UNIVERSITÀ DEGLI STUDI DI PADOVA

Università degli Studi di Padova

Dipartimento di Scienze Oncologiche e Chirurgiche

Scuola di Dottorato di Ricerca in

Oncologia e Oncologia Chirurgica

CICLO XXIV

Role of Arginase 1 and Nitric Oxide Synthase 2 as enzymatic mediators of the immunosuppressive activity in tumor-infiltrating myeloid derived suppressor cells

Direttore della Scuola: Ch.ma Prof.ssa Paola Zanovello

Supervisore: Prof. Vincenzo Bronte

Dottoranda: Dott.ssa Serena Zilio

Anno accademico 2010-2011

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Summary

MDSCs (myeloid derived suppressor cells) are one of the most important immunoregulatory populations involved in the generation of a permissive environment allowing tumor escape, progression and spreading. In physiologic conditions MDSCs protect the organism from exacerbated immune responses in order to protect surrounding tissues from damage. During tumor growth, cancer cells produce TDSFs (tumor derived soluble factors), which induce MDSC accumulation and their acquisition of a persistent suppressive activity. Among the several mechanisms exploited by MDSCs to exert their suppressive function, L-arginine metabolism seems to be fundamental for the alteration of the tumor microenvironment and for the generation of T effector cell dysfunctions.

This work highlights the *in vivo* requirement for ARG1 and NOS2 in the biology of MDSCs during tumor development. MDSCs are known for their phenotypic and functional complexity. It is clear that many factors can shape their identity and function: here we show, indeed that tumor microenvironment can “educate” them progressively. We illustrate that ARG1 and NOS2 are fundamental for MDSC-mediated T cell suppression *in vivo* but also for the expansion of the suppressive subsets of MDSCs. L-arginine metabolism mediated by these enzymes is crucial for tumor spreading and metastasis, as depletion of either enzyme produces a 50-75% reduction in the number of lung metastases. Finally, we show that the effects mediated by the novel drug AT38 are mediated by ARG1/NOS2 reduction and sufficient to improve ACT (adoptive cell therapy) therapeutic approaches. Data here produced represent a body of evidence that could be exploited for improving the efficiency of anti-cancer therapies, and identifies ARG1 and NOS2 as key targets for restoring T cell mediated tumor responses.

Riassunto

Le MDSC rappresentano un’importante popolazione immunoregolatoria coinvolta nella generazione di un microambiente tumorale permissivo, in grado di favorire l’instaurarsi, la crescita e la diffusione del tumore. Le MDSC sono fisiologicamente presenti nell’organismo dove, in caso di infezione o infiammazione si espandono transitoriamente allo scopo di limitare risposte immunitarie eccessive e quindi di evitare danni ai tessuti.

Durante la crescita del tumore, le cellule tumorali secernono fattori solubili in grado di indurre l’accumulo delle MDSC e l’acquisizione del loro fenotipo soppressivo. Tra i diversi meccanismi utilizzati dalle MDSC per svolgere le loro funzioni, il metabolismo dell’aminoacido L-arginina sembra avere un ruolo fondamentale nell’alterazione del microambiente tumorale e nella generazione di linfociti T effettori incapaci di generare un’adeguata risposta anti-tumorale.

In questo lavoro è stato studiato il coinvolgimento degli enzimi ARG1 e NOS2 nell’attività delle MDSC durante lo sviluppo tumorale. Le MDSC sono note per la loro complessità sia fenotipica che funzionale ed ormai è chiaro che queste caratteristiche possono essere plasmate da molti stimoli differenti. In questo lavoro, infatti, è stato dimostrato che durante la sua crescita il tumore è in grado di “educare” le MDSC. Abbiamo inoltre mostrato che ARG1 e NOS2 sono fondamentali non solo per la soppressione *in vivo* dei linfociti T ma anche per l’ espansione delle MDSC. Risulta inoltre evidente come il metabolismo di L-arginina mediato da ARG1 e NOS2 sia cruciale nella per la diffusione delle cellule tumorali e per il processo metastatico. Infatti la deplezione di anche uno solo di questi due enzimi provoca una riduzione del 50-75% nel numero di metastasi polmonari. Infine, abbiamo dimostrato come l’effetto mediato da un nuovo farmaco, AT38, sia dovuto, almeno in parte, alla riduzione di ARG1 e NOS2 e come questo sia sufficiente a migliorare l’efficacia di approcci terapeutici, quali il trasferimento adottivo. I dati riportati in questa tesi rappresentano quindi una solida evidenza del fatto che ARG1 e NOS2 possano rappresentare un rilevante bersaglio terapeutico in grado di aumentare l’efficacia delle terapie anti-tumorali.

Introduction

Despite the enormous increase of knowledge on tumor biology, cancer remains one of the leading causes of death in the Western world. The reason for this resides in the failure to find general strategies for therapy given both the great variability of tumor histotypes and the individual medical history of each patient. One of the long lasting open questions about cancer is why, despite the number of tumor antigens, the immune system fails to efficiently react against them and fully eliminate cancer cells. Since Fifthies, many studies aimed at understanding the function of the immune system in tumor development. Even though many steps in this direction were made, we are still far from having a complete picture of the interaction between cancer cells and immune system. It is now established that there is a dual role of the immune system with respect to cancer development: on one hand it can act as tumor suppressor by recognizing and eliminating cancer cells; on the other hand, however, it can also promote tumor progression by either positive selection of tumor cells particularly fit to survive and spread or by shaping the tumor microenvironment so to favor tumor outgrowth. These opposite functions of the immune system are collectively incorporated in the "theory of cancer immunoediting" (reviewed in (Schreiber, Old et al. 2011; Zamarron and Chen)).

The immune control of cancer is based on the discovery of tumor antigens able to elicit an adaptive immune response against malignant cells, while the pro-tumoral activity of the immune system finds its rational in the correlation between a persistent inflammatory state and a higher incidence of cellular transformation and cancer development. Physiologically, inflammation is required to protect an infected or damaged tissue by recruiting cells able to resolve the insult as well as to isolate that area, in order to prevent spreading of the infection. Generally, the normal tissue function is restored once inflammation is resolved, however in some cases this does not happen and unresolved inflammations have been shown to play a role in initiating and promoting tumor growth. To date, it is generally accepted that an inflammatory

microenvironment is a common feature of all tumors regardless a direct causal relationship between the two conditions. Realistically, the scenario is far more complex than this, since tumors cannot be seen as passive elements in which cell survival is only mediated by some random genetic mutations mediated by a favorable microenvironment: this will not account for the wide incidence of tumor pathologies in the population. Indeed, cancer has also an active role in altering the normal homeostasis of the organism. From this prospective the immune system is also modified by the tumor in such a way to make it dysfunctional to the anti-tumoral response and permissive to the survival and dissemination of cancer cells. In this scenario the key issue is why, if various components of the immune system are infiltrating the tumor site, cancer cells are not always eradicated but rather manage to escape? This is because a sort of equilibrium is established between cancer cells and the immune system in which both are influenced reciprocally. The outcome of this equilibrium can be either tumor escape or tumor rejection depending on the ability of cancer cells to evade the immune system or to shape the microenvironment in ways poorly permissive for the effector function of $CD8^+$ T lymphocytes (Schreiber, Old et al. 2011). Tumors secrete a number of soluble factors that recruit different subsets of helper, effector and regulatory T cells (Treg); these, in concert with myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), can actively modify the tumor environment and thus modulate the cytotoxic activity of the $CD8^+$ T effector cells (Sica and Bronte 2007; Marigo, Dolcetti et al. 2008). Besides reducing the effector arm of the immune system, many events are responsible for tumor escape: as a result of immune editing, cancer cells may lose features that could make them target of the immune system; for example, some of the more common mutations are simply loss of major histocompatibility complex (MHC) class I proteins responsible for antigen presentation to effector T cells, or the alteration of mechanisms involved in antigen processing such as dysfunctional proteasome (Schreiber, Old et al. 2011).

Therefore it appears clear that understanding the nature and the features of the tumor microenvironment is fundamental for identifying new therapeutic targets able to push the equilibrium between malignant and immune cells towards tumor eradication. This

work aims at defining the role of key enzymes involved in the metabolism of L-arginine determining the T cell suppressive function of tumor-induced MDSCs, their effect on tumor outgrowth and dissemination.

The immune regulation as consequence of the evolution of the adaptive immune system

The innate immune system is evolutionary more ancient than the adaptive one. The innate immune system evolved to defend organisms from a limited number of bacterial/viral infections, for this reason it makes use of protective strategies based on receptors with broad reactivity (e.g. toll like receptors (TLRs)), able to recognize conserved molecular signals present on different types of pathogens. Triggering of these receptors causes an inflammatory response which limits the area of the infection and helps resolving it. During evolution, the continuous emergence of new species of pathogens and the high mutation rate of their structures, made innate immunity largely unsuitable to efficiently protect the organism. Organisms needed a protective system able to recognize and to react to this continuously growing number of pathogen-associated antigens. Higher vertebrates acquired the adaptive immune system, characterized by the ability to generate a virtually endless repertoire of receptors specific for all possible combinations of bacterial/viral antigens. This is possible because the antigen receptors are modules encoded by a large number of alleles. These alleles are brought together in a random and imprecise manner; this allows that even receptors build up by the same modules might have different antigen specificity because of the different joining regions. Even though this mechanism ensures an elevated degree of protection against foreign antigens, it could also be a double-edged sword since, due to the high variability of the receptors, either some self-structure could be classified as dangerous or the affinity between antigen receptors and antigens could be so strong to induce exaggerated immune responses leading to autoimmune diseases or severe tissue damage. In order to avoid these unwanted immune responses against self-antigens and to dampen exaggerated

inflammation, a fine and complex network of immunoregulatory pathways evolved. All together, the cellular subsets of immune cells, molecular mechanisms, cytokines and chemokines involved in the regulation of the reactivity of the immune system are called “the regulatory arm” of the adaptive immune system, as opposed to the “effector arm” which collectively includes all the cellular players and molecules active in eliminating the pathogens.

Tumors have learned to manipulate the regulatory arm in order to dampen antitumor responses. This is achieved through the secretion of soluble factors (cytokines, growth factors) modifying both the micro- and macro-environment and recruiting immune regulatory cells at the tumor site. In this way tumors manage to shift the hemopoiesis so that more regulatory cells are generated and, at the same time, influence the quality of the activity of effector cells at the tumor site.

The tumor microenvironment

Cancer is an organ-like structure whose growth and development are dependent on the behavior of recruited cells and cell intrinsic pathway in the tumor. As cancer progress, its behavior changes significantly as a consequence of the crosstalk between tumor cells and the microenvironment. The microenvironment of a developing tumor is composed of proliferating malignant cells, tumor stroma, blood vessels and infiltrating inflammatory cells. The stroma is composed by a various cell populations as fibroblast, vascular smooth muscle, epithelial and immune cells. The immune populations include effector cells from both adaptive and innate immunity as T and B lymphocytes, macrophages, dendritic cell (DCs) and natural killer cells (Whiteside 2008). Because of the dynamic nature of cancer due to the continuous changes in the interaction between cancer cells and the numerous subtypes of population present at tumor site, is often difficult to define precisely the role played by a particular cell type or signaling molecule in promoting or contrasting cancer growth.

In the next paragraphs some features of tumor microenvironment will be described with particular emphasis on cell populations and signaling molecules playing an

important role in tumor progression. Mechanisms determining the establishment of a permissive tumor microenvironment through the suppression of effector mechanisms will be considered in depth.

A feature common to growing tumor masses is the oxygen deficiency termed hypoxia (Chan and Giaccia 2007). This condition is known to induce activation of genes involved in the regulation of cell proliferation, extracellular matrix production, cell adhesion, and other hallmarks of tumorigenesis. The mechanism behind these effects is frequently accomplished through induction of the hypoxia-inducible factor (HIF) family of transcription factors. This family consists of three members, HIF-1,-2, and -3, which act to regulate cellular processes including glucose metabolism, angiogenesis, cell proliferation and tissue remodeling in response to low oxygen levels. Hypoxia has been linked to metastasis formation and poor clinical outcome for patients. In fact, although insufficient oxygen limits tumor cell division, at the same time, it induces cell adaptation selecting for more malignant cells, thus allowing development of a more aggressive behavior. Hypoxia can help increasing the metastatic potential of cancer cells; for example hypoxia-induced HIF-1α expression is sufficient to induce a critical step of the epithelial to mesenchymal transition (EMT) a process favoring the acquisition of metastatic features by cancer cells (Imai, Horiuchi et al. 2003; Esteban, Tran et al. 2006). In line with this pro-metastatic effect, activation of Wnt/beta-catenin signaling pathway, through HIF-1α, can induce prostate cancer cells to be more motile and invasive (Jiang, Luo et al. 2007).

Angiogenesis is another important tumor-driven process linked to hypoxia. Activation of HIF-1α, causes the production of pro-angiogenic factors including the vascular endothelial growth factor (VEGF), angiopoietin 2 (ANG2), PDGF and the fibroblast growth factor (Shweiki, Itin et al. 1992; Hanahan and Folkman 1996; Ruan, Song et al. 2009). In addition to these factors, HIF-1α works to decrease the activity of angiogenic inhibitors, such as thrombospondin, thereby creating a pro-angiogenic environment (Laderoute, Alarcon et al. 2000). Recently, it was shown that HIF-1α can induce activation of two enzymes linked to L-arginine metabolism, arginase 1 (ARG1) and

nitric oxide synthase 2 (NOS2), in a tumor-infiltrating myeloid cells endowed with the ability to suppress effector T cells (Corzo, Condamine et al.).

The tumor microenvironment is not suitable for T lymphocyte functions as demonstrated by number of works indicating that tumor-infiltrating lymphocytes (TILs) have defects in both signal transduction and killing effector systems (Whiteside 1999). In the tumor microenvironment, altered metabolism can have a profound impact on anti-tumor immunity and, more generally, on T cell function. Cancer and host cells, under tumor cell influence, can impair the immune system function by altering the metabolism of simple molecules such as amino acids (L-tryptophan and L-arginine). Amino acid starvation causes the cytosol accumulation of free, uncharged (deacylated) tRNAs that bind the histidyl-tRNA-synthetase-related domain of the amino acid “sensor” general control non-derepressible-2 (GCN2) kinase (Hao, Sharp et al. 2005; Maurin, Jousse et al. 2005). This induces phosphorylation and inactivation of the eukaryotic initiation factor (eIF)2α, one of the key steps in protein translation, leading to an arrest of protein synthesis and finally in the alteration of cell cycle progression (Kimball and Jefferson 2005). The aim of this reaction is to limit amino acids consumption in an already deprived environment, even though the side effect might be a temporary arrest of vital cell functions. GCN2 can be activated by indoleamine 2,3-dioxygenase (IDO), expressed by $CD8^+$ DCs and by plasmacytoid DCs, where it mediates the catabolism of L-tryptophan. GCN2 activation was associated, both in vitro and in vivo, with proliferative arrest, anergy induction, impaired CTL effector function and down-regulation of the TCR ζ-chain in mouse $CD8^+$ T cells (Munn, Sharma et al. 2005; Fallarino, Grohmann et al. 2006). Similar results were obtained studying the effects of L-arginine deprivation. In fact, consumption of L-arginine by ARG1 over-expression in tumor-conditioned macrophages and in MDSCs can also mediate the GCN2 kinase-dependent cell cycle arrest in G_0–G_1 phase and the down-regulation of the ζ-chain of the TCR/CD3 complex in antigen-activated T cells (Rodriguez, Quiceno et al. 2004; Bronte and Zanovello 2005; Rodriguez, Quiceno et al. 2007).

Tumor-induced immune-suppressor cells

As mentioned earlier, an important part of the tumor microenvironment is represented by tumor-infiltrating cellular components of the immune system. These can be subdivided in cell populations belonging to the either the effector or the regulatory arm of the immune system. The relative abundance of these two types of cell populations and their ability to influence each other determine tumor outgrowth or its rejection. Here we will treat the main cell populations found at the tumor site and influenced by the tumor microenvironment. Since the biology of MDSCs is the main topic of this work, they will be discussed in further detail in a separate section.

Regulatory lymphocytes. The regulatory lymphocytes (Treg) are characterized by the expression of CD4/CD25 markers and Foxp3 transcription factor. Foxp3^{+} Treg cells are generated in the thymus but also in the periphery from naïve T cells. Recent studies indicate that Foxp3 function is not determinant for thymocytes to follow the Treg cell lineage but it is necessary to stabilize Treg cell function once cell fate is determined (Gavin, Rasmussen et al. 2007; Lin, Haribhai et al. 2007). Signals triggering Foxp3 expression remain to be determined and different hypothesis are reviewed in (Wing and Sakaguchi). Treg cells actively suppress activation of the immune system and prevent pathological self-reactivity, i.e. autoimmune disease. Treg expansion can be triggered directly by the tumor through some tumor-derived soluble factors (TDSFs) such as TGF-β or indirectly by myeloid cells recruited by the tumor and secreting specific growth factors and chemokines (e.g. CCL2, IL-10 and TGF-β) (Curiel, Coukos et al. 2004). Many studies demonstrated the link between Tregs and tumor-driven immune suppression: for example, *in vivo* depletion of Tregs using anti-CD25 antibody before tumor implantation increased the immune-surveillance and favored tumor rejection in different mouse tumor models (Shimizu, Yamazaki et al. 1999). Further, treatment with an antibody against the Treg glucocorticoid tumor necrosis factor related protein (GITR), to block their inhibitory function without depleting them, reduced tumor growth and increased the number of CD4^{+} and CD8^{+} T cells within the tumor (Ko, Yamazaki et al. 2005).

Even though it seems that Treg might exert their immune-suppressive function in an antigen independent manner, recent evidences show that Treg express T cell receptors (TCRs) with specificity for self antigens (Larkin, Picca et al. 2007). This could be linked to the fact that Treg evolved to control auto-immune responses generated by potentially self-reactive $CD8^+$ T cells that might escape thymic selection (Hsieh, Liang et al. 2004). Apoptotic cancer cells often unmask self antigens which are not presented during the process of thymic selection. In support of this hypothesis, Treg bearing TCRs specific for melanoma antigens have been isolated and found responsible for inducing a local suppression of tumor-infiltrating lymphocytes (Wang, Lee et al. 2004). The mechanism through which Treg exert their suppressive activity seems to be triggered by a membrane signal involving CTLA-4. Treg cells constitutively express high amounts of CTLA-4 (Read, Malmstrom et al. 2000; Salomon, Lenschow et al. 2000). The blockade of CTLA-4 with specific antibody induces the onset of autoimmunity in healthy mice (Read, Malmstrom et al. 2000) and exacerbate diabetes in diabetes-prone NOD mice (Luhder, Hoglund et al. 1998). Moreover the expression of Foxp3 directly induces the up-regulation of CTLA-4 in Tregs by binding the promoter region of *Ctla4* gene (Marson, Kretschmer et al. 2007; Zheng, Josefowicz et al. 2007). Most importantly, in mice lacking CTLA-4, Treg develop lymphoproliferative and autoimmune diseases similarly to Foxp3 deficient mice (Wing, Onishi et al. 2008).

The CTLA-4 binding to the cognate ligands CD80 and CD86 expressed on DCs induces the activation of IDO; this mediates an immunosuppressive response due to tryptophan depletion and the production of pro-apoptotic metabolites called kinurenins (Fallarino, Grohmann et al. 2003; Mellor and Munn 2004) or to the activation of the immune-regulating transcription factor Foxo3 in Treg, which in turn inhibit cytokine secretion by DCs (Grohmann, Orabona et al. 2002; Dejean, Beisner et al. 2009).

The molecular mechanism used by Tregs to induce immune suppression are multiple and often depend on the anatomical site in which Treg exert their functions: some Authors indicate that Tregs expressing IL-10 are rare in the spleen but abundant in the lamina propria of the intestine (Rubtsov, Rasmussen et al. 2008), while others

suggested that a specific program mediated by CD3 and CD46 can activate the production of perforines against cytotoxic T lymphocytes (CTLs) (Grossman, Verbsky et al. 2004). Perforin- or granzyme-expressing Foxp3^{+} cells are rare in the spleen but are abundant in tumor environment (Cao, Cai et al. 2007). Although much evidence shows a clear involvement of Tregs in determining suppression of CTL activity at the tumor site, a significant number of studies suggest that functional immune responses can be achieved also in presence of Tregs. For example, unspecific activation of Tregs induced by APCs stimulated *in vitro* with LPS or CpG ligands do not induce CTL suppression (Pasare and Medzhitov 2003). Moreover Tregs cultured with supernatants of activated APCs, that produce IL-6 in response to TRL activation, did not show any immunosuppressive activity and authors suggest that IL-6 can alter their immunosuppressive activity (Pasare and Medzhitov 2003).

Macrophages. Macrophages are the most abundant population among the tumor-infiltrating leucocytes. Macrophages associated to the tumor microenvironment are collectively called tumor associated macrophages (TAMs). They originate from circulating monocytes that are recruited from tumor through chemo-attractants such as the colony stimulator factor 1 (CSF-1), the chemokines of the CC family (CCL2, CCL3, CCL4, CCL5, CCL8) and the vascular endothelial growth factor 1 (VEGF-1) (Pollard 2004). Once they reach the tumor, monocytes differentiate to TAMs with a relatively immature phenotype, as they express few antigens of differentiated macrophages, for example carboxypeptidase M (CPM) and CD51, and have pattern or cytokine production more similar to that of macrophage precursors: low levels of TNF-α but a constitutive secretion of IL-6 and IL-1, whereas classically activated macrophages express more cytokine receptors, IL-12 and TNF-α (Lewis and Pollard 2006). TAMs preferentially localize to poorly vascularized regions of tumors (Leek, Talks et al. 2002; Lewis and Pollard 2006); indeed, oxygen availability has a role in guiding the microanatomical localization and function of TAMs. Moreover, in TAMs, hypoxia induction of NOS2 and ARG1 can also have important consequences on the suppression of adaptive immunity (Imtiyaz, Williams et al.; Corzo, Condamine et al. 2010).

TAMs have two distinct profiles, depending on their function and markers. In the tumor, TAMs acquire a complete M2 polarization associated with a pro-tumoral activity. M2 macrophages can process and present fragments from apoptotic cells, promote angiogenesis and remodel injured tissues (Mantovani, Sozzani et al. 2002), a completely different asset distinguishing them from classically activated, or inflammatory, M1 macrophages, which are efficient immunity effectors with a markedly anti-tumor activity (Gordon 2003). However recent data obtained from gene expression analysis of TAMs and immature myeloid cells suggest that TAMs represent a unique macrophage population with key properties of M2 cells, which co-express IFN-inducible chemokines (Biswas, Gangi et al. 2006; Gallina, Dolcetti et al. 2006), suggesting a more complex asset. TAMs promote the neo-angiogenesis and tumor proliferation through the secretion of growth factors (e.g. EGF, VEGF) and cytokines (e.g IL-1β) involved in angiogenesis and cells proliferation (Leek, Hunt et al. 2000; Lewis, Landers et al. 2000; Voronov, Shouval et al. 2003). Further, TAMs secrete signaling molecules, proteases, enzymes and other factors (e.g urochinase, plasminogen) involved in the disaggregation of the extracellular matrix, a process that favors the exit of tumor cells from primary lesion and metastatic spreading (Hildenbrand, Dilger et al. 1995). Besides the processes of tissue remodeling necessary for tumor angiogenesis, proliferation and spreading, TAMs can influence the reactivity of the immune system by interfering with the differentiation of myeloid cell precursors to DCs. For example, in tumors the production of IL-6 and M-CSF by both TAMs and cancer cells induces differentiation of myeloid progenitors towards the macrophage rather than the DC lineage. Furthermore, tumor cells express the cyclooxygenase 2 (COX2) enzyme that controls TAM production and release of prostaglandin2 (PGE_2) production, which in turn interferes with DC differentiation and function. Lastly, TAMs produce IL-10 that induces the down-regulation of MHC II co-stimulatory and adhesion molecules on DC surface, thus converting competent DCs into tolerogenic antigen-presenting cells (APCs) (Sombroek, Stam et al. 2002).

