].

Tanaka et al. investigated the *in vivo* antitumor effects of intratumoral (i.t.) administration of dendritic cells (DC) after lowdose chemotherapy using cisplatin + 5-FU [33]. Combination of i.t. injection of DC and systemic chemotherapy induced complete rejection of the treated tumor, MC38 murine adenocarcinoma. The treatment with both anticancer drugs and DC achieved significant inhibition of tumor growth of not only the injected tumor, but also the tumor at the distant site, then significantly prolonged the survival time.

Treatment with DC alone, or chemotherapy alone, generated effective inhibition of tumor growth, but the antitumor effect was marginal compared with that of co-treatment with both anticancer drugs and DC.

We will try to compare the effect in tumor bearer mice of DCs treated with TPT, TPT alone or the combination of TPT and TPT-treated DCs. This approach could permit to reduce TPT dosage during the treatment.

Sustained high doses of chemotherapeutic drugs can cause severe side-effects that include irreversible damage to vital organs and can be themselves carcinogenic. Therefore, alternative or adjuvant therapies are required that are capable of replacing, or reducing the delivery dose of, chemotherapeutic drugs.

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CHAPTER 6

List of publications

- Cristiano Garino, **<u>Francesco Zitelli</u>**, Fabiano Travaglia , Jean Daniel Col sson, Giancarlo Cravotto , and Marco Arlorio (2012) Evaluation of the Impact of Sequential Microwave/Ultrasound Processing on the IgE Binding Properties of Pru p3 in Treated Peach Juice, J. *Agric. Food Chem.*, *60* (35), pp 8755–8762