Immature and dysfunctional DCs. DCs represent the most important class of professional antigen presenting cells (APCs) and for this reason they have fundamental roles in triggering the adaptive immune response at the sites of infection as well as presenting antigens to lymphocytes in the lymph nodes where peripheral tolerance is established. Immature DC (iDC) generation occurs in the bone marrow from hemopoietic stem cells and is due to the exposure to both soluble growth factors (as GM-CSF, FLT3L and IL-3) and cell-cell contact with bone marrow stroma cells (Gabrilovich 2004). To achieve complete maturation, iDCs require further stimuli to acquire the expression of costimulatory molecules, fundamental in activating immune cells such as T lymphocytes. Maturation of DCs is guided by external signals such microbic antigens, danger signals and pro-inflammatory cytokines. Tumor can interfere with the generation of functionally active DCs and this scenario is supported by data showing that tumor are often infiltrated with DCs sharing the phenotype of iDCs (Chaux, Favre et al. 1997). This results in reduced potential of tumor antigen presentation, T cells activation and elimination of cancer cells.

Depending on the kind of stimuli inducing DC maturation and activation, DCs can secrete different patterns of cytokines therefore triggering either a T helper (Th)1 or a Th2 type of immune response. For instance DCs exposed to different pathogens products secrete IL-12, IL-18 and IFN-α driving $CD4^+$ T lymphocytes towards a Th1 polarization, the activation of natural killer (NK) cells and activation of $CD8^+$ T lymphocytes. Upon exposure to IL-10, DCs present a reduced production of inflammatory cytokines and lack of IL-12 synthesis, thus causing the preferential induction of Treg lymphocytes and Th2 immune response (Reis e Sousa 2006). It is known that DCs in stationary state can induce tolerance in $CD4^+$ and $CD8^+$ T lymphocytes causing anergy or deletion (Reis e Sousa 2006). It is therefore clear that DC-dependent downstream effects can be highly modulated by the set of stimuli and chemokines they are exposed to. DCs isolated from the inner tumor masses display an immature phenotype and are not able to induce activation of CTLs. Interestingly DCs isolated from the stroma surrounding the tumor show a more advanced status of maturation and express different sets of surface markers such as CD83 and higher

levels of costimulatory molecules (Allavena, Sica et al. 2000; Pinzon-Charry, Maxwell et al. 2005). This suggests that the tumor microenvironment can influence the degree of DC differentiation and therefore their impact on immune effector cells. This holds true also in humans. Indeed, when human monocytes are treated *in vitro* to induce their differentiation into DCs with IL-4 and GM-CSF, a cytokine abundantly secreted by cancer cells, they become immature iDCs. In absence of maturation signals, these iDCs display an inhibitory effect on the proliferation of $CD8^+$ T cells both in *in vitro* mixed leukocytes reactions and *in vivo*, when injected in healthy volunteers. The fate of DCs is thus determined by the kind of stimulus they receive upon maturation and if they are framed in an immature state, this cannot be rescued by signals present in the healthy organism (Dhodapkar, Steinman et al. 2001). In mouse models, besides GM-CSF, other TDSFs have been shown to interfere with the differentiation of myeloid precursor cells into DCs; they can be cytokines and growth factors such as IL-10 and VEGF, but also gangliosides and prostanoids (Sombroek, Stam et al. 2002; Peguet-Navarro, Sportouch et al. 2003).

DCs are susceptible to different tumor products: IL-10 prevents DCs maturation from myeloid precursors, whereas GM-CSF and VEGF, gangliosides and prostanoids can support the presence of intartumoral APCs with immature phenotype; moreover, some tumor antigens (MUC-1, HER-2/neu) together with polyamine putrescin, spermidin and spermin produced by L-arginine metabolism by ARG1 enzyme prevent the complete maturation of DCs (Pinzon-Charry, Maxwell et al. 2005).

Tie2 expressing monocytes (TEMs). TEMs are a population of monocytes with an important role in tumor angiogenesis. They express the angiopoietin 2 (ANG2) receptor known as TIE2. TEMs were identified in many mouse tumor models as well as in human tumors xenografted in immunodeficient mice such as glioblastoma (De Palma, Venneri et al. 2003; De Palma, Venneri et al. 2005). More recently the presence of TEMs was demonstrated also in tumor and in peripheral blood of cancer patients (Venneri, De Palma et al. 2007). In mouse, TEMs can be distinguished by TAMs by the expression of SCA1 and TIE2 markers and because of their marked proangiogenic

activity and preferential localization in areas of neoangiogenesis (De Palma, Venneri et al. 2005). In mice, circulating TIE2 monocytes are $CD11b^{+}Gr\text{-}1^{low/neg}$ (De Palma, Venneri et al. 2005) while in humans they express CD14, CD16 and CD11c (Murdoch, Tazzyman et al. 2007; Venneri, De Palma et al. 2007). A direct demonstration of the pro-angiogenic properties of TEMs was provided with experiments of "genetic suicide" in which the selective elimination of TEMs induced a dramatic reduction of tumor angiogenesis. Moreover, in the same study, co-injection of TEMs and tumor cells induced a higher vascularization during tumor growth compared with tumor cells injected with $TIE2^{-}$ monocytes (De Palma, Venneri et al. 2005). Up to date it is not completely clear whether TEMs are recruited by tumors; indeed circulating human TEMs do not express CCR2, the receptor for CCL2, the chemokine with chemoattractive properties for monocytes (Venneri, De Palma et al. 2007). It is probable that ANG2, trough the interaction with the TIE2 receptor, can be involved in TEM recruitment: two studies showed that *in vitro,* ANG2 stimulate the migration of $TIE2^{+}$ monocytes (Murdoch, Tazzyman et al. 2007; Venneri, De Palma et al. 2007). Tumor blood vessels up-regulate the production of ANG2 and this can represent the signal for TEMs recruitment (Fiedler, Scharpfenecker et al. 2004). Moreover, hypoxic condition induces up-regulation of TIE2 expression in TEMs, thus making them more sensitive to ANG2 recruitment in tumor regions oxygen deprived (Murdoch, Tazzyman et al. 2007; Venneri, De Palma et al. 2007). ANG2 modulates also the cytokine production in TEMs and causes a lower TNF-α production and elevated level of TNF-α can induce apoptosis in tumor and endothelial cells (Balkwill 2009). Probably the down-regulation of TNF-α near the $ANG2^{+}$ tumor vessels promotes the formation of the new vessels by enhancing the survival of both tumor and endothelial cells, therefore supporting angiogenesis and metastasis. In presence of hypoxia, ANG2 also inhibits the secretion of IL-12, an antiangiogenic cytokine, by TEMs (Murdoch, Tazzyman et al. 2007) and this can represent an additional mechanism by which tumors can modulate the antiangiogenic activity of macrophage population.

Myeloid Derived Suppressor Cells. The term myeloid derived suppressor cells (MDSCs) indicates a cellular population that expands and accumulates upon different pathogenic stimuli such as infection, chronic inflammation, autoimmune disease or tumor growth. The expansion of this cell population has been described both in humans and mice (Gabrilovich, Bronte et al. 2007). The analysis of various murine tumor models and genetic backgrounds revealed that MDSCs accumulate in different districts such as the bone marrow, blood, and the spleen (Serafini, De Santo et al. 2004; Serafini, Borrello et al. 2006). In mice this population is characterized by the expression of CD11b and Gr-1 markers and low levels of markers for mature myeloid lineage. An additional signature of these cells is the up-regulation of genes involved in L-arginine metabolism and production of reactive oxygen and nitrogen species (ROS and RNS, respectively). Besides the expression of surface markers or specific enzymes, however, MDSCs are defined by their ability to suppress, both *in vivo* and *in vitro,* the proliferation and cytotoxic activity of the effector T cells (Gabrilovich, Bronte et al. 2007; Dolcetti, Marigo et al. 2008; Marigo, Dolcetti et al. 2008; Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg and Sinha 2009). In healthy mice, CD11b^{+}Gr-1^{+} cells are physiologically present in the bone marrow, where they represents 40-50% of all cells, whereas only a minor fraction resides in the spleen (about 5% of total splenocytes) and circulate in the blood. Although these cells are CD11b/Gr-1 positive, they do not show a significant suppressive activity ex vivo on CD8^{+} T cells, since T-cell functions can be inhibited only when the ratios between myeloid cells and T cells is very high and far from being physiologic. MDSCs derive from myeloid precursors but some differences exist between steady-state and pathologic myelopoiesis. In the process of normal hemopoiesis, in fact, hematopoietic stem cells give rise to the myeloid multipotent precursor (MP) cells. This differentiation is initially triggered by different cytokines, such as GM-CSF, IL-3, M-CSF, SCF and FLT3L. MPs give rise to DCs and immature myeloid cells (iMCs). In healthy organisms iMCs exit from the bone marrow and reach secondary lymphoid organs, where they rapidly differentiate into granulocytes, macrophages and monocytes.

Under pathologic conditions a block in maturation of iMCs occurs, leading to accumulation of MDSCs (Gabrilovich and Nagaraj 2009; Geissmann, Manz et al.). During inflammatory responses, MDSCs transiently expand first in the bone marrow and then in secondary lymphoid organs, where they probably act as modulators of the immune response, to avoid tissue damages or autoreactivity due to hyper-activation of T cells. When the stimulus ceases, because the tissue damage is resolved, MDSCs quickly differentiate and the normal T cells function is restored. When inflammation is not resolved or it is associated to tumor growth, the prolonged and ingravescent stimulation of the immune system causes a progressive accumulation of myeloid cells both in lymphoid organs and at the tumor site (Gallina, Dolcetti et al. 2006; Kusmartsev and Gabrilovich 2006; Marigo, Dolcetti et al. 2008). MDSCs induced under these conditions fail to terminally differentiate and seem to get “stuck” half way through the differentiation process, retaining some features of immature precursor cells. Macrophages, monocytes, granulocytes, and DCs, which are not fully functional, end up altering the reactivity of the immune system, through mechanisms that will be discussed later. Some examples in which MDSCs can accumulate and alter the immune system functionality are summarized in Table 1.

Condition	Phenotype	Immune dysfunction	Reference
TUMOR			
Trasplantable tumors: colon carcinoma (CT26, C26), melanoma (B16), fibrosarcoma (C3, 15-12RM)	CD11b/Gr-1	Yes	Kusmartsev, Li et al. 2000; Salvadori, Martinelli et al. 2000; Gabrilovich, Velders et al. 2001; Liu, Van Ginderachter et al. 2003; Kusmartsev, Nagaraj et al. 2005)
Trasplantable mammary carcinoma (TS/A), pulmonary carcinoma	CD11b/Gr-1/CD31	Yes	(Bronte, Chappell et al. 1999; Apolloni, Bronte et al. 2000)
Trasplantable tumors: colon carcinoma (C26-GM), mammary carcinoma (4T1), fibrosarcoma (MCA203)	CD11b/Gr-1/F4/80/IL-4Rα	Yes	(Gallina, Dolcetti et al. 2006; Dolcetti, Peranzoni et al. 2010; Marigo, Bosio et al. 2010)
Trasplantable tumors: thymoma (EL-4, EG7), lymphoma (BW-Sp3) colon carcinoma (MCA26)	CD11b/Gr-1/F4/80/CD115^{int}	Yes	(Kulbe, Hagemann et al. 2005; Movahedi, Guilliams et al. 2008; Molon, Ugel et al. 2011)
Trasplantable tumors: ovaric carcinoma	CD11b/Gr-1/CD80	Yes	(Yang, Cai et al. 2006)ang, Cai et al. 2006)
Spontaneus mammary tumor (BALB/neu T)	CD11b/Gr-1/CD31	Yes	(Melani, Chiodoni et al. 2003; Melani, Sangaletti et al. 2007)
INFECTION			
Candida Albicans	CD11b/Gr-1/CD80	Yes	(Mencacci, Montagnoli et al. 2002)
Trypanosoma Cruzi, Porphyromonas gingivalis	CD11b/Gr-1	Yes	(Giordanengo, Guinazu et al. 2002; Ezernitchi, Vaknin et al. 2006)
Schistosoma mansoni	CD11b/Gr-1/CD16	Yes	(Marshall, Jankovic et al. 2001)
IMMUNE STRESS			
Superantigeni (Staphylococcal enterotoxin A)	CD11b/Gr-1/LFA-1	Yes	(Cauley, Miller et al. 2000)
Schistosome oligosaccharide (Lacto-N-neotetraose)	CD11b/Gr-1/F4/80	Yes	(Terrazas, Walsh et al. 2001)
Vaccination with recombinant virus	CD11b/Gr-1/CD31	Yes	(Bronte, Wang et al. 1998; Apolloni, Bronte et al. 2000)
CHRONIC INFLAMMATION/AUTOIMMUNITY			
Experimental autoimmune encephalomyelitis (EAE)	CD11b/Ly6C^{high}/F4/80/CD93	Yes	(Zhu, Bando et al. 2007)
Experimental autoimmune uveoretinitis	CD11b/Gr-1	Yes	(Kerr, Raveney et al. 2008)
Chronic eczema	CD11b/Gr-1/CD31	Yes	(Marhaba, Vitacolonna et al. 2007)
Gut chronic inflammation	CD11b/Gr-1	Yes	(Haile, von Wasielewski et al. 2008)
OTHERS			
Cyclophosphammide treatment (recovery phase)	CD11b/Gr-1/CD31	Yes	(Angulo, de las Heras et al. 2000; Pelaez, Campillo et al. 2001)
Medullary chimeras	CD11b/Gr-1/Ly6-CCD11b/Sca-1	Yes	(Billiau, Fevery et al. 2003)
Graft-versus-host desease	CD11b	Yes	(Bobe, Benihoud et al. 1999)
Traumatic stress	CD11b/Gr-1/CD31CD11b/Gr-1	Yes	(Makarenkova, Bansal et al. 2006; Munera, Popovic et al. 2010)

Table 1: Example of MDSC accumulation in different experimental pathologies

Signaling molecules and tumor micro-environment

Inflammation is a key component of tumor microenvironment and represents the seventh hallmark of cancer. It is known that chronic inflammatory conditions increase the risk of developing cancer and genetic events that cause neoplastic transformation induce cancer cells to produce inflammatory mediators. Tumor-related inflammation is linked to the ability of tumors to create a permissive environment in which myeloid cells are recruited and re-educated to favor tumor growth and metastasis formation. Chemokines and cytokines are probably the main factors involved in these processes.

Chemokines. Chemokines are small molecular-weight (8-14 kDa) chemotactic cytokines that bind receptors associated to G proteins (Tanaka, Bai et al. 2005). Chemokines are grouped in four sub-families (CXC, CC, CX3C, C) based on the positioning of the conserved two N-terminal cystein residues of the mature protein. According to their function chemokines are distinguished in homeostatic, which control leukocyte homing and lymphocyte recirculation under normal conditions, and inflammatory chemokines, produced in response to inflammatory and immune stimuli (Mantovani 1999). Chemokines and their receptors have been identified as mediators of chronic inflammation that play a key role in the initiation and progression of many tumor types (Balkwill, Charles et al. 2005; Ali and Lazennec 2007; Mantovani 2009). Tumor growth and dissemination is the result of dynamic interaction between tumor cells and other components of tumor environment. In this regard, chemokines are emerging as key mediators in the homing of cancer cells to metastatic sites and recruitment of cells at the tumor site. Different chemokines can specifically attract various populations of the immune system depending on the presence on their surface of the chemokine-specific receptor. Generally CXC chemokines attract neutrophils and lymphocytes, while CC chemokines attract lymphocytes and monocytes (Balkwill and Mantovani 2001). The recruitment of monocytes to tumors can lead to an accumulation of TAMs. In breast carcinoma patients the expression of CCL2 (also known MCP-1) and CCL5 (RANTES) correlates with an advanced stage of pathology, early relapse and poor prognosis (Balkwill 2004). CCL3 and CCL4 are also involved in

monocyte recruitment and can be related to high levels of TAMs in tumors (Mantovani, Sica et al. 2004) Tumor cells from many human carcinomas, such as ovaric and pancreatic carcinoma, breast and papillary thyroid carcinoma, express chemokines receptors that regulate the proliferation of these cells (Colombo and Mantovani 2005). CCL2 is one of the most important chemokines present in the tumor microenvironment. It is known to be a potent chemoattractant for macrophages and monocytes, which can favor angiogenesis and metastasis (Ben-Baruch 2006; Soria and Ben-Baruch 2008). CCL2 pro-tumoral ability seems to be dependent on recruitment of MDSCs that favor neoplastic growth in synergy with macrophages (Huang, Lei et al. 2007). This cytokine seems to be part of a reciprocal activation circuit with IL-6 in $CD11b^+$ cells isolated from peripheral blood of metastatic prostate carcinoma patients: these two molecules protect MDSCs from apoptosis inducing anti-apoptotic proteins and promoting a phenotype similar to alternatively activated, M2-type macrophages (Roca, Varsos et al. 2009). The activity of chemokines can be regulated not only by the expression of specific receptors but also by post-translational modifications which can modify the interaction properties between the chemokine and its receptor. For example, our group showed that CCL2 can be modified by the nitration mediated by reactive nitrogen species (RNS) produced at the tumor site. CCL2 nitration impairs the interaction with its receptor CCR2 expressed on T cells, which therefore cannot enter and eliminate tumor cells (Molon, Ugel et al.).

In MDSCs many of these factors converge on signaling pathways regulating cell proliferation, survival and differentiation such as, for example, those controlled by members of JAK (Janus Kinase) protein family and signal transducer and activator of transcription 3 (STAT3) transcription factors. Indeed, STAT3 is, to date, one of the main factors responsible for MDSC expansion. The activation of STAT3 is strongly increased in MDSCs isolated from tumor-bearing mice compared to the same cells isolated from healthy mice (Nefedova, Nagaraj et al. 2005). To prove that STAT3 is sufficient to induce MDSC expansion, hemopoietic progenitors where cultured in presence of supernatants derived from tumor cells. This treatment induced activation of JAK2 and STAT3 pathways and led to the *in vitro* expansion of MDSCs (Nefedova, Huang et al.

2004). By using either conditional STAT3 knock out (KO) mice or selective inhibitors of this transcription factor a reduction of MDSCs expansion and an increase in T lymphocytes response was observed (Kortylewski, Kujawski et al. 2005; Nefedova, Nagaraj et al. 2005). STAT3 activation is also associated with increased survival and proliferation of bone marrow progenitors, which is likely due to the expression of cyclin D1, MYC and surviving (Fukuda and Pelus 2006; Leslie, Lang et al. 2006; Zhang, Nguyen-Jackson et al. 2010). It is clear that the abnormal and persisting activation of STAT3 in myeloid progenitors prevents their differentiation in mature myeloid cells hence promoting MDSCs expansion (Foell, Wittkowski et al. 2007).

Cytokines. Cytokines are small cell-signaling molecules secreted by a number of different cell types including cancer cells and immune cells. Cytokines are used extensively in intercellular communication coordinating different cellular activities such as proliferation, differentiation and cell death. In the tumor micro-environment an intense cytokine production has been reported to influence the behavior of leukocytes, epithelial and endothelial cells. The cytokines involved in MDSC expansion and activation can be divided in two main groups. The first group comprises cytokines mainly produced by cancer cells mediating MDSC expansion as VEGF, SCF, GM-CSF, G-CSF, M-CSF, IL-6, IL-10, IL-12, MMP9 (Talmadge 2007; Gabrilovich and Nagaraj 2009). Many of these factors converge on STAT3 pathway fundamental for MDSCs because it is implicated in all major features of these cells, such as expansion, proangiogenic activity, accumulation and suppressive function (Condamine and Gabrilovich 2010; Sonda, Chioda et al. 2011). The second group of cytokines include factors produced by activated T cells and tumor stroma that drive MDSC activation, such as INF-γ, IL-4, IL-13 and TGF-β that activate pathways involving STAT1, STAT6 and NF-κB (Gabrilovich and Nagaraj 2009). In this paragraph we will outline some of these cytokines and their role in MDSCs biology.

Two of the most important cytokines involved in both MDSCs induction and activation are GM-CSF and IL-6. Recently our group demonstrated that bone marrow cells cultured with these two cytokines acquire phenotype and functions similar to tumor-

induced MDSCs (Marigo, Bosio et al. 2010). **GM-CSF** is a glycoprotein of 14 kDa able to stimulate, in combination with other factors, myeloid precursors proliferation and their differentiation to different cellular types such as monocytes/macrophages, dendritic cells, granulocytes and osteoblasts (Sebollela, Cagliari et al. 2005). GM-CSF is the cytokine that most likely promotes the accumulation of tumor-associated MDSCs. Indeed, GM-CSF is produced by approximately 30% of all human tumors and by many murine tumor cell lines; its production correlates with the accumulation of $CD11b^+$/$Gr\text{-}1^+$ cells (Bronte, Chappell et al. 1999). In squamous head and neck carcinoma the amounts of secreted GM-CSF and the number of immature $CD34^+$ cells that accumulate in primary tumor mass are considered a negative prognostic factors: patients with high GM-CSF production and accumulation of $CD34^+$ cells show a relevant incidence of relapses and metastasis (Young, Young et al. 1990). However, these data contrast with evidences that tumor cells engineered to produce GM-CSF act as potent antitumor vaccine in pre-clinical tumor models (Dranoff, Jaffee et al. 1993); further, in addition to G-CSF, these cells accelerate the bone marrow reconstruction after bone marrow transplantation, and in association with chemotherapeutic drugs increase the toxicity against leukemias. Nevertheless, immunosuppressive properties of GM-CSF and G-CSF are described in some studies on patients in which cytokine-mediated therapy produced an increase of $CD14^+$ cells associated with suppression of T $CD4^+$ response against allo-antigens (Mielcarek, Graf et al. 1998). Many evidence seems thus to suggest a dicothomic effect of GM-CSF; on one side, it acts as immune stimulant, but on the other side it can be immunosuppressive. In recent studies, where antitumor vaccines were tested, tumor-bearing mice were treated with a constant dose of antigen and with increasing doses of GM-CSF. These studies demonstrated the existence of a systemic threshold over which GM-CSF not only lost its efficiency as immune-stimulator but also caused *in vivo* immunosuppression through the recruitment of MDSCs (Serafini, Carbley et al. 2004). It is not clear whether high doses of GM-CSF activate macrophages and other myeloid cells to release immunosuppressive factors or whether high doses act simply on MDSC mobilization. Recent data seem to indicate that GM-CSF is necessary for both the recruitment of

MDSCs and the acquisition of their suppressive functions (Dolcetti, Peranzoni et al. 2010; Marigo, Bosio et al.); (Schmidt submitted).

IL-6 is a cytokine involved in several processes, both physiologic and pathologic, as inflammation, haemopoiesis, bone metabolism and resorption, immune response and carcinogenesis. High levels of IL-6 are found in squamous head and neck carcinoma, esophageus carcinoma and cervix/uterus carcinoma and usually correlate with bad prognosis (Partridge, Chantry et al. 1991; Grimm, Six et al. 2005). By acting on myeloid precursors, T and B lymphocytes, IL-6 produces different immunoregulatory effects: it influences APC maturation promoting defective DCs through a mechanism involving STAT-3 (Chomarat, Banchereau et al. 2000; Diehl and Rincon 2002; Park, Nakagawa et al. 2004).

VEGF is secreted by the majority of tumors, high levels of this cytokine are often associated with bad prognosis. In a murine breast tumor dependent on the specific expression of the Her2/neu oncogene, blood levels of VEGF correlate with neoplastic progression and with MDSC accumulation in the spleen and blood of tumor bearing animals (Melani, Chiodoni et al. 2003). The same group also demonstrated that using an inhibitor of MMP9, a metalloprotease regulating the bio-availability of VEGF, it is possible to obtain a reduction in MDSC accumulation and restore the immune anti-tumor response induced by immunization (Melani, Sangaletti et al. 2007). *In vivo* administration of VEGF inhibited DC differentiation and caused accumulation of $CD11b^+/Gr\text{-}1^+$ cells (Gabrilovich, Ishida et al. 1998; Gabrilovich, Velders et al. 2001). Another study showed that the activation of VEGFR2 was able to induce expansion of myeloid cells in bone marrow in response to an increase in GM-CSF levels (Larrivee, Pollet et al. 2005). Recently, the effect of the clinical treatment with anti-VEGF (VEGF-Trap) was evaluated with regard to different cellular populations in the blood of patients with solid tumors in advanced stage; although this treatment was able to increase the circulating mature DCs, it was not able to alter significantly other myeloid populations and among them MDSCs, thus preventing rescue of the immune response (Fricke, Mirza et al. 2007). These data suggested a secondary or rather indirect role of VEGF in the MDSC recruitment in human and mouse.

IL-10 is a Th2 type cytokine found at the tumor site and in serum of patients with different neoplastic pathologies. This cytokine produces effects on immune system and influences T lymphocytes proliferation, Th1 cytokine production, antigen presentation and cytotoxicity (Curiel, Coukos et al. 2004). It was observed that DCs treated with IL-10 induce tolerance in $CD4^+$ and $CD8^+$ T lymphocytes (Steinbrink, Graulich et al. 2002).

TGF-β is a cytokine whose deregulation is associated to fibrosis and cancer. Tumor secreted TGFβ-1 recruits circulating monocytes from peripheral blood and stimulates their secretion of pro-angiogenic factors as IL-8 and VEGF (Kaminska, Wesolowska et al. 2005). TGF-β is spontaneously secreted by pancreatic carcinoma where mediates the suppression of immunity response and induces a systemic shift toward Th2 response, inefficient against tumors (Bellone, Smirne et al. 2006).

INF-γ is mainly produced by activated T cells and blockade of IFN-γ abolishes MDSC-mediated T-cell suppression (Kusmartsev and Gabrilovich 2005; Movahedi, Guilliams et al. 2008). IFN-γ activates the transcription factor STAT1, involved in up-regulation of ARG1 and NOS2 in MDSCs present in the tumor microenvironment. MDSCs from $Stat1^{-/-}$ mice failed to up-regulate ARG1 and NOS2 and therefore could not inhibit T-cell responses (Kusmartsev, Nagaraj et al. 2005). IFN-γ produced by both activated T cells and MDSCs induced the up-regulation of NOS2 in MDSCs that, in concert with ARG1, was implicated in the suppressive function of MDSCs (Gallina, Dolcetti et al. 2006).

IL-4 binds to a multi-chain receptor whose alpha chain (IL-4Rα) is shared between IL-4 and IL-13 signaling pathway in MDSCs. Activation of the IL-4R signaling pathway in freshly isolated MDSCs or MSC-2, a myeloid cell line, by IL-4 induced the expression of ARG1 (Bronte, Serafini et al. 2003) (Rutschman, Lang et al. 2001). IL-4Rα signaling activates STAT6, whose deficiency blocks the activation of ARG1 in MDSCs (Sinha, Clements et al. 2005; Gallina, Dolcetti et al. 2006).

A factor that in the last years was identified as a responsible for the accumulation and activation of MDSCs is the prostaglandin E2 (**PGE_2**) produced by COX2 enzyme from arachidonic acid. In mouse, this inflammatory mediator seems to be crucial in some tumor models for the recruitment of MDSCs. Interference with the signaling through the receptors for PGE_2 (EP1, EP2 and EP4) reduce both numbers the suppressive

activity of MDSCs. A similar effect can be reproduced *in vivo* by the administration of a COX2 inhibitor (Sinha, Clements et al. 2007). In another murine tumor model, Rodriguez and colleagues proved that tumor-produced PGE_2 can induce ARG1 through the EP4 receptor in tumor infiltrating MDSCs (Rodriguez, Hernandez et al. 2005). In a similar way, the inhibitor of COX2, Celecoxib, not only reduces the number of MDSCs in chemically-induced tumors, but it is also able to normalize the expression levels of ARG1 e NOS2 enzymes in treated animals (Talmadge, Hood et al. 2007).

Pro-tumoral activity of MDSCs

During tumor growth the continue production of cytokines, growth factors and chemokines stimulates hematopoiesis resulting in a progressive accumulation of MDSCs in the blood, spleen and tumor microenvironment. MDSCs belong to an inflammatory network supporting tumor growth with different mechanisms both at systemic and local level (Coussens and Werb 2002; Balkwill and Coussens 2004; Chioda, Peranzoni et al. 2011).

The suppression of the CTL-mediated immune responses is not the only pro-tumoral strategy exerted by MDSCs since these cells are also able to support directly tumor growth. For example, the activation of the enzyme ARG1 generates the production of polyamines that contribute to cancer cells proliferation (Schmielau and Finn 2001; Belting, Borsig et al. 2002). The MDSC-mediated generation of reactive e potentially mutagenic molecules such as nitric oxide (NO), ROS and RNS could induce DNA damage in the surrounding tissues favoring neoplastic transformation. In the last years, evidences indicate that MDSCs are involved in angiogenesis and metastasis formation, functions already associated also to other hematopoietic cells present in tumor infiltrate as TAMs and TEMs (De Palma, Murdoch et al. 2007; Murdoch, Muthana et al. 2008). Indeed, some TDSFs, as the chemoattractant proteins S100A8 and S100A9, can induce $CD11b^+$ cells accumulation in the pre-metastatic lung; here these MDSCs act as a “radio beacon” for circulating tumor cells thanks to the production of TNF-α, TGF-β and macrophage inflammatory protein-2 (Chioda,

Peranzoni et al.). In mouse tumor models, MDSCs secrete high quantity of MMP9 that, by regulating the bio-availability of VEGF, acts as pro-angiogenic factor. Moreover, under control of TDSFs, MDSCs can enter the tumor endothelium, differentiating in endothelial-like cells (Yang, DeBusk et al. 2004). In addition, failure of anti-cancer therapies based on treatments with antibodies against VEGF is often associated with the presence of large amounts of MDSCs, suggesting a counteracting role of this population (Shojaei, Wu et al. 2007). Lastly, in two models of adenocarcinomas, MDSCs were shown to promote local tumor invasion and metastatization, a process mediated by the activity of different metalloproteases (Yang, Huang et al. 2008; Marigo, Bosio et al. 2010).

Immunosuppression

Suppression of the immune effector cells is the main and relevant property of MDSCs. We and others hypothesized a model of stepwise induction in the immunosuppression mediated by these cells during neoplastic growth (Peranzoni, Marigo et al. 2007; Dolcetti, Marigo et al. 2008; Marigo, Dolcetti et al. 2008; Peranzoni, Zilio et al. 2010). As it shown in Fig. 1, in the early phases of the recruitment of myeloid cells, from the bone marrow and the spleen, only a moderate number of MDSCs accumulate in the tumor micro-environment. There MDSCs encounter and process tumor associated antigens (TAAs). MDSCs loaded with these antigens can reach draining lymph nodes where they interact with $CD8^+$ T cells causing an early stage of CTL tolerance against TAAs. Tumor growth triggers progressively an increasing accumulation of MDSCs which can inhibit immune response both against TAAs and non-self antigens presented by APCs. In this phase MDSCs can activate other regulatory populations as Treg whose function is to help maintaining T cell suppression. At later stages, the massive presence of MDSCs and other immune populations induce a generalized immunosuppressive state targeted not only to $CD8^+$ T cells, but also to $CD4^+$ T lymphocytes, NK and natural killer T (NKT) cells.

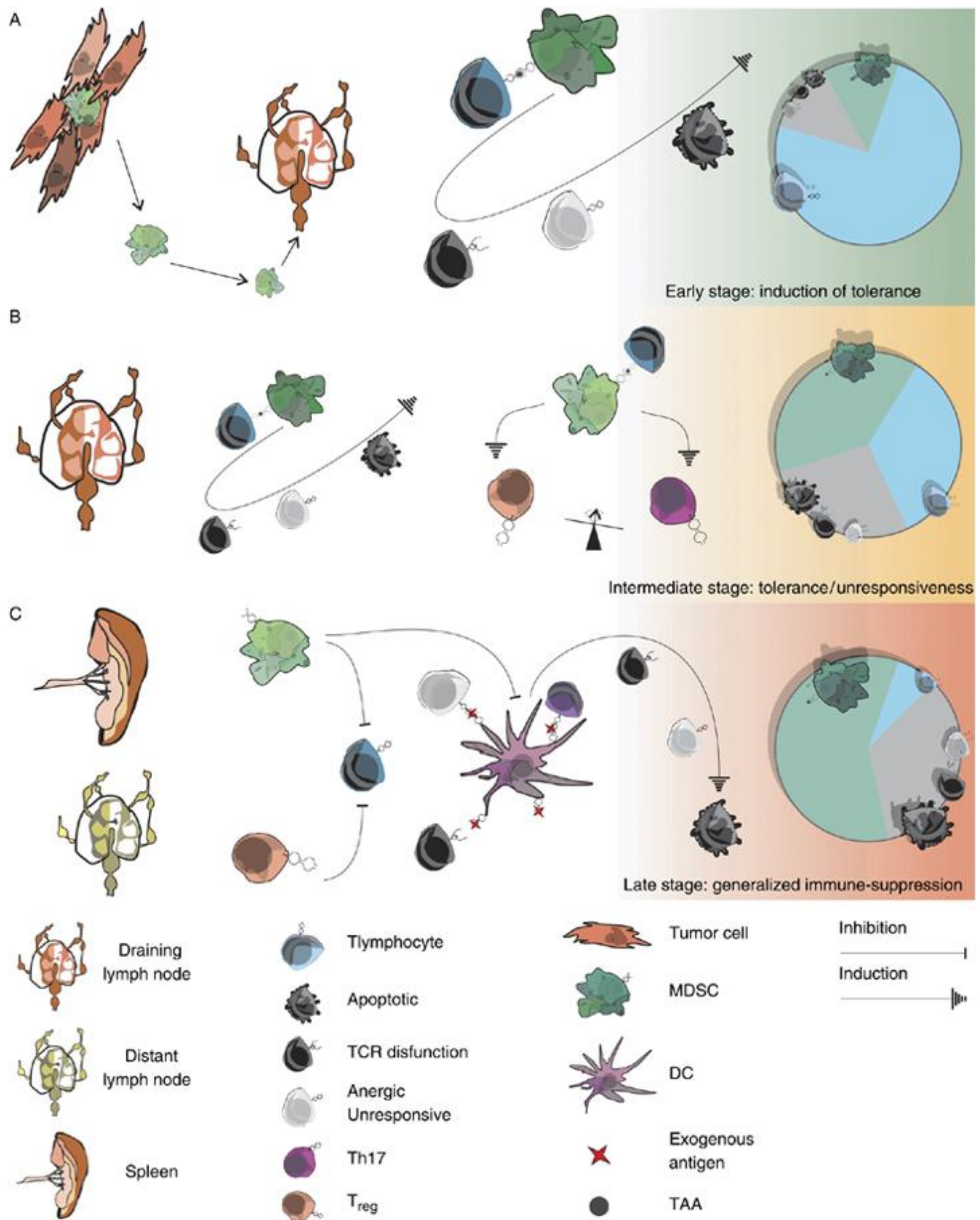

A
Early stage: induction of tolerance
B
Intermediate stage: tolerance/unresponsiveness
C
Late stage: generalized immune-suppression
Draining lymph node
Distant lymph node
Spleen
Tlymphocyte
Apoptotic
TCR disfunction
Anergic Unresponsive
Th17
Treg
Tumor cell
MDSC
DC
Exogenous antigen
TAA
Inhibition
Induction

Figure 1. Working hypothesis for a time- and topologically-structured model of tumor-induced tolerance and immune suppression. (A) In response to various TDSFs MDSCs are mobilized from hematopoietic sites and infiltrate tumor mass where they encounter TAAs. MDSCs presenting TAAs are redirected to tumor draining lymph nodes (TDLNs). The small fraction of MDSCs found in TDLNs (marked in the schematic pie chart on the right side of the picture) are involved in the early steps of tolerance induction by presenting TAAs to naive CD8 T cells through some short-range mechanisms. Such focused 'poisoning' can induce TCR dysfunction, a state of anergy, or apoptosis in T cells. (B) Tumor growth leads to a progressive increase of the number of MDSCs in intermediate stages; moreover, these two inter-related phenomena engage in a self-amplifying circuit. MDSCs, geared up, can now exert an MHC-independent immune suppressive pathway, both against TAAs specific T cells and exogenous antigens presented in TDLNs by APCs. Other regulatory immune populations such as Treg cells and Th17 lymphocytes, activated by MDSCs, could contribute to induce tolerance and unresponsiveness. (C) As exemplified in the pie chart, MDSCs become overwhelming in the late stage and could induce a generalized (i.e. no longer restricted to TDLNs) immune suppression to the detriment of $CD8^+$ and $CD4^+$ T cells and against both TAAs and exogenous antigens, in an antigen-dependent but MHC-independent manner. Other innate immune elements such as NK and NKT cells can act in concert with MDSCs, Treg cells, and Th17 lymphocytes to induce a widespread state of immune suppression. From (Marigo, Dolcetti et al. 2008)

Phenotype and subpopulations of MDSCs

MDSCs are a heterogeneous population that comprises granulocytes, monocytes/macrophages and myeloid precursors at different maturation stages. In mice, MDSCs have been generally described by the staining with anti-CD11b and anti-Gr-1 antibodies. In the last years many studies have tried to clarify which cell subset in the MDSC macropopulation is responsible for the inhibitory effect on T lymphocytes (Peranzoni, Zilio et al. 2010). At first researchers tried to define markers that univocally distinguish suppressive fraction of MDSCs; this was the case of the alpha chain of IL-4 and IL-13 receptor (IL-4Rα) (Gallina, Dolcetti et al. 2006), M-SCF receptor (Huang, Pan et al. 2006) or the co-stimulatory molecule CD80 (Yang, Cai et al. 2006). However, deeper analysis revealed that the expression of these molecules was dependent on the tumor type and hence these markers were not suitable to pinpoint suppressive

MDSCs. MDSCs comprise cell subsets that display different intensity of anti-Gr-1 staining. Indeed, anti-Gr-1 antibody, commonly used to study these cells, allows to identify at least two sub-populations based on its expression, Gr-1^{high} and Gr-$1^{int/low}$ (Terabe, Matsui et al. 2003; Gallina, Dolcetti et al. 2006; Van Ginderachter, Meerschaut et al. 2006; Fichtner-Feigl, Terabe et al. 2008).

As anti-Gr-1 antibody recognizes, albeit with different affinity, two molecules of the Ly6 gene family, Ly6G and Ly6C (Fleming, Fleming et al. 1993; Nagendra and Schlueter 2004; Sunderkotter, Nikolic et al. 2004), some groups tried to separate MDSCs by exploiting the expression of these two molecules.

Two different laboratories have physically separated MDSCs from the spleen of tumor bearing mice exploiting Ly6G and Ly6C expression, obtaining two cell fractions named PMN-MDSC (polymorfonucler MDSC) and MO-MDSCs (mononuclear MDSCs) (Movahedi, Guilliams et al. 2008; Youn, Nagaraj et al. 2008). PMN-MDSCs are described as $CD11b^{+}$Gr-1^{high}Ly6G^{+}Ly6$G^{low/int}$ and display a suppressive function on $CD8^{+}$ T cells response. These cells expressed ARG1 and suppressed antigen-specific $CD8^{+}$ T cells mainly through a ROS-mediated mechanism (Youn, Nagaraj et al. 2008), while according to another work suppression was not recovered by ROS, ARG1, or NOS2 inhibitors, but required instead IFN-γ acting through a not yet indentified STAT1-independent pathway (Movahedi, Guilliams et al. 2008). The MO-MDSCs are indicated as $CD11b^{+}$Gr-1^{int}Ly6G^{-}Ly6C^{high} and, at least in some tumor models, express also markers typical of the monocytic lineage such as F4/80, CD115, 7/4, and CCR2. The suppressive function of this subpopulation is mostly NOS2-mediated and, in part, IFN-γ-dependent (Movahedi, Guilliams et al. 2008; Youn, Nagaraj et al. 2008).

New studies have recently re-examined the relative role of these cellular fractions by using a separation criterion based on the intensity of anti-Gr-1 staining. Although the specificity might be an issue, anti-Gr-1 mAb is still useful since it allows to distinguish several cell subsets. Indeed, it is possible to subdivide the MDSC population in at least three fractions, Gr-1^{high}, Gr-1^{int} and Gr-1^{low}, with peculiar phenotypic and functional characteristics, both in chronic inflammation and cancer (Greifenberg, Ribechini et al. 2009; Dolcetti, Peranzoni et al.). The Gr-1^{high} population comprises Ly6G^{high}

granulocytes and has a weak suppressive activity on antigen-specific or allogenic T response; on the contrary, Gr-1^{int} and Gr-1^{low} fractions, which are mainly composed by immature myeloid cells with ring-shaped nucleus and monocytes, are responsible for lymphocyte inhibition in both models (Greifenberg, Ribechini et al. 2009; Dolcetti, Peranzoni et al.). An indirect contribution of Gr-1^{high} population in suppression cannot be excluded; these cells indeed release INF-γ and are able to suppress the CTL response when present in high concentration (over 30% of lymphocytic culture) *in vitro* (Dolcetti, Peranzoni et al.).

A similar distinction between mononuclear and polymorphonuclear fractions is also described for tumor-infiltrating MDSCs. In two different tumor models 90% of MDSCs were constituted of CD11b$^+$Gr-1^{low}F4/80$^+$IL4Rα^+CCR2$^+$CX3CR1$^+$ monocytes, with markers of both M1 and M2 macrophages, while the remaining were Gr-1^{high}F4/80^{low} cells (Umemura, Saio et al. 2008). In another work, the polymorphonuclear component of MDSCs was found to be recruited at tumor site by C5a complement fragment, while the mononuclear one is induced by the same molecule to produce ROS and RNS (Markiewski, DeAngelis et al. 2008).

Human MDSCs

The human MDSC phenotype is still not completely defined. Indeed, the absence of a counterpart for murine Gr-1 marker has hampered a clear definition of MDSCs. In humans, the variability of the MDSC fractions is even higher than that described in mice, most likely for the higher degree of complexity of the anatomy and ontogeny of the considered tumors. Nevertheless, as in mice, many studies have defined the existence of two MDSCs populations, one granulocytic and one monocytic.

A granulocytic MDSC population, defined by CD11b$^+$/CD115$^+$/CD14 phenotype and ARG1 expression, was described in patients with renal cell carcinoma (Zea, Rodriguez et al. 2005). These cells were further defined as activated CD66b$^+$VEGFR1$^+$CD62L^{low}CD16^{low} granulocytes releasing ARG1 in the plasma following degranulation (Rodriguez, Ernstoff et al. 2009). In patients with non-small cell lung

carcinoma, MDSCs were described as $CD11b^+CD14^-CD15^+CD33^+IL\text{-}4R\alpha^+IFN\text{-}\gamma R^+$ cells; they expressed ARG1 and NOS2 and caused reduction of T cell TCR expression by impairing the stability of CD3ζ chain in T lymphocytes (Liu, Wang et al.).

On the contrary, monocytic MDSCs were identified in patients with metastatic prostate carcinoma, multiple myeloma and head and neck carcinoma (Serafini, Meckel et al. 2006). A monocytic population of $CD14^+/CD11b^+/HLA\text{-}DR^{low/neg}$ MDSCs that mediated suppression through the release TGF-β, was also described in melanoma patients (Filipazzi, Valenti et al. 2007). These MDSCs were significantly expanded in metastatic melanoma patients in contrast with healthy donors. In addition, as described in mice, this population was significantly expanded in non-responding patients who had received cancer vaccines based on GM-CSF (Filipazzi, Valenti et al. 2007). By analyzing $CD14^+$ monocytes in the blood of 111 hepatocellular carcinoma patients, a subpopulation of $HLA\text{-}DR^-$ monocytes was described that significantly increased in both peripheral blood and tumor infiltrate of the patients; these cells suppressed autologous T cell proliferation and had high arginase enzymatic activity (Hoechst, Ormandy et al. 2008). Interestingly, these MDSCs induced an IL-10 secreting, $CD4^+CD25^+Foxp3^+$ Treg population when co-cultured with autologous T cells (Hoechst, Ormandy et al. 2008).

IL-4Rα, a MDSC marker found in many murine tumor models, is expressed by both monocytic and granulocytic MDSCs in melanoma and colon carcinoma patients, but its expression correlates with a suppressive activity only in the monocitic/macrophagic $CD14^+$ fraction (Mandruzzato, Solito et al. 2009). Despite these descriptions of "mature" MDSCs, some studies have focused the attention on cells at earlier stage of differentiation. Indeed, in patients with renal cell carcinoma the MDSCs isolated from the peripheral blood belonged to the $Lin^-/HLA\text{-}DR^-$ fraction; these cells showed both a monocytic and granulocytic phenotype and displayed a suppressive mechanism mediated by NO and ROS. *In vitro,* these cells can be forced to complete differentiation by treatment with trans-retinoic acid (ATRA); their differentiation resulted in loss of their suppressive activity, supporting the fact that in humans, as in mice, a crucial requisite for MDSC suppressive activity resides in their immature phenotype

(Kusmartsev, Su et al. 2008). Moreover, in human mammary carcinoma the accumulation of Lin^{-}/HLA-DR^{-}CD11b^{+}CD33^{+} MDSCs correlates with the stage of cancer progression and with the metastatic spreading (Diaz-Montero, Salem et al. 2009). Recently, G-CSF and GM-CSF were found to induce the expansion of human MDSCs *in vitro* from the bone marrow of healthy donors. These cells comprise promyelocites and myeloblasts characterized by a low level of CD11b expression and absence of CD16 marker. The same authors demonstrate also that this immature population is the only responsible for immune suppression of MDSCs and that more mature subsets were completely devoid of suppressive activity (Solito, Falisi et al.).

Tumor associated metabolism and suppression

The metabolism of L-arginine and L-tryptophane can alter the functionality of immune system either directly by limiting their availability or indirectly through the accumulation of downstream metabolites. The metabolism of arachidonic acid has many effects on tumor progression by influencing the expression of cytokines, chemokines or their receptors; it can also act directly as toxic agent for the cells of innate and adaptive immune system.

L-triptophan metabolism. L-tryptophan is metabolized by two enzymes that mediate the same reaction, i.e. the oxidative cut of 2,3 double bond of indolic ring of this aminoacid. The tryptophane 2,3 deoxigenase (TDO) is an enzyme constitutively expressed in the liver and does not respond to any regulative signal linked to the immune system. Instead, the enzyme indolamine 2,3, deoxygenase (IDO) is finely regulated by a large panel of immune signals (Mellor and Munn 2004). IDO physiologically guarantees the immune tolerance of the mother versus the fetus during pregnancy (Munn, Zhou et al. 1998). Both in human and mouse, under the influence of tumor signals, IDO is expressed in different cellular populations regardless of whether they express it physiologically or not. IDO is expressed in monocyte-derived macrophages in response to IFN-γ mediated activation, in CD123^{+}/CCR6^{+} DCs and in cells with a CD14^{-}/CD83^{+}/CD80^{+}/CD86^{hi}/HLA-DR^{hi} phenotype (Grohmann, Fallarino et

al. 2003; Mellor and Munn 2004). In both human and tumors, IDO is also constitutively expressed in CD123$^+$CCR6$^+$CD19$^+$ plasmacytoid dendritic cells (pDCs) present in tumor-draining lymph nodes, but not in healthy lymph nodes. One hypothesis is that these IDO$^+$ pDCs reprogram the lymph node environment into a tolerogenic one, preventing the correct activation of T lymphocytes. In patients with breast, prostate, colon and brain cancer IDO expression is enhanced as consequence of the aberrant expression in the tumor mass of its transcriptional regulators, such as Bin1 (Muller, DuHadaway et al. 2005). On the contrary, activation of the innate immunity can de-regulate IDO in many cellular populations; this process is triggered by the binding of oligonucleotides CpG (CpG-ODN) to the appropriated toll like receptors (TLR), which activates alternative signaling pathways distinct from those controlled by type I and II IFNs (Wingender, Garbi et al. 2006). The immunosuppressive role of IDO was challenged by the observation that non adherent DCs CD123$^+$/CCR6$^+$ expressed IDO only upon IFN-γ stimulation and that this was not sufficient to prevent both the activation of resting T lymphocytes and the suppression of activated T cells. Since the behavior of these IFN-γ-treated DCs was observed in cells obtained from healthy subjects as well as from patients affected by multiple sclerosis, it was questioned whether IDO activation was *per se* signature of immunosuppressive cells (Terness, Chuang et al. 2005). To date the literature relative to the role played by IDO in immunosuppression is still controversial. Two immunosuppressive mechanisms involving IDO have been proposed: the first should target effector cells functionality; the second should induce APCs to express tolerogenic signals, such as CTLA-4, and therefore affect their ability to activate effector cells (Mellor and Munn 2004). Several studies demonstrated that T lymphocyte functionality suffers for both depletion of L-tryptophan and the accumulation of IDO metabolites, known as kyneurin, which cause proliferative arrest and T cells apoptosis (Grohmann, Fallarino et al. 2003). It was also demonstrated that IDO is expressed both in primary tumors and tumor cell lines and that the administration of specific IDO inhibitors in different tumor models allowed the recovery of tumor-dependent immune suppression (Uyttenhove, Pilotte et al. 2003).

Arachidonic acid metabolism. Lipids play a critical role in immune-regulation both as pro-inflammatory and anti-inflammatory molecules. They modulate the expression of various immunoregulatory factors. Arachidonic acid is an unsaturated fatty acid that needs to be mobilized by phospholipase A2 before being delivered to one of the three biochemical pathways for which it is a substrate: cyclooxygenase, lipooxygenase and cytocrome P450 arachidonic acid monooxygenase. Arachidonate is the principal precursor of a large variety of immunological active lipids: the 15 lipooxygenase produces lipoxines, cyclooxygenase produces prostaglandins and 5-lipooxigenase produces leucotriens (Cabral 2005). Lipids can act both as autocrine and paracrine signals and, depending on the cellular context and on the differentiation state of the cells responding to the signal, they can determine opposite effects. PGE_2 is a prostaglandin, one of the most studied derivative of the arachidonic acid; it can act in concert with pro-inflammatory cytokines as TNF-α, IL-1β and IL-6 to drive DC maturation. On the opposite side in DCs, PGE_2 can induce the expression of IL-10 and IL-12 with the consequent reduction of CCL-3 and CCL-4 expression determining the acquisition of an immune-suppressive effect (Gualde and Harizi 2004).

Cyclooxygenase, one of the enzymes acting on arachidonic acid, has three isoforms: COX1, COX2 and COX3. COX1 is involved in the transition from $CD4^-/CD8^-$ to $CD4^+/CD8^+$ of T cells during thymic maturation (Williams, Mann et al. 1999); COX3 produces anti-inflammatory prostanoids (Gasparini, Longo et al. 2003); COX2 is over-expressed in a lot of different tumors: epithelial adenoma, the mouse model of multiple gut neoplasia, the human colorectal cancer and some pre-neoplastic lesions such as leukoplakia, the actinic cheratosis, the intra-epithelial prostatic neoplasia and the *in situ* carcinoma of bladder and breast (Williams, Mann et al. 1999; Gasparini, Longo et al. 2003). For its expression COX2 was linked to mechanisms of tumor-induced immune suppression. Indeed, in tumor microenvironment COX2 over-expression induces DCs to secrete IL-10 and TGF-β, which drive Tregs activation (Akasaki, Liu et al. 2004). PGE_2 produced by COX2 can directly inhibit T cell activity by increasing the cAMP levels (Uotila 1996). COX2 is also induced in endothelial cells and cancer cells by IL-1β secreted by TAMs and other pro-inflammatory sub-populations (Kuwano, Nakao et al.

2004). In patients with lung cancer, COX2 is over-expressed both in tumor and stromal cells, where its transcription was found to parallel VEGF mRNA levels. Thus in human, COX2 up-regulation is linked to tumor driven angionesis and increased expression of both genes is associated with an unfavourable prognosis of disease (Nefedova, Huang et al. 2004; Yuan, Yu et al. 2005).

L-arginine metabolism

Arginase 1 (ARG1). The hydrolysis of L-arginine to L-ornithine and urea by arginase (ARG) is a key catabolic process in evolution, as testified by the ubiquity of this enzyme across species (Jenkinson, Grody et al. 1996). In mammals, two arginase isoenzymes exist designated ARG1 (or liver arginase) and ARG2 (or renal arginase). The two isoforms differ in tissue and subcellular localization: ARG1 is a cytosolic enzyme abundant in hepatocytes while ARG2 is a mitochondrial isoform constitutively expressed in renal cells, neurons, and enterocytes. ARG most important function is the ammonia detoxification through the urea cycle in the liver but only ARG1 is involved in this process. In fact, complete deletion of *Arg1* gene in mouse is lethal; animals are born normally but only survive 10–12 days, before succumbing of hyperammonemia showing symptoms of decerebrate posture, encephalopathy and tremors in the extremities (Iyer, Yoo et al. 2002). On the other hand complete absence of *Arg2* does not cause any particular abnormalities (Shi, Morris et al. 2001).

Arg1 expression can be induced also in myeloid cells, macrophages, DCs (Munder, Eichmann et al. 1999; Mayer, Bartz et al. 2008) and granulocytes (Munder, Mollinedo et al. 2005) by type Th2 cytokines (Munder, Eichmann et al. 1999), as IL-4/IL-13, TGF-β (Boutard, Havouis et al. 1995), and GM-CSF (Jost, Ninci et al. 2003). Also, the cytokine IL-21 acts as an amplifier of Th2-mediated ARG1 induction by increasing the expression of the IL-4Rα and IL-13Rα1 chains in mouse macrophages (Pesce, Kaviratne et al. 2006).

The transcriptional regulation of *Arg1* is the result of a very complex network of transcriptional regulators activated by a numerous set of cellular and extracellular

signals. *Arg1* expression in immune cells could be both independent (Gray, Poljakovic et al. 2005; Munera, Popovic et al.) or independent (El Kasmi, Qualls et al. 2008) on STAT-6 activation. Th2-mediated induction of *Arg1* in macrophages is regulated by the coordinated action of the transcription factors acting on the enhancer region placed 3 Kb upstream of the promoter region. Among the transcription factors controlling ARG1 expression are PU.1, STAT-6 and CCAAT/enhancer-binding protein beta (C/EBPβ) (Gray, Poljakovic et al. 2005) (Pauleau, Rutschman et al. 2004). C/EBPβ is probably the converging point of different pathways regulating *Arg1* transcription: for example in rats hepatocytes, it controls *Arg1* expression in response to glucocorticoids and glucan (Gotoh, Chowdhury et al. 1997). In myeloid cells, C/EBPβ represents the cross-road controlling *Arg1* expression stimulated by cAMP levels, LPS and hypoxia (Albina, Mahoney et al. 2005). Rauh et al. demonstrated that the Src-homology 2 (SH2)-containing inositol-5′-phosphatase SHIP1 inhibits the inducibility of the *Arg1* gene in macrophages. Indeed in SHIP1$^{-/-}$ mice, virtually all macrophages are skewed towards the M2 phenotype expressing high levels of ARG1 (Rauh, Sly et al. 2004; Rauh, Ho et al. 2005). ARG1 can also be induced in mouse macrophages by the activation of the innate immunity in presence of mycobacterial infections, through a STAT6-independent but C/EBPβ-dependent mechanism triggered by a not fully identified TLR and the MyD88 pathway (El Kasmi, Qualls et al. 2008; Marigo, Bosio et al. 2010).

The role of ARG1 in determining the suppressive activity of MDSCs is documented in different mouse tumor models (Bronte, Serafini et al. 2003; Kusmartsev, Nefedova et al. 2004; Rodriguez, Quiceno et al. 2004; Kusmartsev and Gabrilovich 2005; Gallina, Dolcetti et al. 2006) as well as in human pathologies. Earlier studies on ARG1-mediated suppressive mechanisms focused the attention on the ability of this enzyme to sustain tumor growth by the production of polyamines and by the impairment of NO-mediated anti-tumor cytotoxic response (Bronte and Zanovello 2005). Subsequently, it became clear that the role of ARG1 in tumor progression was far more complex. MDSCs can suppress T cell immune functions by constitutive expression of ARG1 with the consequent L-arginine depletion in the microenviroment (Bronte, Serafini et al. 2003; Liu, Van Ginderachter et al. 2003; Kusmartsev, Nefedova et al. 2004; Rodriguez,

Quiceno et al. 2004; Gallina, Dolcetti et al. 2006; Serafini, Borrello et al. 2006). L-arginine depletion determines the down-regulation of the ζ chain of the CD3 protein complex associated with the T cells receptor (TCR), destabilizing its expression on the surface membrane. The incorrect assembly of TCR alters the signal transduction pathway downstream the receptor as demonstrated, both *in vivo* and *in vitro*, in mouse tumor models and patients with renal carcinoma (Rodriguez, Zea et al. 2003; Baniyash 2004; Rodriguez, Quiceno et al. 2004; Zea, Rodriguez et al. 2004). Furthermore, depletion of essential amino acids, as L-arginine can influence biosynthesis of DNA and consequently causing cell cycle arrest in G_0-G_1 phase (Rodriguez, Quiceno et al. 2007). In this scenario it is clear how important is the role of ARG1 in cells infiltrating the tumor microenvironment: L-arginine depletion can modulate the reactivity of T cells by destabilizing the TCR and inhibit their proliferation, which is sensitive to the bioavailability of this essential aminoacid (Bronte and Zanovello 2005).

A second effect of ARG1 activity is the production of RNS and ROS. These highly reactive molecules intervene in distinct but correlated mechanisms leading to $CD8^+$ T cell suppression by MDSCs (Bronte, Serafini et al. 2003; Kusmartsev, Nefedova et al. 2004). RNS and ROS can cooperate, for example, in the generation of peroxynitrite from O_2^- and NO under conditions of L-arginine starvation (Bronte, Serafini et al. 2003; Gallina, Dolcetti et al. 2006; Sica and Bronte 2007). Peroxynitite induces protein modification such as nitration of tyrosin residues inducing multiple effects as dysfunction in IL-2 receptor signaling in $CD8^+$ T cells or impairment of chemoattractive properties of some chemokines as CCL2 (Bronte and Zanovello 2005; Molon, Ugel et al. 2011). In table 2, different disease models are reported in which ARG1 is involved and its mechanism of action.

Disease model	Mechanism	Species	Reference
DEPLETION OF ARGININE/REDUCED SYNTHESIS OF NITRIC OXIDE			
Allergen-induced hyperresponsiveness	Deficiency of cNOS/nNOS-derived NO	gp	(Meurs, McKay et al. 2002);
Trypanosoma brucei brucei infection	Reduced synthesis of NO due to high arginase activity in macrophages	m	(Gobert, Daulouede et al. 2000 ; Duleu, Vincendeau et al. 2004)
Leishmania mexicana infection	Parasite-encoded arginase: Suppression of macrophage microbicidal activity by reducing NO production via arginine depletion	m	(Gaur, Roberts et al. 2007)
Psoriasis	Arginase overexpression in psoriatic epidermis – reduction of anti-proliferative NO	hu	(Bruch-Gerharz, Schnorr et al. 2003)
Apoptosis	Induction of arginase II in macrophages upon phagocytosis of apoptotic cells	m	(Johann, Barra et al. 2007)
Cystic fibrosis	Sputum arginase activity inversely correlated with exhaled NO	hu	(Grasemann, Schwiertz et al. 2005)
Toxoplasma gondii infection	Stat6-dependent macrophage arginase induction: depletion of L-arginine	m	(Rutschman, Lang et al. 2001; El Kasmi, Qualls et al. 2008)
Mycobacterium tuberculosis infection	TLR-mediated induction of macrophage arginase I: L-arginine substrate competition	m	(El Kasmi, Qualls et al. 2008)
SYNTHESIS OF COLLAGEN VIA PROLINE			
Schistosomiasis	Th2-mediated induction of arginase I	m	(Hesse, Modolell et al. 2001)
Lung allograft fibrosis	TGF-β-mediated induction of arginase; inhibition of arginase = reduction of fibrosis	r	(Liu, Drew et al. 2005)
SYNTHESIS OF POLYAMINES			
Helicobacter pylori (H.p.) infection	Inhibition of iNOS translation = NO production via spermine (generated via arginase-ODC pathway) – persistence of H.p.	m	(Bussiere, Chaturvedi et al. 2005)
Helicobacter pylori infection	Induction of macrophage apoptosis via arginase-ODC-pathway: spermidine/spermine synthesis	m	(Gobert, Cheng et al. 2002)
Leishmania infantum/major infection	N^{ω}-hydroxy-L-arginine: inhibition of arginase = infection control	m	(Iniesta, Gomez-Nieto et al. 2001; Kropf, Fuentes et al. 2005; Muller, Hailu et al. 2008)
Colitis	Protective role of arginase within the intestinal inflammatory milieu	m	(Gobert, Cheng et al. 2004)
Breast adenocarcinoma	Macrophage arginase: providing polyamines for growth of tumour cells	hu	(Chang, Liao et al. 2001)
INHIBITION OF PROTEIN SYNTHESIS DUE TO ARGININE DEPLETION			
Pseudorabies infection	Reduced synthesis of viral structural proteins and folding	m	(Wang, Kao et al. 2005)
INHIBITION OF T CELL ACTIVATION VIA ARGININE DEPLETION			
Tumours	Tumour progress: correlation with TCRζ down-regulation	m/hu	(Rodriguez, Quiceno et al. 2004; Sinha, Clements et al. 2005; Zea, Rodriguez et al. 2005)
Colon carcinoma/fibrosarcoma	L-arginine limitation → ROS production via NOS reductase domain → Suppression of T cell activation	m	(Bronte, Serafini et al. 2003; Kusmartsev, Nefedova et al. 2004; Molon, Ugel et al. 2011)
Pregnancy	correlation with TCRζ down-regulation	hu	(Kropf, Baud et al. 2007)
Helicobacter pylori infection	H. pylori arginase: down-regulation of T cell TCRζ	hu	(Zabaleta, McGee et al. 2004)
Hepatitis B	Inhibition of CD8 T cell proliferation and IL-2 production	hu	(Das, Hoare et al. 2008)
Trauma	correlation with TCRζ down-regulation	m	(Makarenkova, Bansal et al. 2006)
HOST PROTECTION IN INFECTIOUS DISEASE			
Acute Schistosoma mansoni infection	Down-regulation of detrimental Th1 inflammatory response	m	(Herbert, Holscher et al. 2004)
Heligmosomoides polygyrus infection	Intestinal Worm expulsion	m	(Anthony, Urban et al. 2006)
Nippostrongylus brasiliensis infection	Intestinal smooth muscle hypercontractility: worm expulsion	m	(Zhao, Urban et al. 2008)

Table 2: Examples of pathogenetic association of arginase and downstream metabolic consequences with different diseases.

CD, cluster of differentiation; gp, guinea pig; hu, human; IL, interleukin; iNOS, inducible nitric oxide synthase; m, murine; NO, nitric oxide; ODC, ornithine decarboxylase; r, rat; ROS, reactive oxygen species; TCR, T cell receptor; TGF, transforming growth factor; Th, T helper; TLR, toll like receptor. Modified from (Munder 2009)

Nitric oxide sinthase 2 (NOS2). Nitric oxide synthase (NOS) catalyses the reaction of L-arginine and oxygen to produce L-citrulline and nitric oxide (NO), a short-life gas. NOS acts as dimeric enzymes, each monomer contains two distinct catalytic domains: the N-terminal oxygenase domain and C-terminal reductase domain. The N-terminal is the binding site for L-arginine and oxygen in addition to the BH_4 and HEME, while the C-terminal is the binding site for NADPH, FMN and FAD. NO production is illustrated in Figure 2. Three isoforms of NOS enzyme exist, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). A fourth mitochondrial-specific isoform (mtNOS) has been described (Elfering, Sarkela et al. 2002). These isoforms are expressed in different tissue and cell types and display about 50% sequence identity. The catalytic activity of NOS isoforms varies as they are characterized by quite different transcriptional regulation. NOS1 and NOS3 are constitutively expressed in neurons and endothelial cells, their transcription is calcium/calmodulin-dependent and they generate very low amount of NO (picomolar-nanomolar range) only for short time windows. On the other hand, NOS2 is induced by cytokines, is calcium/calmodulin independent and can be expressed essentially in all cells type generating locally high quantities of NO (micromolar range) even for long time periods (Nathan and Xie 1994; Vodovotz, Kwon et al. 1994).

NO is a molecule critical for a number of different cellular pathways and its role in tumor biology is still matter of debate: on one hand, NO might exert a cytotoxic effect on cancer cells, but at the same time it can interfere with many signaling pathways in the effector T cells causing their inhibition.

Anti-tumor potential evoked by the expression of NOS2 was found by studying a

human colorectal adenocarcinoma cell line. Cells from the primary tumor and lymph nodal metastases were shown to have calcium-independent NOS2 activity (Radomski, Jenkins et al. 1991). Depletion of NOS activity, with specific inhibitors, increased the metastatic potential of these cells. Comparison studies on the characteristics of several non-metastatic or highly metastatic clones, obtained from a bulk K1735 melanoma cell line, revealed that the non-metastatic clones generally expressed much higher levels of endogenous NO (Dong, Staroselsky et al. 1994).

Evidence supporting a pro-tumoral effect of NO was produced by Ishii and co-workers, who reported that NO production could lead to the activation of various matrix metalloproteinases involved in tumor driven tissue remodeling (Ishii, Ogura et al. 2003). In human melanoma C32TG cells, MMP-1, -3, -10 and -13 were transcriptionally enhanced when exposed to increasing doses of NO donor, SNAP. Further investigation confirmed that MMP-1 activation was transcriptionally enhanced by NO via the ERK and p38 MAPK pathways, and that these pathways were highly activated during tumor inflammation, resulting in tumor progression (Wu, Akaike et al. 2001).

In mice, NOS2 is expressed in immune cells as macrophages, DCs, and NK cells. While ARG1 is regulated by Th2 cytokines, NOS2 expression is mainly triggered by Th1 cytokines, in particular INF-γ and LPS but also other inflammatory messengers such as IL-1, TNF, IFN-α and INF-β. These cytokines regulate different transcriptional regulators like NF-κB, IFN-regulatory factor 1 (IRF-1) and STAT-1.

The immunoregulatory role of NO released by MDSCs was inferred by treatments of immortalized MDSCs with NOS2 inhibitors which resulted in a reduction of immunosuppression; similar data were obtained when MDSCs were isolated from $Nos2^{-/-}$ (Nos2KO) and tested for their ability to suppress T cell proliferation and activity (Mazzoni, Bronte et al. 2002). The effects of NO in T cell suppression are not associated with early events triggered by antigen-mediated TCR activation but with secondary events linked to the alteration of the IL-2 signaling pathway.

NO can negatively regulate proteins involved in intracellular signaling both directly through post-translational s-nitrosylation of key cysteine residues, which alter the functional properties of the targeted proteins. NO is also an intercellular messenger

sensed by G-proteins, it activates the soluble guanylyl cyclase that, depending on the cell type, can drive adaptive/developmental changes requiring protein synthesis (Bingisser, Tilbrook et al. 1998; Fischer, Palmetshofer et al. 2001). In T lymphocytes, NO blocks the activation by phosphorylation of regulatory proteins (e.g. JAK1, JAK3, STAT5, ERK and AKT) involved in the three principal signaling pathways controlling cell proliferation through the IL-2 receptor (Pericle, Kirken et al. 1997; Mazzoni, Bronte et al. 2002). Studies carried out in human T cells revealed similar control of NO on IL-2 transcription and release from activated T lymphocytes (Macphail, Gibney et al. 2003).

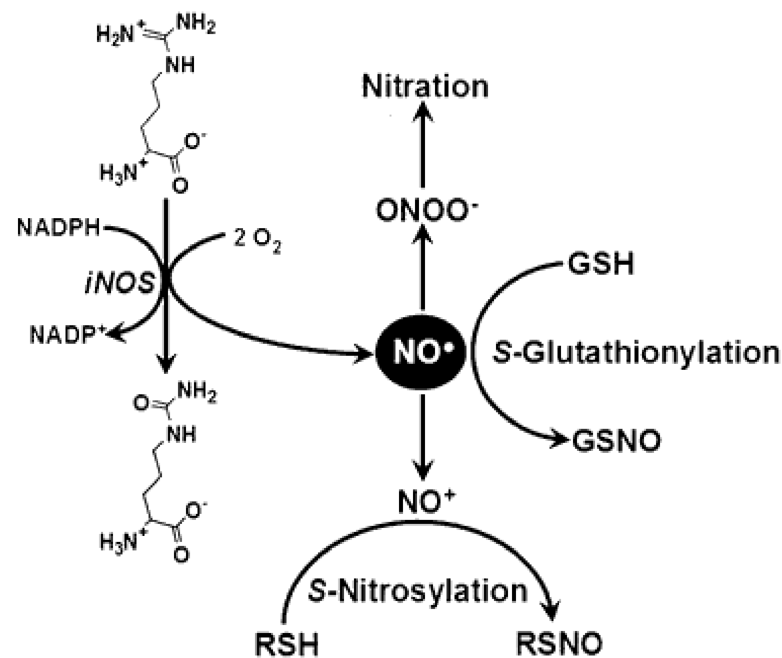


Figure 2: NO chemistry and main downstream redox reactions with biological substrates.

NO is synthesized endogenously from L-arginine, NADPH and oxygen. NO freely diffuses creating concentration gradients across subcellular compartments. Redox or additive reactions with constituents of cellular microenvironment convert NO to a number of NO species (RNS), which in turn, dictate the biological effects of NO (Singh and Gupta).

Cooperation between NOS2 and ARG1 in immune-suppression.

L-arginine metabolism in macrophages was traditionally used as important parameter to discriminate between classical and alternative activation of these cells. As previously mentioned, Th1 type cytokines, (IFN-γ, TNF-α), or microbial lipopolysaccharide (LPS), determine classical activation (M1) of macrophages. M1 macrophages produce pro-inflammatory cytokines, support microbicidal activity and cytotoxic T cell activation; further they show high levels of NO production due to enhancement of NOS2 activity and inhibition of ARG1. At the contrary, Th2 type cytokines (IL-4, L-10, IL-13), TGF-β and glucocorticoids induce the alternative activation of macrophages (M2), which are essential for humoral immunity, allergic and anti-parasitic responses, tissue repair/remodeling and fibrosis; in these cells NOS2 expression is inhibited while ARG1 synthesis is promoted. However, this maturation dicotomy based on the reciprocal regulation of ARG1 and NOS2 represents an extreme simple vision that cannot explain the complex ARG1/NOS2 dependent mechanisms described for MDSC function. These cells, could express both enzymes and co-express macrophage markers of both classic and alternative activation (Gallina, Dolcetti et al. 2006). With respect to the variety of stimuli present in tumor-microenvironment and to co-existence of different MDSC sub-populations, the phenomenon of immunosuppression could very well be the results of both single and combined action of both ARG1 and NOS2 (Bronte and Zanovello 2005; Dolcetti, Peranzoni et al. 2010; Marigo, Bosio et al. 2010). The cooperation between these two enzymes can be explained on biochemical basis: depletion of intracitoplasmatic L-arginine, caused by over-expression of ARG1 in MDSCs, can modify NOS2 activity, producing the superoxide anion (O_2^-), a phenomenon known as "uncoupled NOS reaction" (Xia and Zweier 1997; Xia, Roman et al. 1998; Bronte, Serafini et al. 2003). The O_2^- reacts with NO to generate RNS/ROS, such as the peroxynitrite ($ONOO^-$) and the hydrogen peroxyde (H_2O_2). Peroxynitrite produced by the reaction between O_2^- and NO is a highly reactive oxidant agent that can pass through the membranes and damage different biological targets. Peroxynitrite can act as intra- and extra-cellular messengers and induce post-translational modifications on tyrosine, cysteine,

methyonine or tryptophan residues. These modifications influence different biological activities, such as enzymatic activation/inactivation, protein-protein interaction, cellular differentiation and proliferation (Schopfer, Baker et al. 2003; Radi 2004). The involvement of peroxynitrite in the mechanisms of T cell suppression MDSCs was initially demonstrated by Kusmartsev e colleagues (Kusmartsev, Li et al. 2000). In the extracellular environment, peroxynitrite can induce apoptosis in human T lymphocytes. Moreover, activated T lymphocytes are more susceptible than “resting” lymphocytes to this effect (Brito, Naviliat et al. 1999). Recently, it was demonstrated that peroxynitrite can alter the recognition of the MHC class I-antigen complex by TCRs; this is due to the nitration of some tyrosines in the TCR chain and the CD8 co-receptor molecule, which change the binding specificity of the lymphocyte antigen receptors (Nagaraj, Gupta et al. 2007). Tumor-infiltrating T lymphocytes (TILs) isolated from human prostatic adenocarcinoma have a reduced ability to respond to antigenic stimulation, which was linked to high levels of nitrated tyrosines. The neoplastic prostatic epithelium over-express both ARG2 and NOS2 and TILs isolated from these tumors display high levels of nitro-tyrosines and are poorly responsive to stimulation. The content of nitro-tyrosines can be decreased by a combination of inhibitors for the two enzymes, allowing also a functional rescue of effector T lymphocytes (Bronte, Kasic et al. 2005). These evidences indicate that ARG1 and NOS2 could represent important key players exploited by tumors to induce local immunosuppression and that the rescue of T lymphocytes responsiveness could be achieved by simultaneous inhibition of these two enzymes.

Because of the rapid reactivity of O_2^- with others molecules largely present in the cells, such as water (H_2O), MDSCs produce high quantities of the ROS hydrogen peroxide (Bronte, Serafini et al. 2003) (Kusmartsev and Gabrilovich 2003; Kusmartsev, Nefedova et al. 2004). Hydrogen peroxide can diffuse through the cell membrane acting as cellular messenger. In tumor-bearing animals ROS act both inhibiting DC maturation and favoring MDSC survival/accumulation (Kusmartsev and Gabrilovich 2003), and suppressing T cell function and proliferation. Lymphocyte-MDSC interaction in presence of antigen increases the ROS production from myeloid cells, with a IFN-γ-

independent mechanism and probably mediated by integrines (CD11b, CD18 and CD29), since the blockade with specific antibodies of these molecules reduce both ROS production and T lymphocytes suppression (Kusmartsev, Nefedova et al. 2004). Most likely, also the CD3ζ chain is target of ROS: oxidative stress due to MDSC activity can reduce the expression of CD3ζ chain in tumor bearing mice (Otsuji, Kimura et al. 1996). In patients with advanced stages of cancer, a population of low density granulocytes producing H_2O_2 was found responsible for the down-regulation of the CD3ζ chain on the surface of T lymphocytes, decrease in cytokine secretion and T cell unresponsiveness (Schmielau and Finn 2001). In addition ROS, similarly to peroxynitrite, have also a role in inducing apoptosis of antigen-activated T lymphocytes. This can be due to the deregulation of intracellular levels of anti-apoptotic protein Bcl2 and to the NF-κB-mediated increase of FasL level (Hildeman, Mitchell et al. 2003).

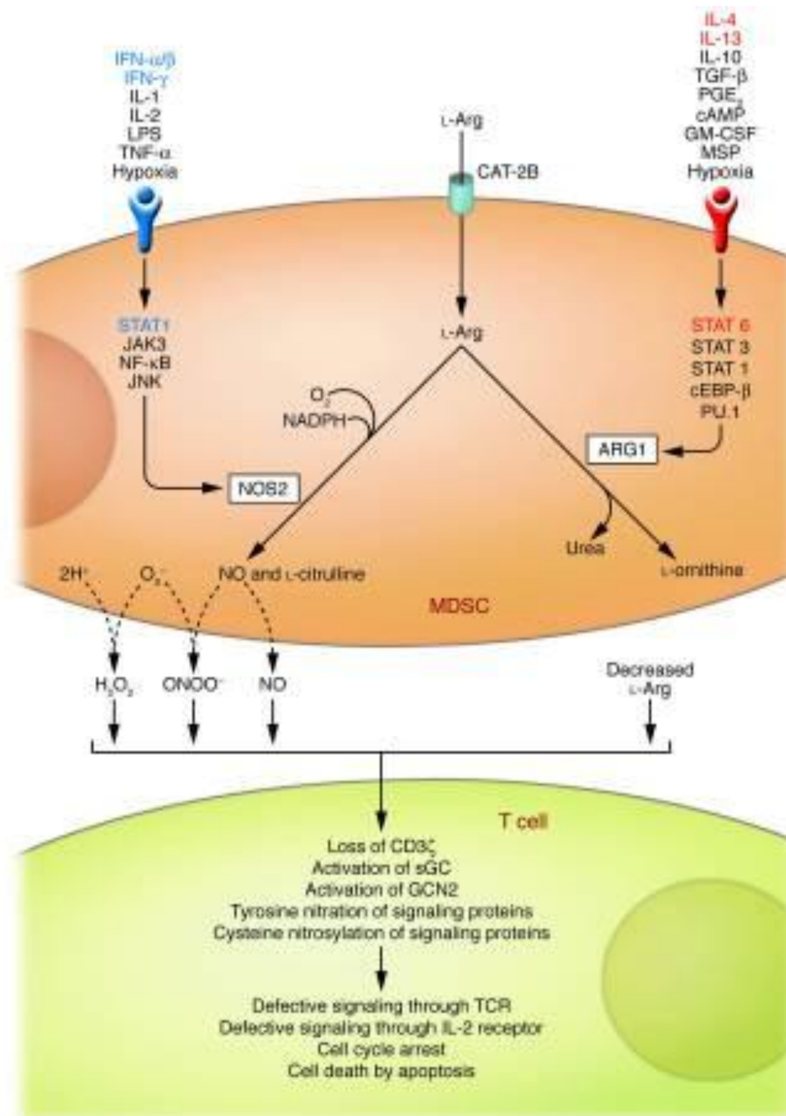


Figure 3: Inhibitory effects of MDSC L-arginine metabolism on antigen-activated T cells.

L-arginine enters MDSCs through a cationic amino acid transporter (CAT-2B) and is mainly metabolized by the inducible forms of NOS and ARG (i.e., NOS2 and ARG1, respectively) although the contribution of other isoforms cannot be ruled out. Depending on the balance between these enzymes, depletion of extracellular L-arginine concentration, NO release, and enhanced production of reactive oxygen and nitrogen species (for example, O_2^- and H_2O_2, and $ONOO^-$, respectively) can ensue. T cells that are activated in the MDSC-conditioned environment stop proliferating and eventually die by apoptosis through pathways involving activation of general control nonderepressible 2 (GCN2) and soluble guanylate cyclase (sGC); tyrosine nitration and S-cysteine nitrosylation of various proteins; loss of CD3ζ; and interference with the IL-2R signaling pathway (reviewed in ref. (Bronte and Zanovello 2005). c/EBP-β, CCAAT enhancer–binding protein β; MSP, macrophage-stimulating protein. From (Sica and Bronte 2007)

Therapeutic targeting of MDSCs

MDSC population is an important actor in immunosoppressive circuits during tumor growth. Because of this, many groups are studying different pharmacological approaches that aim to eliminate these cells or to modulate their function. These therapies are proposed as support to anti-tumor immunotherapy and can act by interfering with different aspects of MDSC biology as recruitment and accumulation in secondary lymphoid organs, maturation and activation. Some strategies, as the use of either anti-Gr-1 depleting antibodies or chemotherapeutic agent such as gemcitabine, aim to eliminate in a non selective way the $CD11b^+/Gr\text{-}1^+$ population; these studies showed some encouraging results leading to restoration of anti-tumor T cell responses (Bronte, Chappell et al. 1999; Terabe, Matsui et al. 2003; Suzuki, Kapoor et al. 2005). However, these treatments need to be evaluated cautiously because they eliminate not only MDSCs but also other myeloid cells. MDSC recruitment can also be limited by interfering with STAT3: this is the case of the Sunitinib, an inhibitor of tyrosin-kinase activity, which reduces the number of MDSCs in patients with renal carcinoma (Xin, Zhang et al. 2009), or COX2 inhibitors, which were shown to reduce both the number and the activity of MDSCs in preclinical models (Talmadge, Hood et al. 2007; Ko, Zea et al. 2009). Another interesting approach, useful especially in the initial phases of tumor development, when MDSCs accumulation is still low, is the conversion of these cells to mature APCs. The 1-α25-diidroxyvitamin D3, a D3 vitamin metabolite, is able to reduce MDSC induction, favoring their maturation to mature DCs, principally blocking the release of GM-CSF (Young, Halpin et al. 1993; Lathers, Clark et al. 2004). Using trans-retinoic acid (ATRA), it is also possible to obtain a similar effect on MDSC maturation with an improvement of anti-tumor immune response (Kusmartsev, Cheng et al. 2003).

Lastly, another possible therapeutic approach is the block of MDSC activity, mainly by inhibition of ARG1 and NOS2 enzymes. Specific inhibitors of these two enzymes actually cannot be administrated *in vivo* because of the severe side effects that they can cause. Nevertheless, some drugs used in clinic for others pathologies have recently shown an efficacy in altering the activity of these two enzymes, eventually resulting in an increase in antitumor response. For example, the nitro-aspirin can interfere with the NOS pathway, because of the NO-donor group, and probably with the pathways of ARG1 through its salicylic portion, that operate indirectly by inhibiting STAT6-mediated signals triggered by IL-4 and IL-13 which are potent activator of ARG1 in MDSCs. (De Santo, Serafini et al. 2005). During last years our group

designed and identified a new molecule, named AT38, able to inhibit the expression of both ARG1 and NOS2 (Molon, Ugel et al.). In addition, phosphodiesterase-5 inhibitors (PDE5) improve the T lymphocytes recruitment at the tumor site and reduce NOS2 and ARG1 expression in MDSCs when administered *in vivo* to tumor-bearing mice (Serafini, Meckel et al. 2006). Thanks to recent studies about sub-populations of MDSCs and their mechanisms of action, it will be possible to design therapeutic strategies aimed to inhibit, physically or functionally, only the real suppressive MDSC population, reducing, in this way, the consequences of a unwanted myelo-ablation achieved with current chemotherapeutic drugs such as gemcitabine.

Aim

Tumors establish strict connections with the surrounding tissues creating a "microenvironment" which support tumor progression. The release of soluble factors by cancer cells also condition distant anatomical sites, to sustain the demand of myeloid cells necessary for tumor neovascularization and metastatic spreading. This tumor-dependent "macroenvironment" includes also myeloid-derived suppressor cells (MDSCs) which have a powerful inhibitory activity on anti-tumor T cell responses. Since the tumor macro- and micro-environment dampen immune responses, it is necessary to understand how MDSCs are recruited selectively to tumor site. Further it is important to clarify what is MDSCs contribution in shaping a microenvironment-barrier to T cell function and recruitment. Targeting MDSCs could represent a powerful anti-cancer therapy in order to facilitate immune attack of malignant cells. Previously, ARG1 and NOS2 were identified as key enzymatic activities responsible for the functionality of MDSCs. Although pharmacological inhibition of NOS2 and ARG1 has already been investigated by our group and others, inhibitors may have off-targets effects and the levels ARG1/NOS2 metabolites may still be functional at levels which are not detectable by common assays. With this work we aimed at defining the mechanisms controlling MDSCs biology in mouse models KO for ARG1, NOS2 or both. We also wanted to characterize ARG1^{+} cells and understand what stimuli could drive its expression, for this purpose we made use of a transgenic reporter mice expressing YFP following ARG1 gene activation. Lastly, we addressed the role of ARG1/NOS2 in tumor development to rule out their role in the various phases of this process: tumor outgrowth, tumor escape or metastatic spreading.

Materials and Methods

Mice

Eight-week-old C57BL/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice and congenic CD45.1 ($Ly5^+$) were purchased from Charles River Laboratories. OT-1 transgenic mice in C57BL/6 background (C57Bl/6-Tg(TCRαTCRβ))1100mjb) that bear a Vα2Vβ5.1-5.2 $H2K^b$ restricted-TCR specific for ovalbumine peptide $OVA_{257\text{-}264}$ $CD8^+$ T lymphocytes were purchased from Charles River Laboratories. OT-1/CD45.1 F1 mice were obtained by crossbreeding OT-1 and CD45.1 mice. Pmel-1 transgenic mice in C57BL/6 background, that bear a Vα1Vβ13 $H\text{-}2^b$ restricted TCR specific for murine and human melanoma peptide $gp100_{25\text{-}33}$ on $CD8^+$ T lymphocytes, were provided by Dott. N. Restifo (Surgery Branch, National Institutes of Health, Bethesda, MD). CL4 transgenic mice in BALB/c background that bear a Vβ8 TCR $H\text{-}2^d$-restricted TCR specific for $HA_{512\text{-}520}$ peptide on T $CD8^+$ lymphocytes, are derived were obtained from L. Sherman (The Scripps Research Institute, La Jolla, CA). $Nos2^{-/-}$ deficient mice were from Jackson Laboratories. $Tie2cre^{+/+}$, $Arg1^{flox/flox}$, $Nos2^{-/-}$;$Arg1^{flox/flox}$ and $Nos2^{-/-}$;$Arg1^{flox/flox}$;Tie2cre and YARG mice, that express the YFP protein under control of *Arg1* promoter, were a gift from P.J. Murray (Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee). For simplicity in this thesis $Arg1^{flox/flox}$;$Tie2cre^{+/-}$ mice were named ArgKO, $Nos2^{-/-}$ mice were named NosKO and $Nos2^{-/-}$;$Arg1^{flox/flox}$;$Tie2cre^{+/-}$ mice were named Arg/NosKO. Animal care and experiments were approved by the institutional review board of Istituto Oncologico Veneto. All mice were maintained under specific pathogen-free conditions in the animal facilities of the Istituto Oncologico Veneto. Mice were inoculated s.c. on the left flank with tumor cells, and tumor growth was monitored every 2 days by caliper measurement.

Cell lines

MBL-2 lymphoma and EG.7, the EL4 thymoma cell line transfected with chicken ovalbumin (OVA) are derived from C57BL/6 mice (aplotype H-2^b). EG.7 cell line was cultured in complete medium supplemented with geneticin antibiotic (G418, Gibco) at of 0,4 mg/ml. C26-GM (aplotype H-2^d) was derived from C26 colon carcinoma cells genetically engineered to release GM-CSF (Bronte, Serafini et al. 2003) This cell line was cultured in complete medium supplemented with geneticin antibiotic at concentration of 0,8 mg/ml. MCA-MN, a primary cell line of fibrosarcoma derived from C57BL/6 mice that spontaneously forms metastasis in lungs was a gift of Antonio Sica (Istituto Humanitas, Milan, Italy). All cell lines were cultured in DMEM 10% FBS supplemented with 2mM L-glutammine, 10 mM HEPES, 20 μM 2β-ME, 150 U/ml streptomycin, 200 U/ml penicillin.

EG.7 cells was injected i.p. or s.c. at doses of $0{,}3x10^6$ and $0{,}5x10^6$ cells/mouse, respectively. C26-GM was injected in the inguinal fold at the dose of $0{,}5x10^6$ cells/mouse. MCA-MN was injected intra-muscle at the dose of $5x10^4$ cells/mouse. MSC-2 are a cell line originated from Gr-1^+ splenocytes from mice immunized with a recombinant vaccinia virus encoding mouse IL-2 (Apolloni, Bronte et al. 2000). These cells up-regulate the expression of *Nos2* and *Arg1* when treated with IFN-γ or IL-4, respectively.

Cytokines and synthetic peptides

Recombinant murine GM-CSF (40 ng/ml final concentration), IL-6 (40 ng/ml final concentration), IL-4 (100 ng/ml final concentration) and IFN-γ (25 ng/ml final concentration) were purchased from Peprotech Inc. Peptides. $OVA_{257\text{-}264}$ (SIINFEKL) aplotype H-2^b-restricted, $hgp100_{25\text{-}33}$ (KVPRNQDWL) aplotype H-2^b and $HA_{512\text{-}520}$ (IYSTVASSL) aplotype H-2 L^d-restricted were synthetized by JPT. All lyophilized peptides were resuspended in DMSO (Sigma-Aldrich) and stored at –20°C until used.

Inhibitors, AT38 and peroxynitrite scavengers

L-NMMA was used 100 mM and was purchased from Merk. MnTBAP was used at a final concentration of 500 µM and were purchased from Calbiochem.

AT38 for *in vivo* administration was dissolved in carbossi-methylcellulose (CMC) 1%. Mice received 30 mg/kg/days of AT38 for 7 days. For *in vitro* experiments, AT38 was dissolved in DMSO, subsequently diluted in RPMI and used at a final concentration of 25 or 50 µM.

Organ cryoconservation and slice preparation

Mice were euthanized and organs were explanted. Immediately after explantation, organs were fixed in 3,7% formaldehyde for 3 hours at 4°C. After fixation organs were dehydrated by solution with increasing sucrose concentration for some days (PBS 20% sucrose and PBS 30% sucrose). Organs were included when they sank to the bottom of the tubes. After dehydration, organs were included in optimal cutting medium (OCT), frozen on liquid nitrogen vapors and stored at -80°C. Frozen organs were cut with a cryostat (Leica) in 10 µm-thick slices which were stored at room temperature.

Immunofluorescence staining

Organ slices were rehydrated in PBS for at least 10 minutes. Samples were fixed 5 minutes in 3,7% formaldehyde at RT and washed; unspecific binding site were blocked with PBS 10% FBS 0,02% tween20 (blocking solution) and primary antibodies were incubated O.N. at 4°C in blocking solution. Slices were washed 3 times for 8' in PBS 0,02% tween20 and conjugated secondary antibodies were added and incubated for 2 hours at 37°C or O.N at 4°C. Slices were washed 3 times for 8' in PBS 0,01% tween20 and once in PBS. Nuclear staining was performed with DAPI (Invitrogen) for 10 minutes at RT. Slices were mounted with ProLong® Gold Antifade Reagent (Invitrogen) and analyzed with a Leica confocal microscopy. The primary antibodies used were rat anti-mouse CD11b (BD Bioscences), goat anti-mouse liver Arginase and rabbit anti-mouse NOS2 (Abcam). Secondary antibodies used were all purchased from Jackson Immune Research.

Wester Blot

Cells were collected and rinsed once in PBS, then immediately frozen in liquid nitrogen. The samples were dissolved in 15 µl Laemmli buffer and denatured for 10 min at 98°C. The samples were separated electrophoretically on a 12% SDS-PAGE gel and transferred onto an Immobilon P membrane (Millipore). The immunoblots were probed with anti-ARG1 or anti-NOS2 (Abcam) Abs. Secondary HRP-conjugated Abs were obtained from GE Healthcare.

Spleen and tumor disaggregation

Mice were euthanized and spleens and tumors were collected. Spleens were mechanically disaggregated and filtered with nylon mesh filter. Splenocytes were centrifuged and red blood cells were lysed with a hypotonic solution. Tumors were cut in small pieces with a scissor; pieces were covered with a digestive solution composed of collagenase IV (1 mg/ml) hyaluronidase (0,1 mg/ml) and DNase (0,03 KU/ml) and incubated at 37°C; every 10 minutes tumors were mechanically disaggregated using a 5 ml pipette. After 1 hour, cells were collected and washed in complete medium twice to remove all digestive solution.

Immunomagnetic sorting

Total $CD11b^+$ cells were isolated from the tumor mass through an anti-CD11b antibody conjugated with magnetic microbeads (Miltenyi Biotec). $1x10^8$ cells were resuspended in 900 µl sorting buffer (PBS 0,5% BSA 2mM EDTA) and 100 µl anti-CD11b microbeads were added. Samples were incubated at 4°C for 15’ and washed with sorting buffer. Samples were resuspended in 500 µl sorting buffer and eluted with LS columns according to manufacturer instructions (Miltenyi Biotec).

Cytofluorimetric staining and analysis

After hypotonic lysis (when necessary) of red blood cells, $5x10^5$-$1x10^6$ cells were washed in PBS and incubated with an anti-Fc-γ receptor (2.4G2 clone) for 10 minutes at 4 °C to reduce unspecific binding. Samples were then stained with antibodies of

interest or their relative isotype controls for 20 minutes at 4°C, then they were washed in PBS and resuspended in 300 µl of PBS for cytofluorimetric analysis. These antibodies were used: anti-CD11b-PerCPCy5.5, anti-Gr-1-APC, anti-CD8a-PE, anti-CD11c-PE anti-Ly6G-PE, anti-CD11b PE from Biologend, the antibodies, anti-F4/80-APC from AbD Serotec; anti-CD45.1 PE and anti Thy1.1 PE from eBioscience. To analyze cell viability, cells were incubated with 7-AAD dye for 10 minutes at RT and then directly resuspended in 300 µl of PBS. Samples were acquired with a FACS Calibur cytofluorimeter (BD Biosciences) and analyzed with FlowJo (Tree Star, Inc.) software.

CFSE cell labeling

For T $CD8^+$ lymphocytes proliferation assay, OT-1/CD45.1 splenocytes were labeled in PBS with 1 µM of Carboxyfluorescein Succinimidyl Ester (CFSE) for 5 minutes at 37 °C (Invitrogen Molecular Probe). The staining was blocked by adding 1/5 volume of FBS, cells were then washed twice with PBS 2% FBS and resuspended in culture medium. The same protocol was used for MDSCs derived from bone marrow culture.

Mixed lymphocyte peptide culture (MLPC)

For proliferation assay, MLPC culture on C57BL/6 background was prepared by mixing γ-irradiated C57BL/6 splenocytes with OT-1/CD45.1 splenocytes. In order to obtain 1% OVA-specific CFSE labeled lymphocytes in the final culture (typical ratio 20:1). $6x10^5$ cells were plated in flat-bottom 96-well plates (BD Falcon) and stimulated for 3 days with 1 µg/ml $OVA_{257-264}$ peptide. For chromium release assay, C57BL/6 splenocytes were mixed with pmel-1 splenocytes, that have $CD8^+$ T lymphocytes specific for gp100 peptide, in order to obtain 1% gp100-specific CTLs in the final culture. $6x10^5$ cells were plated in flat-bottom 96-well plates (BD Falcon) and stimulated for 5 days with 1 µg/ml $hgp100_{25-33}$ peptide. Where required MDSCs (derived from immunomagnetic sorting or from bone marrow cells cultures) were added as a third part in cultures at decreasing percentages (24%, 12%, 6%, 3% and 1,5%). For ^{51}Cr release assay performed without sorted MDSCs, cultures were prepared by mixing splenocytes from C57BL/6 or KO tumor-bearing mice with pmel-1 splenocytes as described above. Cultures were

maintained at 37°C with 5% CO_2 in RPMI medium supplemented with 10% heat-inactivated FBS Superior (Biochrom), 2 mM L-glutammine, 1 mM Na-pyruvate, 150 U/ml streptomycin, 200 U/ml penicillin, 20 μM 2β-mercaptohetanol.

MDSCs generation from bone marrow cells

Tibiae and femurs of C57BL/6 and KO mice were removed with sterile techniques and bone marrow cells were flushed with medium. The red blood cells were lysed with hypotonic solution. To obtain MDSCs from bone marrow cultures, $2{,}5x10^6$ cells were plated in 100 mm petri dishes (Falcon, Becton Dickinson) in 10 ml of RPMI medium supplemented with GM-CSF (40 ng/ml) and IL-6 (40 ng/ml). Cultures were incubated for 4 days at 37°C with 5% CO_2. Cells from the non-adherent and adherent fraction were removed by rinsing the dishes with PBS 2mM EDTA. Cells were then washed and resuspended in medium for culture or PBS for staining and CFSE labeling.

Cytotoxicity evaluation with ^{51}chromium release assay

Lytic activity of MLPCs was analyzed with a 5 hour cytotoxicity assay. MBL-2 cells were used to test cultures of C57BL/6 background. MBL-2 were either pulsed or not with $hgp100_{25\text{-}33}$ peptide (1 µg peptide/$1x10^6$ cells). Target cells were incubated 1 hour at 37°C with 100 µCi of $Na_2{}^{51}CrO_4$ (PerkinElmer). Cells were then washed and resuspended in RPMI 5% FBS medium, at the concentration of $2x10^4$/ml; 100 µl of this suspension were plated in triplicates in U-bottom 96-well microplates in presence of 100 µl of scalar dilutions of effector cells derived from MLPCs. Microplates were centrifuged for 1 minute and incubated at 37°C for 5 hours. After the incubation, 30 µl of supernatant were taken from each well and transferred to scintillator-coated microplates (LumaPlate, Perkin Elmer). When plates were dried the emitted radioactivity was measured with a γ counter (Top Count, PerkinElmer). The percentage of specific lysis was calculated from each well with the following formula:

$$\%\ of\ lysis = 100 * \frac{(Rexp - Rmin)}{(Rmax - Rmin)}$$

Where R_{exp} is the experimental release of each well of effector/target co-colture, R_{min} is the spontaneous release of target cells in the absence of effector cells and R_{max} is the maximum release obtained after forced lysis of cells with 0,5% SDS. 30% lytic unit ($L.U._{30}$) were calculated as the number of effector cells able to induce 30% lysis of 2000 target cells. The percentage of $L.U._{30}$ was calculated as the ratio between $L.U._{30}$ of experimental groups and L.U.30 of the control group (MLPC without MDSCs).

Proliferation assay

After three days of culture (MLPC as described above), cells were collected and stained with anti-CD8 and anti-CD45.1 antibodies. The percentage of proliferating cells was determined by evaluating the CFSE dilution within the $CD8^+$/$CD45.1^+$ population using FlowJo software (Tree Star, Inc.).

Metastasis induction

In order to induce lung metastasis, C57BL/6 mice and KO transgenic mice were intra-muscle injected with $1x10^5$ MCA-MN cells (B6: n=22; ArgKO: n=9; NosKO: n=8; Arg/NosKO n=9). After 26 days, when tumors exceeded 200 mm^2, mice were euthanized and lungs were collected and fixed in Bouin solution (a picric acid, formalin and glacial acetic acid saturated solution). Number of lung metastasis was counted blindly.

Immunohistochemestry

Tumors were fixed in PLP fixative (paraformaldehyde/lysine/periodate), cryoprotected and frozen as described above. The samples were cut with a cryostat (6 µm) and fixed with acetone for 3 min. The primary antibodies used were: anti-nitrotyrosine (1:400; Millipore), anti-CD3 (1:50; Dako), anti-CD8 (1:20; eBioscience), anti-CD45.1 (1:100; BioLegend). The appropriate secondary antibodies were used. Immunoreactivity was visualized with 3,3-diaminobenzidine (DAB; Sigma-Aldrich). Sections were counterstained with hematoxylin and mounted in Eukitt. For each specimen, $CD3^+$, $CD8^+$ and $CD45.1^+$ cells were counted by two different operators in 20 randomly selected fields of each slide at high magnification (400×). The percentage of the area that was immunoreactive-positive was calculated for 20 randomly selected fields of each slide at high magnification (400×).

Adoptive cells therapy (ACT)

C57BL/6 mice were s.c. injected with $0,5x10^6$ EG.7 cells. After 7 days, when tumor area reached approximately 300 mm^3, mice were transferred i.v. with $2x10^6$ antigen experienced T lymphocytes specific for OVA-I antigen. After 6 hours from ACT, mice were injected i.p. with human recombinant IL-2 (30,000 IU); IL-2 was administrated twice a day for three consecutive days. AT38 was prepared as described and was administrated i.p. for 4 days before and 3 days after ACT (30mg/kg/day; twice a day). Tumor volume was monitored every two days with a caliper and mice were euthanized when tumors reached 1000 mm^3.

OVA-I specific CTLs were prepared from OT-I splenocytes. Splenocytes were plated in 6-well plates in presence of 1 µg/ml OVA-I peptide and 20 IU/ml IL-2. Cultures were maintained for 7 days in complete medium supplemented with 20 IU/ml IL-2 (Novartis, Basel, Switzerland). Data were presented as the percentage of survival after ACT.

Arg1 and Nos2 induction in MSC-2 cell line

MSC-2 cells were cultured for 4 days in the presence of either 100 ng/ml IL-4 or 25 ng/ml IFN-γ. Cells were then treated or not with 25 or 50 µM AT38 for 24 and 48 hours. Whole-cell extracts were obtained from 2 x 10^5 cells. Cells were collected and rinsed once in PBS, and then immediately frozen in liquid nitrogen.

Statistic analysis

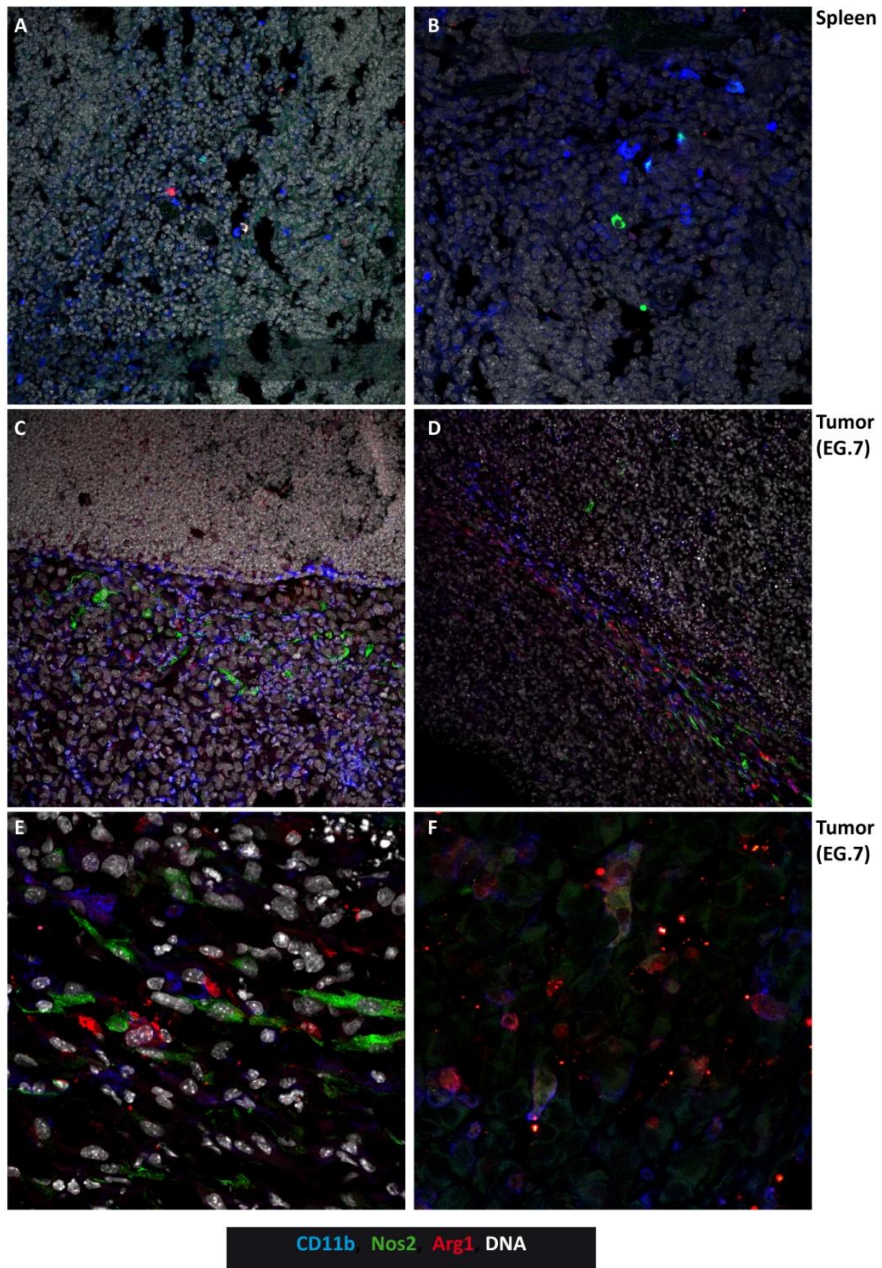
Values are reported as mean ± standard error (SE). Survival experiments are reported as Kaplan-Meyer curves and significance was determined with log-rank test. Student's *t*-test was performed on parametric groups. Values were considered significantly with $p \leq 0{,}05$ and are indicated as *=$p \leq 0{,}05$; **=$p \leq 0{,}01$ and ***=$p \leq 0{,}001$.

Results

ARG1 and NOS2 are preferentially expressed in intra-tumor myeloid cells

Since ARG1 and NOS2 have been shown to be key players in regulating immune suppression by MDSCs, first we attempted to identify which anatomical compartment and to what extent could contain MDSCs expressing ARG1 and/or NOS2. Our previous data based on Affimetrix® profiling of tumor versus splenic MDSCs, already highlighted an over-expression of *Arg1* and *Nos2* in intra-tumoral MDSCs, though it was not clear whether the two enzymes could be co-expressed by the same cell type or their expression was restricted to distinct cell subsets. We thus analyzed the distribution of ARG1 and NOS2 by immunofluorescence (IF) staining of thin frozen tissue sections obtained from either tumor masses or spleen of tumor bearing mice. C57BL/6 mice were injected with EG.7 tumor cells. Approximately 18 days after tumor injection, mice were sacrificed, organs were collected and fixed in formalin. Samples were stained with anti-CD11b, anti-ARG1 and anti-NOS2 antibodies and analyzed with a confocal microscopy. As expected, in the spleen of healthy mice only a small percentage CD11b$^+$ cells is present that did not express neither ARG1 nor NOS2 (Figure 4). In the spleen of tumor bearing hosts, the accumulation of CD11b$^+$ cells was evident but the expression of ARG1 and NOS2 was restricted to few isolated cells. This was somehow surprising because previous data from our group showed that the mechanism used by splenic MDSCs to induce T cell suppression was mediated by both ARG1 and NOS2 (Gallina, Dolcetti et al. 2006). This apparent discrepancy could be due to the functional assays, since splenocytes are cultured for five days and these conditions could influence *Arg1* and *Nos2* expression. However, in tumor mass, it was possible to find a sizeable number of CD11b$^+$ cells positive for both enzymes. Considering their morphology, it seems that these two enzymes are particularly abundant in macrophages, as previously described (Mantovani, Sozzani et al. 2002),

even though they were suspected to be expressed separately, i.e. ARG1 in M1-type and NOS2 in M2-type macrophages. We could detect few cells in which the two enzymes were weakly expressed at the same time. From these data, we can conclude that the expression of ARG1 and NOS2 is particularly enriched in tumor-infiltrating myeloid cells. Most likely the cells expressing these two enzymes are TAMs and, in general, the expression of the two enzymes is mutually exclusive. However it is also possible to identify a limited number of cells expressing both. It remains to be established whether these cells represent a particular cell type with a proper functionality or a transition state between M1 and M2 macrophages, including immature cells like MDSCs. The differential expression of ARG1 and NOS2 in tumor-infiltrating MDSCs with respect to those found in the spleen was also confirmed by Western blotting analysis (Figure4. G). Our data show that regardless the tumor model, ARG1 and NOS2 can be detected only in $CD11b^+$ MDSCs sorted from the tumor mass and not from those isolated from the spleen of tumor bearing animals.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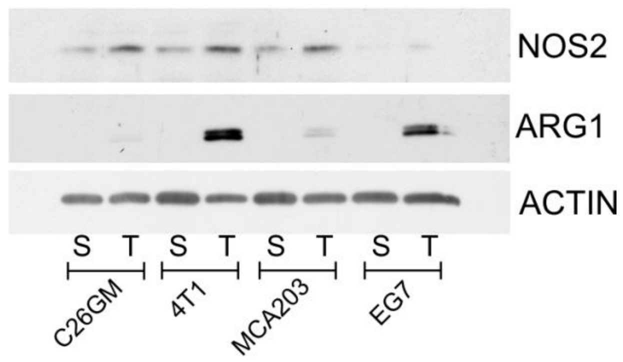
CD11b Nos2 Arg1 DNA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S T S T S T S T
C26GM
4T1
MCA203
EG7

Figure 4: ARG1 and NOS2 are preferentially expressed in tumor-associated CD11b$^+$ cells.

Thin cryosections of spleens from healty **(A)** and tumor-bearing **(B)** mice and tumors from WT EG.7 tumor-bearing mice **(panel C-F)** were stained with anti-CD11b (blue), anti-NOS2 (green), anti-ARG1 (red) and were counterstained with DAPI (white). Panel **A-D:** 40x magnification; panel **E-F:** 63x magnification. **(G)** Western blot analysis for ARG1, NOS2 of CD11b$^+$ cells isolated from spleen and tumor of mice injected with different tumor cells both in BALB/c (4T1 and C26GM) and C57BL/6 (MCA203 and EG.7) mouse backgrounds. Anti-actin antibody was used as endogenous control (S: Spleen; T: Tumor).

During tumor progression, MDSCs acquire an increasing suppressive potential which is dependent on ARG1/NOS2.

Tumor produces a number of factors that can induce the expansion of MDSCs in secondary lymphoid organs and in the tumor mass (see introduction). It is known that the MDSCs accumulation follows tumor growth, however it was not clear whether tumor-infiltrating MDSCs display a suppressive activity as soon as reaching the tumor or whether their function could be progressively “instructed and improved” by inhabiting the tumor microenvironment. In order to answer this question we setup kinetics experiments in which we induced EG.7 tumor in mice and isolated tumor-infiltrating MDSCs at different time points. First, we induced ascites by intra-peritoneal (i.p.) injection of EG.7 in wild type mice. We chose the peritoneum as injection site because of the easier way to recover a significant number of MDSCs, even after only few days post injection. Mice were sacrificed at different time points: 7, 12 and 21 days. CD11b^{+} cells were isolated by immunomagnetic sorting and tested for their ability to suppress T lymphocyte proliferation. Suppression of CD8^{+} T lymphocytes proliferation is one of the distinct features of MDSCs. To test this we performed a proliferation assays in which different concentrations of MDSCs were added as third part to a MLPC. Splenocytes from OT-I mice were labeled with the CFSE dye and cultured with MDSCs isolated from either ascites or the spleen of tumor-bearing hosts. After three days of culture, all cells were collected and analyzed by FACS. In order to trace specifically OT-I T cells, culture were labeled with anti-CD45.1 and anti-CD8 antibodies.

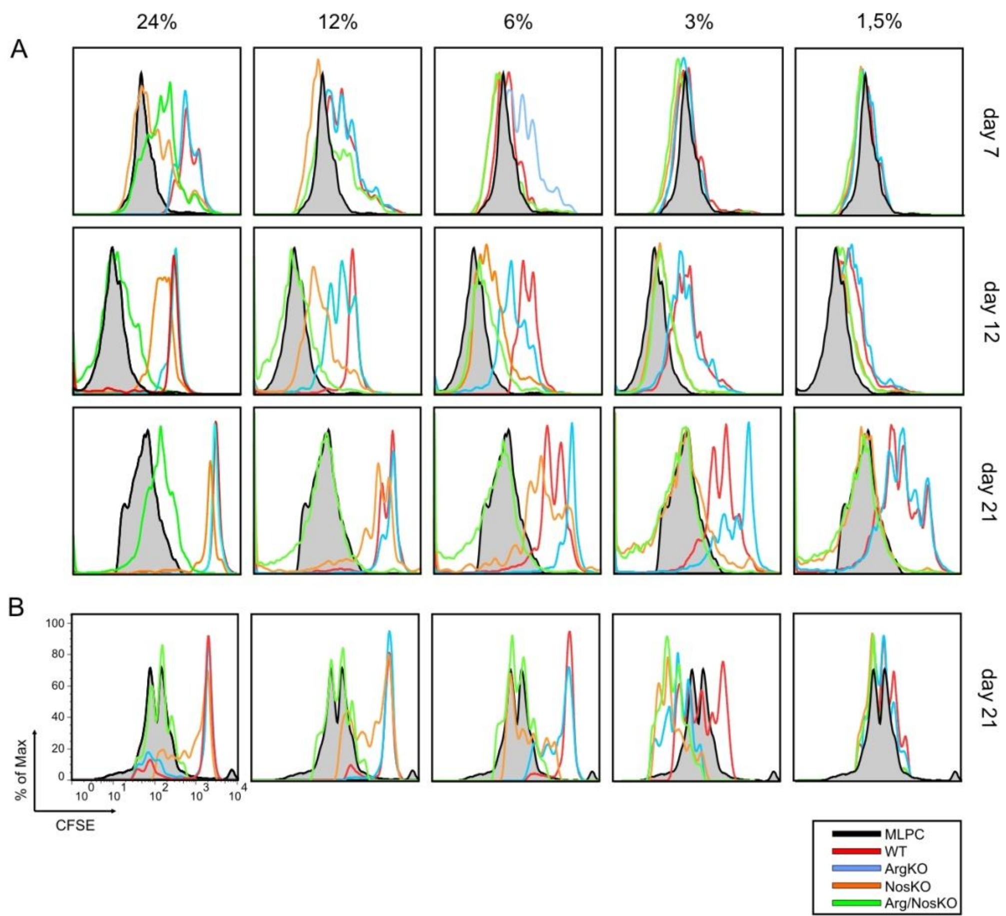
Already starting at day 7, MDSCs from wild type (WT) mice (red line) show a moderate suppressive activity on CD8^{+} T cell proliferation. At this stage, the inhibitory function in only observed when higher ratios of MDSCs:effector cells were used (24%) but it was rapidly lost at lower ratios. The suppressive activity of MDSCs increases along with tumor development, as it can be appreciated by comparing the graphs obtained when only 6% of the total culture was represented by MDSCs. It is clear that the proliferation profile of CD8^{+} T cells is qualitatively different and while in presence of day 7 MDSCs

almost all T cells underwent proliferation, MDSCs from day 21 abrogated completely lymphocyte proliferation.

Next, we wondered whether the increase of suppressive activity could be explained by the activity of ARG1 and/or NOS2. In previous works, chemical inhibitors of these enzymes were employed so the results could be biased by "off-target" effects of the drugs. To address this issue in a more appropriate experimental setting, we made use of transgenic mice were *Arg1* could be specifically deleted in the myeloid lineage (ArgKO mice, blue line). When *Arg1* was deleted in MDSCs, T cell proliferation was essentially suppressed at any time point considered at the levels seen with the WT MDSCs, indicating that ARG1 alone is not sufficient to confer the suppressive activity. Next, we performed the same experiments in mice null for *Nos2* (Nos2KO, orange line). When MDSCs NosKO were used, T cell inhibition was impaired only at high MDSCs:effector cells ratios and starting from late time points (day 21). In other words, MDSCs poorly suppressed T cell proliferation and only at day 21 and when present in high concentrations (24%), suggesting that a conspicuous part of the suppressive activity mediated by tumor-infiltrating MDSCs was NOS2 dependent. Finally, we tested MDSCs from mice deficient for both ARG1 and NOS2 (Arg/NosKO, green line). Under these conditions, MDSCs never acquire a suppressive activity. From these data is clear that timing is very important for acquisition of MDSCs suppressive activity and during progression (from day 14 post tumor injection) ARG1 and NOS2 appear to cooperate to maintain the immunosuppressive machinery.

The EG.7 tumor grown as ascites is fairly aggressive and compared with the same tumor injected subcoutaneously (s.c.) and grown as solid mass. Further, ascites do not really represent the same microenvironment of the solid tumors since the peritoneum possess a high number of blood vessels which are not present in growing solid tumors. For this reason we decided to compare the suppressive activity of MDSCs during s.c. tumor growth. As shown in Figure 5B for day 21, we obtained the same results described for the EG.7 tumor grown in ascites. We further quantified these results (Figure 5C) in terms of proliferating $CD8^+$/$CD45.1^+$ T cells as percentage of the control MLPC. Statistics on three independent experiments confirmed data obtained for the

ascites model. These findings suggest that, regardless the anatomical site, MDSCs accumulate and develop their suppressive activity along with tumor growth. Moreover MDSCs full suppressive potential requires both ARG1 and NOS2 enzymatic activity, although NOS2 contribution seems to be the main factor responsible for MDSC-mediated inhibition of T cell proliferation.


24%
12%
6%
3%
1,5%
A
B
day 7
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day 21
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% of Max
CFSE
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Arg/NosKO

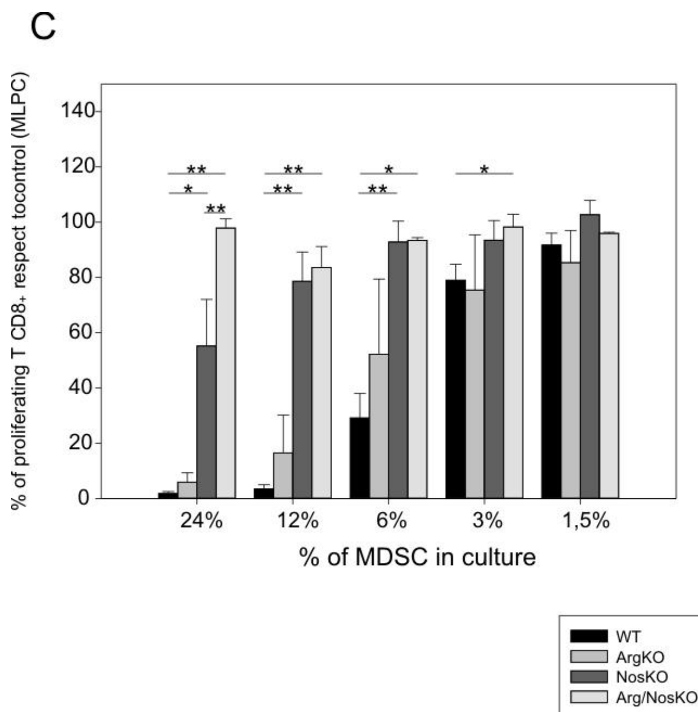

C
% of proliferating T CD8+ respect tocontrol (MLPC)
% of MDSC in culture
24%
12%
6%
3%
1,5%
WT
ArgKO
NosKO
Arg/NosKO

Figure 5. The suppressive activity of MDSCs is reduced in NosKO and abrogated in Arg/NosKO mice. CFSE-labeled $CD8^+$ $CD45.1^+$ T lymphocytes were stimulated with the OVA-I peptide for 3 days and cultured with MDSCs from either WT or KO tumor-bearing mice isolated from either peritoneal ascites **(A)** or subcutaneous masses **(B). (C)** Amount of proliferating $CD8^+$/$CD45.1^+$ T cells in samples after three days of culture with peptide and MDSCs derived from each mouse strain. The percentage is calculated considering the number of proliferating $CD8^+$/$CD45.1^+$ T cells in the culture relative to control MLPCs, set as 100%. T cell proliferation in presence of Arg/NosKO MDSCs is significantly increased compared to MLPCs with WT MDSCs, at all concentrations but 1,5% (24% WT vs Arg/NosKO: p=0.002; 12% WT vs Arg/NosKO: p=0.0013; 6% WT vs Arg/NosKO: p=0.011; 3% WT vs Arg/NosKO: p=0.048);T cell proliferation between cultures with NosKO MDSCs and Arg/NosKO MDSCs is significantly different when MDSCs are 24% of the culture (p=0.005). (Student's *t* test, *, P ≤ 0,05; **, P ≤ 0,01.***, P ≤ 0,001) Values represent the mean ± standard error (SE) of three independent experiments.

Nos2 KO determines a differential MDSC recruitment in the spleen of tumor bearing hosts.

The previous data suggest that during tumor growth, MDSCs acquire progressively the ability to suppress T cells and that this is mostly dependent on NOS2. However, this phenomenon could have many explanations. NOS2 activity could be necessary to suppress directly T cell proliferation or NOS2 might regulate differentiation of suppressive MDSCs, and NOS2 deficiency lead to a qualitatively different population of MDSCs. For these reasons, we sought to investigate whether the reduced suppressive activity of NosKO- and Arg/NosKO- derived MDSCs could be due to a differential accumulation of MDSCs. The main sites where MDSCs accumulate during tumor development are the spleen and the tumor mass, thus we analyzed the progressive accumulation and phenotype of MDSCs in either WT mice or mice KO for *Arg1*, *Nos2* or both.

Spleens and tumor masses of WT and transgenic mice were explanted when tumor reached 200 mm^2 and single cell suspensions of splenocytes and tumor cells were labeled with anti-CD11b and anti-Gr-1 antibodies. We recently published that it is possible to identify three MDSC subsets based on the Gr-1 expression (Gr-1^{high}, Gr-1^{int} and Gr-1^{low}) with functionally distinct features (Dolcetti, Peranzoni et al.). When we analyzed the tumor infiltrating MDSCs, no significant alteration in their number or Gr-1 distribution was observed regardless the depletion of ARG1 and/or NOS2. The only statistically significant difference was the reduction of Gr-1^{int} MDSCs among $CD11b^+$ cells in Arg/Nos2 KO mice (Figure 6B). This is in line with the suppressive defect shown by these MDSCs (Figure 5), since the Gr-1^{int} subset is mainly responsible for T cell inhibition. However it is not sufficient to explain the defects seen when only NOS2 was depleted.

Next we analyzed the accumulation of the MDSCs in the spleen. As shown in Figure 6C-D, a significantly reduced number of the total $CD11b^+$ cells can be observed only in Arg/NosKO mice (p=0,013). However, the relative distribution of the Gr-1^{high}, Gr-1^{int} and Gr-1^{low} subset varied depending on the genetic background considered.

Arg/NosKO display a significantly lower number of all subpopulations with the Gr-1^{low} subset being under-represented when compared to the wild type distribution. Single *Nos2* deletion yielded only a defect in the Gr-1^{low} group, whereas *Arg1* single deletion did not cause significant changes. Interestingly, the relative abundance of the Gr-1 fractions was pretty different between spleen- and tumor-derived CD11b^{+}/Gr-1^{+} cells. As previously reported, Gr-1^{high} cells are mainly polymorphonuclear cells, Gr-1^{int} cells comprise monocytes and myeloid precursors while the Gr-1^{low} fraction is enriched in monocytes and macrophages (Dolcetti, Peranzoni et al. 2010). Thus it seems that the population mainly affected by NOS2 depletion comprises monocytes and macrophages. It is intriguing that, while we registered functional defects in MDSCs isolated from tumor masses, depletion of ARG1/NOS2 affected the accumulation of these cells in the spleen where the expression of ARG1/NOS2 is limited.

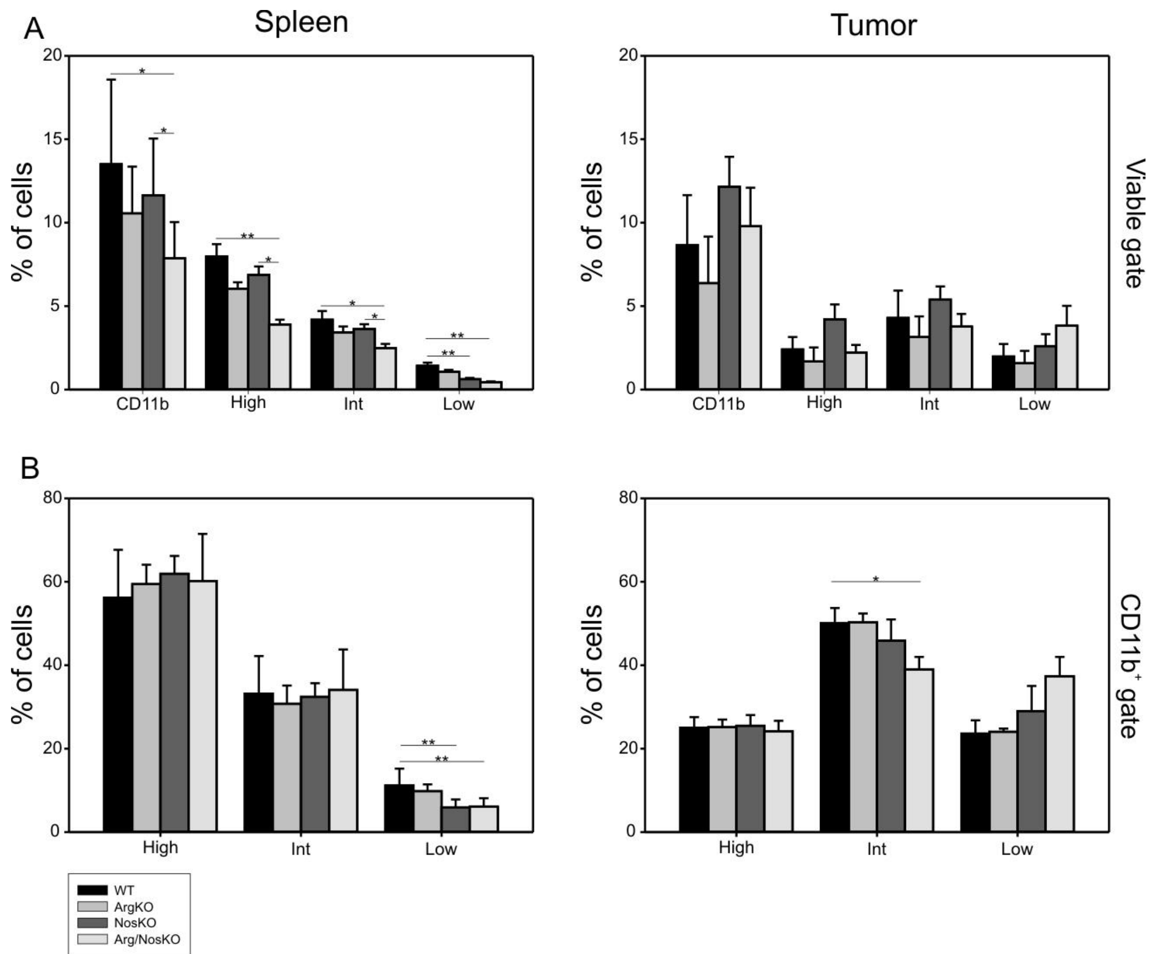


Figure 6. Arg/NosKO mice have a reduced number of MDSC subsets in the spleen.

(A) Accumulation of $CD11b^{+}$/$Gr\text{-}1^{+}$ cells in the tumor and **(C)** the spleen of WT and transgenic mice. The percentage of cells is calculated on the number of viable cells. When compared to WT mice, in the spleen of Arg/NosKO mice there is a significant decrease in $CD11b^{+}$ cells (p=0.013) and all $Gr\text{-}1^{+}$ fractions ($Gr\text{-}1^{high}$ p=0.005; $Gr\text{-}1^{int}$ p=0.028; $Gr\text{-}1^{low}$ p=0.001). The $Gr\text{-}1^{low}$ subset is reduced also in NosKO mice (p=0.001). Among $CD11b^{+}$, $Gr\text{-}1^{high}$ and $Gr\text{-}1^{int}$ cells the difference between NosKO and Arg/NosKO mice is statistically significant ($CD11b^{+}$ p=0.026; $Gr\text{-}1^{high}$ p=0,025; $Gr\text{-}1^{int}$ p=0.019) There is no significant differences in tumor-infiltrating MDSCs derived from either wild type or transgenic animals. **(B-D)** The percentage of cell subsets is calculated as fraction of the $CD11b^{+}$ population. In NosKO and Arg/NosKO mice, there is a significant reduction of the splenic $Gr\text{-}1^{low}$ subset (p=0.001 and p=0.003, respectively) compared to WT mice. (Student's *t* test, *, P ≤ 0.05; **, P ≤ 0.01.***, P ≤ 0.001) Values represent the mean ± SE of three independent experiments.

MDSCs from NosKO and Arg/NosKO have a reduced suppressive activity on CD8⁺ T lymphocyte cytotoxic function.

One of the most important functions of MDSCs consists in blocking not only the proliferation but also the cytotoxic activity of effector T cells. To test the role of ARG1 and NOS2 also in this MDSC property, we performed a short term ^{51}Cr release assay. Briefly, we setup cultures in which antigen-specific CD8^{+} T cells were stimulated with the specific peptide in presence of decreasing MDSC concentrations. At the end of the culture period, the cytotoxic activity of effector CD8^{+} T cells was measured as the amount of ^{51}Cr release induced in labeled target cells. Values are presented as 30% lytic units (L.U.$_{30}$), a parameter indicating the lytic potential of the lymphocytes that allows normalization of results across different experiments. MDSCs were isolated by immunomagnetic sorting with anti-CD11b beads from the tumor masses and the spleen of mice in which EG.7 cells were injected s.c. 21 days before. In cultures, where either WT or ArgKO MDSCs were added, it was possible to appreciate a strong immunosuppression of the cytotoxic activity mediated by peptide-activated lymphocytes. Conversely, when NosKO- or Arg/NosKO-MDSCs were added to the cultures, CD8^{+} T cells maintained their ability to eliminate target cells, with some differences among these groups. In fact, at higher concentrations (24%) NosKO-MDSCs partially suppressed effector cells, Arg/NosKO-MDSCs did not inhibit T cell activity at any concentration, but rather they seemed to stimulate CTL cytolytic potential (Figure 7A).

Since we unveiled a NOS2-dependent differential MDSC accumulation in the spleen (Figure 6), we also performed the cytotoxicity assays on MDSCs isolated from the spleen of WT and KO mice. In this case MDSCs were not isolated by immunomagnetic sorting, since in the spleen there were not contaminant cancer cells that could interfere with T cell function. In these cultures, total splenocytes were stimulated with the immunogenic peptide. As shown in Figure 7B, MDSCs present in the spleens of NosKO and Arg/NosKO mice failed to suppress efficiently the cytolytic activity of T effector cells. However, as shown for MDSCs isolated from tumor, while NosKO-MDSCs retained some suppressive activity, Arg/NosKO-MDSCs did not.

Taken together these data show that *ex vivo* MDSCs isolated from both tumor and spleen can efficiently suppress T cell proliferation and cytotoxicity, and that the inhibition of these mechanisms is dependent on NOS2. It is interesting to notice that even though single depletion of ARG1 in MDSCs is not sufficient to alter their effect on T cells, deletion of both enzymes is necessary to completely abrogate MDSC-dependent suppression. Further, while it is easy to explain the contribution of ARG1 and NOS2 in effector functions of tumor-derived MDSCs, it is puzzling to reconcile functional data obtained with splenic MDSCs which barely express ARG1 and NOS2. Our results could indicate that either the expression of ARG1 and NOS2 can be up-regulated during 5 day cultures by stimuli coming from activated T cells, as previously suggested (Gallina, Dolcetti et al. 2006), or else these enzymes do not directly mediate MDSC suppression but rather they act through unknown secondary pathways.

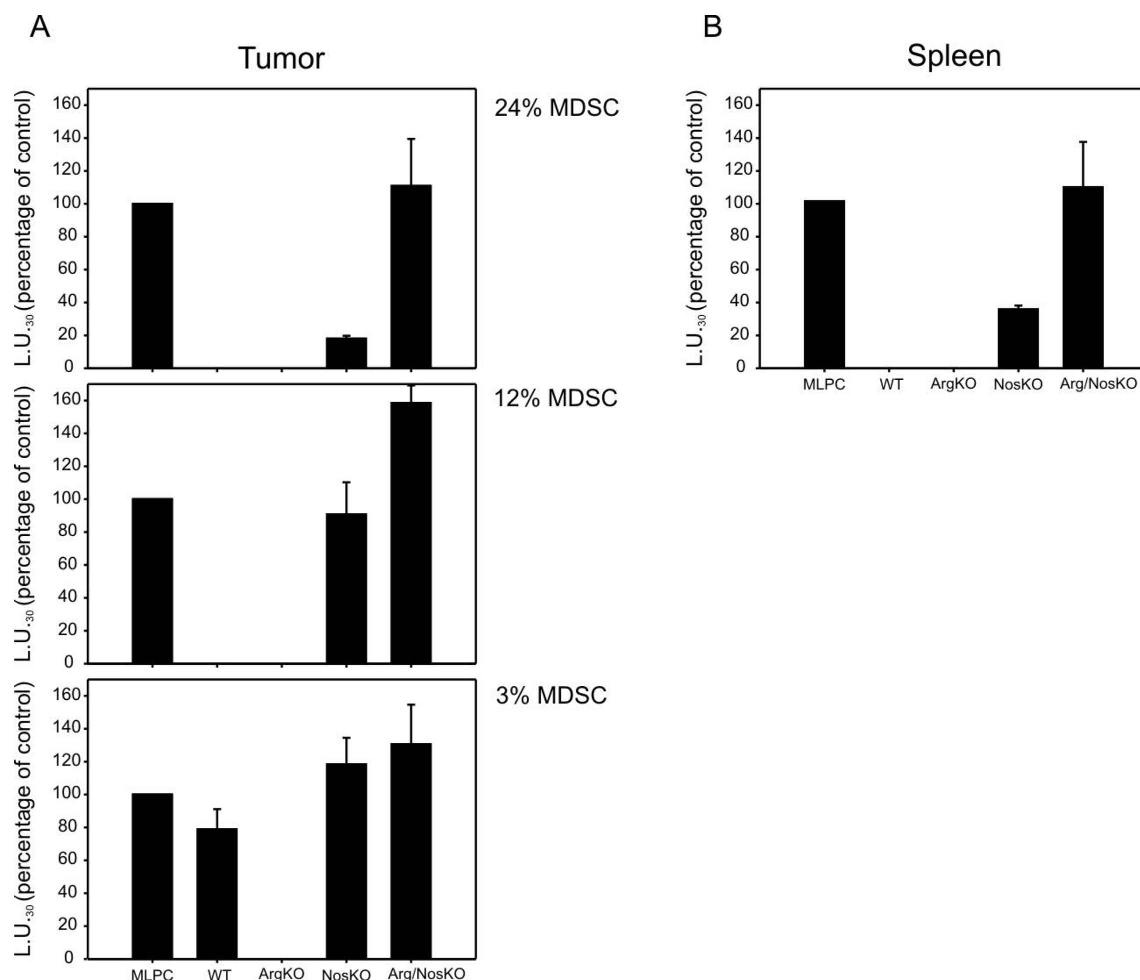


Figure 7. MDSCs from NosKO and Arg/NosKO fail to suppress cytotoxic activity of T lymphocytes.

(A) T lymphocytes with a TCR specific for gp100 melanoma antigen, obtained from pmel mice, were cultured in the presence of MDSCs isolated by immunomagnetic sorting from tumors of WT and KO mice and stimulated with gp100 immunodominant peptide. After 5 days of culture, the cytolytic activity of T cells was tested in a ^{51}Cr release assay. Values are described as lytic unit 30% (L.U.$_{30}$), a representation of the lytic potential of T lymphocytes. **(B)** Suppressive effect on T lymphocytes of splenic MDSCs induced in WT and KO EG.7 tumor-bearing mice.

ARG1 and NOS2 activity do not interfere with the in vitro differentiation of MDSCs from bone marrow.

In the previous data, we considered the contribution of ARG1 and NOS2 in MDSCs isolated from tumor bearing hosts. The effects of ARG1 and NOS2 activities were measured both in the tumor and in the spleen. Even though these genes are strongly expressed only in the tumor microenvironment, we could not rule out that these enzymes were not required for systemic differentiation/expansion of MDSCs. We thus addressed whether ARG1 and/or NOS2 could control *in vitro* differentiation of MDSCs from the bone marrow (BM) of either wild type or KO mice.

Recently our group developed a protocol for the generation of MDSC-like cells from the BM of healthy animals (BM-MDSCs). BM-MDSCs are phenotypically and functionally similar to tumor-induced MDSCs. BM cells were isolated from femurs of WT and KO healthy mice and cultured for four days in presence of GM-CSF and IL-6. After *in vitro* BM-MDSCs differentiation, we tested the phenotype and the suppressive activity of these cells. Results are described in Figure 8. Given the differences in the relative accumulation of Gr-1 subsets measured in the spleen of tumor bearing KO mice, we sought to investigate whether this result could be explained by defects in cell differentiation from BM precursors. In order to obtain a more comprehensive picture, cells were stained with anti-CD11b, anti-Gr-1, anti-F4/80 and anti-CD115 (or M-CSF receptor) antibodies. The cytofluorimetric analysis in Figure 8 shows that depletion of ARG1 and/or NOS2 did not change the amount of CD11b cells, nor the relative distribution of the Gr-1^{hi}, Gr-1^{int} and Gr-1^{low} subsets. Since ARG1 and NOS2 are fundamental for the function of alternatively or classically activated macrophages (Mantovani, Sozzani et al. 2002), we investigated whether the absence of either enzyme could also interfere with the correct maturation of macrophages. In Figure 8A macrophages are indicated as F4/80^{high}/$CD115^{+}$ cells and no significant differences could be measured in the numbers and phenotype of this population, regardless the genetic background considered.

Next, we tested the suppressive activity of BM-MDSCs in a T lymphocyte proliferation assay. As shown in Figure 8B, wild type (WT) WT-BM-MDSCs and Arg-BM-MDSCs did

not display any suppression on T cell proliferation while NosKO- and Arg/NosKO-derived BM-MDSCs did not acquire suppressive potential, at least under the conditions tested. Differently from *ex vivo* MDSCs, BM-MDSCs suppressive activity was entirely dependent on NOS2, since no difference in the suppressive activity was observed when NosKO- and Arg/Nos-BM-MDSCs were compared on a cell per cell basis.

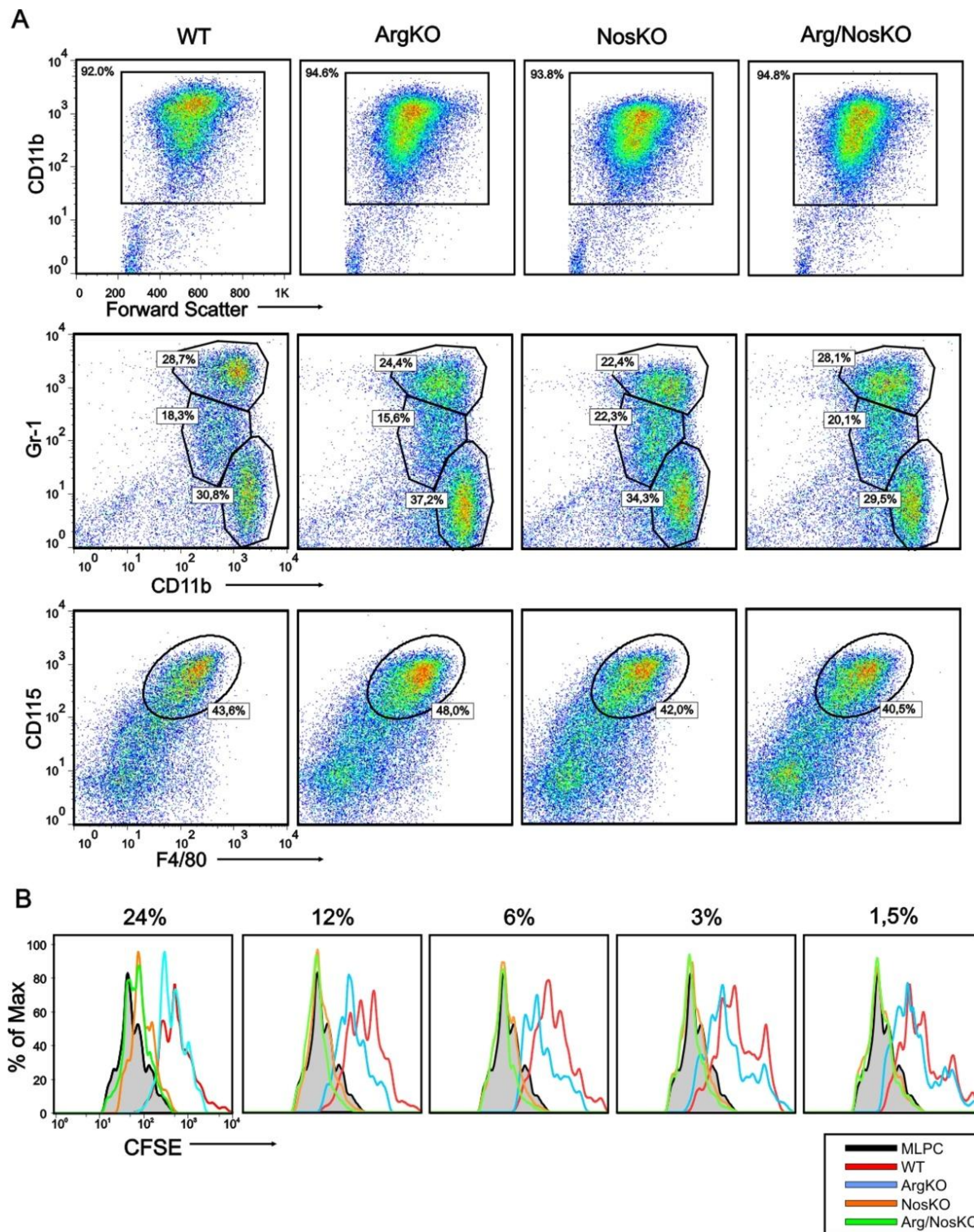


Figure 8. Absence of ARG1 and/or NOS2 did not alter the *in vitro* differentiation of MDSCs from bone marrow but interfered with the acquisition of the suppressive activity.

BM cells from healthy, WT and transgenic mice were cultured for four days with 40 ng/ml of GM-CSF and IL-6 cytokines. Cells were then collected and labeled with anti-CD11b, anti-Gr-1, anti-F4/80 and anti-CD115 antibodies **(A). Top:** percentage of $CD11b^+$ cells after differentiation with cytokines. **Middle**: CD11b/Gr-1 staining and percentage of Gr-1^{high} Gr-1^{int} and Gr-1^{low} fractions. **Bottom**: F4/80/CD115 staining and percentage of double positive cells (macrophages/monocytes). Differentiated cells were also tested for their suppressive activity on T lymphocyte proliferation **(B)**.

ARG1 and NOS2 depletion does not affect in vitro proliferation of CD11b$^+$/Gr-1$^+$ MDSC subsets

BM-MDSCs derived from animals lacking ARG1 and/or NOS2 do not show alteration in their subpopulation composition. Since it has been reported that L-arginine depletion can affect cell proliferation determining cell cycle arrest, we wanted to investigate the proliferation potential and the viability of BM-MDSCs derived from WT or KO mice. BM cells were obtained from femur, CFSE labeled and differentiated in presence of GM-CSF and IL-6. During each of the 4 days of the differentiation protocol, cells were collected and the CSFE labeling was analyzed together with the CD11b and Gr-1 markers by cytofluorimetry. In wild type BM-MDSCs, the three CD11b$^+$/Gr1$^+$ (hi, int, low) subpopulations have different rates of proliferation which reflect the respective degree of differentiation among these cells. Only the Gr-1$^{int/low}$ subsets show high proliferation rate, which increases during the culture. The same proliferative patterns were obtained for BM-MDSCs KO for ARG1 and/or NOS2, suggesting that these enzymatic activities do not play roles directly on MDSC proliferative rates.

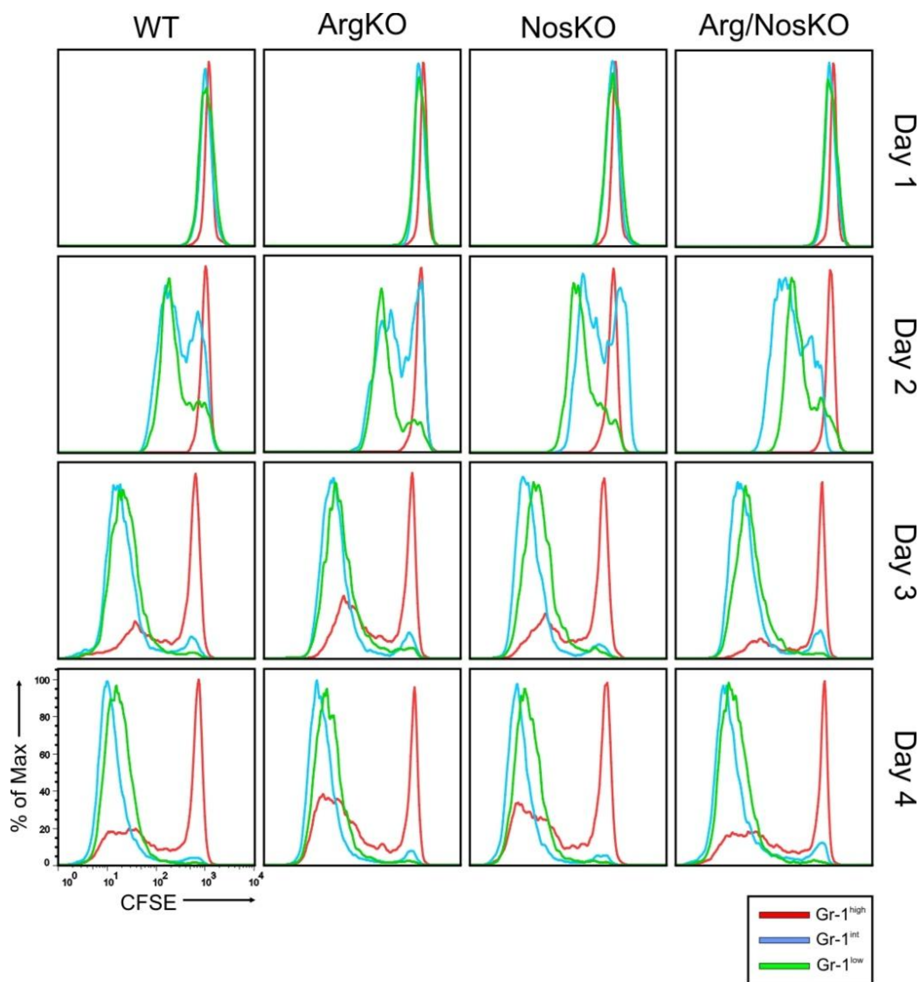


Figure 9. ARG1 and NOS2 did not interfere with *in vitro* proliferation of MDSCs subset.

Freshly isolated BM-cells were labeled with CFSE and cultured as described in Figure 5 for the *in vitro* generation of BM-MDSCs. Every day, the proliferative potential of MDSC subsets Gr-1^{hi}, Gr-1^{int} and Gr-1^{low} were analyzed and monitored as CFSE dilution. In KO strains there were no appreciable differences in proliferation potential of BM derived MDSC precursors in comparison with WT mice.

ARG1 and NOS2 are involved in tumor spreading and metastatic formation

MDSCs have been shown to favor tumor growth, by inhibiting T cell function, promote tumor vascularization and influence the metastatic process (Ben-Baruch 2006; Soria and Ben-Baruch 2008). However, the molecular mechanisms underlying this complex network of events are not clearly defined. Since TAMs have been proposed to be responsible for tumor invasion and spreading (Qian and Pollard)and we found that ARG1/NOS2 depletion affected the accumulation of macrophages, we wanted to elucidate whether ARG1 and NOS2 had a role in tumor spreading and distant metastasis formation. For this reason we used MCA-MN tumor, a fibrosarcoma spontaneously forming lung metastasis following intramuscular injection (Sica, Saccani et al. 2000). MCA-MN were inject in the posterior leg of the animals. After 26 days, when tumors exceed a volume of 200 mm^2, mice were sacrificed, lungs were explanted and secondary metastasis were counted. In wild type animals the average of metastasis was of about 120, while in the KO mice for ARG1 and/or NOS2 we observed a dramatic decrease of metastases (Figure 10A). In particular, single ARG1 depletion was sufficient to halve the metastases, and effect similar to that obtained with NOS2 depletion. The fact that in Arg/NosKO mice we did not observe any additive or rescue effect suggests that the two enzymes might act on the same final pathway in a linear cascade. Interestingly, the loss of metastatic potential, was not linked to defects in the growth of the primary tumor (Figure 10B).

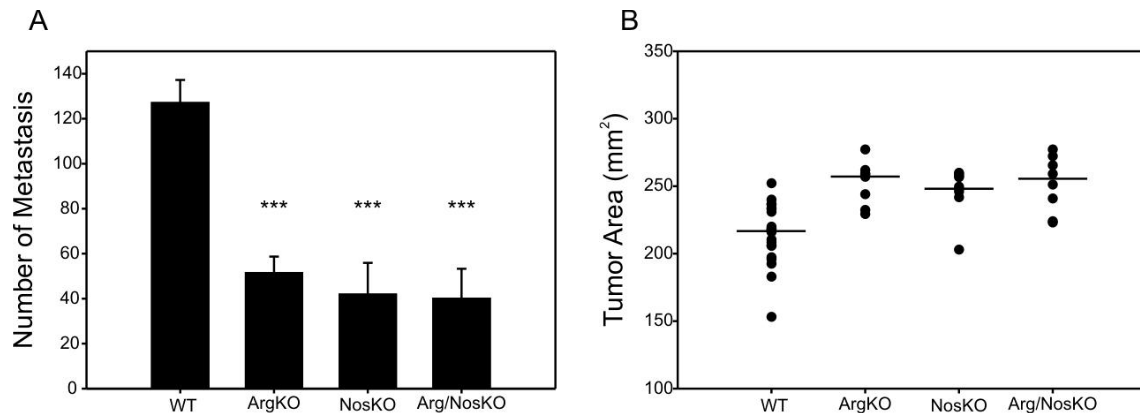


Figure 10. Lung metastasis number is decreased in ArgKO, NosKO and Arg/NosKO mice

(A) Number of lung metastases following intramuscular injection of MCA-MN sarcoma cells in WT and KO mice. All KO strains showed a significant reduction of the number of metastases compared to WT (in each case p=0.001; B6, n=21; ArgKO, n=8; NosKO, n=9 ; ArgNosKO, n=8) **(B)** Primary tumor volume at sacrifice. (Student's *t* test, *, P ≤ 0.05; **, P ≤ 0.01.***, P ≤ 0.001).

ARG1-expressing cells share characteristic with TAMs and TEMs

The previous set of data helped us in determining the requirements for ARG1 in the metastatic process and, together with NOS2, in controlling the suppressive activity of MDSCs. In order to understand the mechanism of ARG1 action in MDSCs, we attempted to identify *in vivo* the cells expressing this enzyme. For this purpose, we used reporter animals in which the yellow fluorescent protein (YFP) expression is under control of the *Arg1* promoter. In these mice, the YFP is cloned downstream of the internal ribosome entry site (IRES) element at the 3'UTR of the *Arg1* gene. This allows YFP transcription when *Arg1* transcription is activated. These reporter animals are called YARG mice. Given the kinetic experiments described in Figure 5, we decided to inject i.p. the EG.7 tumor in YARG mice in order to obtain a sufficient number of MDSCs to carry out the cytofluorimetric analysis. Tumor-infiltrating cells were collected 18 days post injection and various markers for the myeloid lineage were used to identify the phenotype of ARG1-YFP$^+$ cells. Results are reported in Figure 11. The YFP$^+$ cells represent about the 5% of total cells recovered from the ascites and they are all positive for CD11b$^+$. These cells belong to all the Gr-1 subsets but the majority is Gr-1$^{int/low}$. They express also F4/80$^+$ and CD115$^+$ markers typical of monocytic/macrophagic lineage. This phenotype is also confirmed by the low number of cells expressing the Ly6G chain of the Gr-1, which normally is present on granulocytic cells. Angiopoietin receptor 2 (TIE2) is a marker expressed among other cells by a particular subpopulation of monocytes called TEMs involved in angiogenesis and tumor spreading. TIE2 is moderately expressed by ARG1-YFP$^+$ cells, therefore these cells display phenotypic features that remind of but are not fully consistent with TEMs. However, these data need further functional characterization to nail clearly the identity and the role of ARG1-expressing MDSCs.

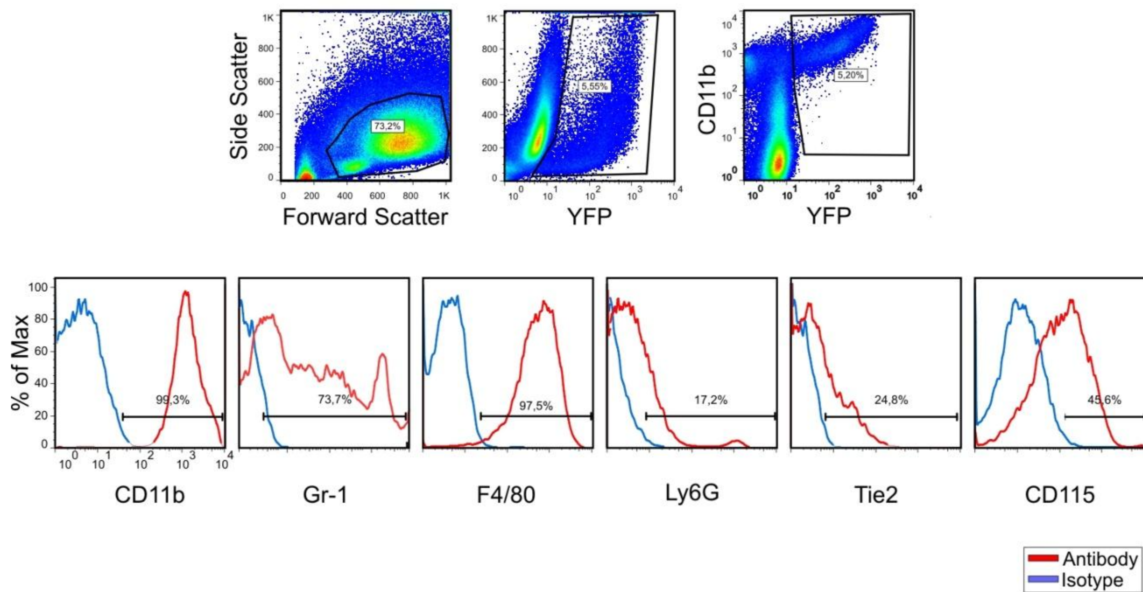
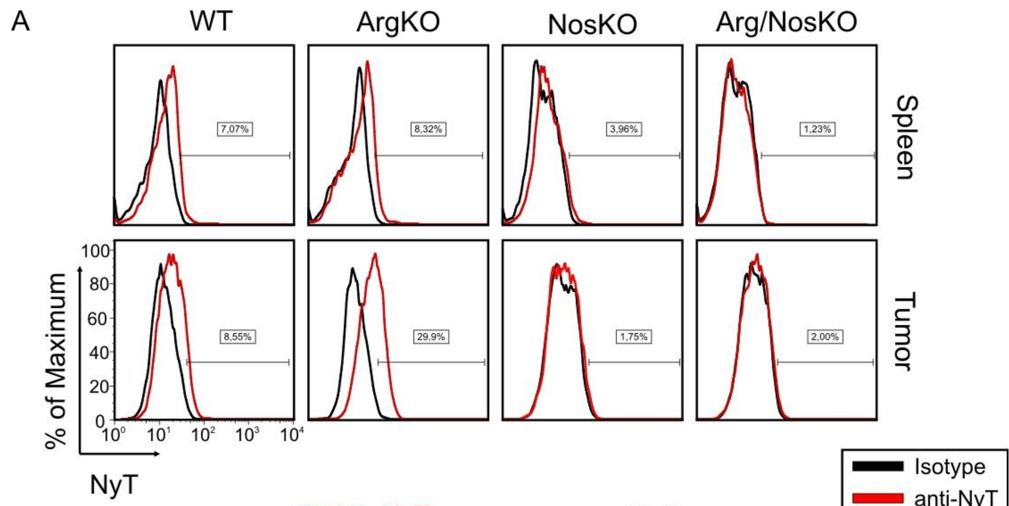


Figure 11. YFP-ARG1-expressing cells share phenotypic characteristics with TAMs and TEMs

Ascites were collected from the peritoneal cavity of YARG mice, 18 days following injection of EG.7 cells. Cell suspensions were stained with anti-CD11b, anti-Gr-1, anti-F4/80 anti-Ly6G, anti-Tie2 and anti-CD115 antibodies. **Top** panel shows the percentage of YFP$^+$ cells calculated on SSC vs FSC morphological gating. In the **bottom** panels the percentage of positive YFP$^+$ cells are reported for each marker together with the appropriate control isotype.

MDSCs derived from tumors of Nos2KO and Arg1/Nos2KO mice are unable to induce nitration of tyrosine residues.

As discussed in the introduction, decreased level of L-arginine induced by ARG1 in extracellular compartment induces the uncoupling of NOS2, which start to produce superoxide anion. Superoxide reacts with NO to produce various RNS, such as peroxynitrite, that can nitrate tyrosine residues of different proteins (Xia, Roman et al. 1998; Bronte, Serafini et al. 2003). We investigated whether the absence of these two enzymes was sufficient to abolish the production of RNS, measured as the staining with an anti-nitrotyrosine (NyT) antibody. For this purpose, we prepared cell suspensions from the spleen and tumors of WT, ArgKO, NosKO or Arg/NosKO mice. These cells were cultured in complete medium for 48 hr, then collected, stained with anti-NyT antibody and analyzed by FACS. Data are presented in Figure 12. While MDSCs derived from WT or ArgKO mice display a moderate but clear amount of MDSCs positive for nitrotyrosines, in absence of NOS2 (NosKO, Arg/NosKO) nitrotyrosine were undetectable in MDSCs. These data suggest, as expected, that the mechanism of nitration/nitrosylation of MDSCs is fully NOS2-dependent. Furthermore, we also stained for NyT and CD11b thin cryosections of EG.7 tumors developed in the different genetic backgrounds (Figure 12B). Even with this method we could confirm that, in absence of NOS2, nitrotyrosine positivity was lost specifically in MDSCs (CD11b^{+}, blue) while it could still be detected among tumor cells. It must be reminded that tumor cells derive from a NOS2 competent mice hence nitrotyrosine could arise for the endogenous production or RNS, as observed in mouse and human tumors (Goto, Haruma et al. 1999; Kato, Miyazaki et al. 2000).


A
WT
ArgKO
NosKO
Arg/NosKO
Spleen
Tumor
7,07%
8,32%
3,96%
1,23%
8,55%
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1,75%
2,00%
% of Maximum
NyT
Isotype
anti-NyT

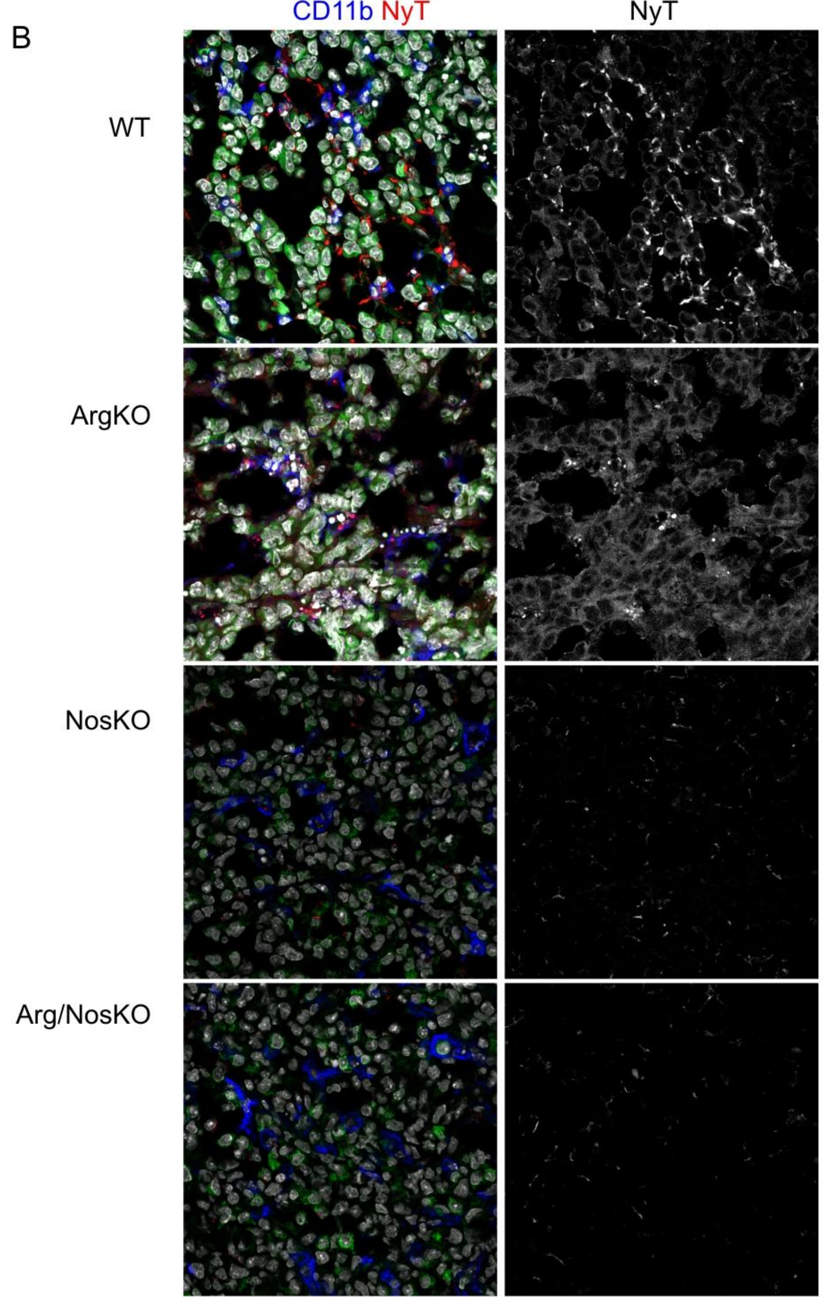

B
CD11b NyT
NyT
WT
ArgKO
NosKO
Arg/NosKO

Figure 12. MDSCs derived from tumors of Nos2KO and Arg1/Nos2KO mice are unable to induce nitration of tyrosine residues

(A) Single cell suspension from the spleen and the tumor of WT or KO mice were cultured for 48h in complete medium, collected and stained with anti-nitrotyrosine antibody (NyT) or the appropriate isotype. **(B)** Thin tumor cryosections from WT and KO mice were stained with anti-CD3 (green), anti-CD11b (blue) and anti-NyT (red) antibodies (63X magnification). The right panel shows the split channel for NyT (white).

AT38 causes a decrease in ARG1 and NOS2 protein level in MSC-2 cell line

Our group developed and characterized a drug, AT38, with the aim to reduce the RNS production by MDSCs. Starting from our past experience with nitroaspirin (De Santo, Serafini et al. 2005), AT38 was designed to interfere with both ARG1 and NOS2. To confirm this dual activity, we used an immortalized MDSC line, the MSC-2 (Apolloni, Bronte et al. 2000). These cells, when treated with either IFN-γ or IL-4, up-regulate the NOS2 and ARG1 expression, respectively. We induced the expression of the two enzymes in MSC-2 and we treated them with AT38 for 24 or 48 hours. Cells were collected and a WB analysis was performed (Figure 13). The treatment with AT38 caused down-regulation of both ARG1 and NOS2 proteins.

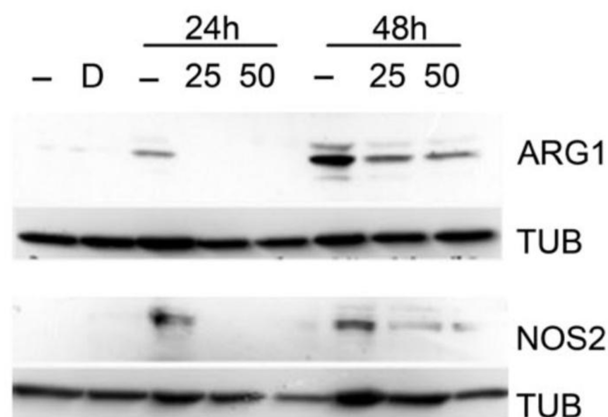


Figure 13. AT38 causes a decrease in ARG1 and NOS2 protein levels in MSC-2 cells.

MSC-2 cells were treated with either IL-4 or INF-γ to induce *Arg1* or *Nos2* expression, respectively. After being cultured in the presence of 25 µM or 50 µM AT-38, cells were collected after 24 and 48 hours. Whole cell protein extracts were prepared and Western blotting were performed using specific antibodies for mouse ARG1 or NOS2. Control lanes (-) and (D) indicate samples without treatment or in presence of DMSO used to dissolve AT38, respectively. The same membranes were probed with an anti-tubulin antibody (TUB) as loading control.

The proliferation of antigen activated CD8$^+$ T lymphocytes depends on the levels of peroxynitrite.

We next compared AT38 treatment with the effects of a NOS2 specific inhibitor (L-NMMA) and a peroxynitrite scavenger (MnTBAP) on T lymphocyte proliferation in a CFSE dilution assay. BALB/c mice were injected with C26GM colon carcinoma and after 9 days, animals were euthanized and spleen explanted. CFSE-labeled, CD8$^+$CD45.1$^+$ T lymphocytes carrying a TCR specific for hemoagglutinin (HA) were admixed with splenocytes from either tumor-free or tumor-bearing mice and stimulated with the $HA_{512-520}$ peptide for 3 days. This assay allowed us precisely quantifying the number of cell cycles performed by the activated, antigen-specific CD8$^+$ T cells. In the tumor-conditioned spleen, AT38 was more effective than the other inhibitors in restoring the number of cell divisions induced by the antigen stimulation in TCR transgenic CD8$^+$ T lymphocytes, whereas L-NMMA and MnTBAP supported only a limited number of cell cycles in T cells (Figure 14). Taken together these observations suggest that the effect of AT38 on restoring T cell proliferation is most likely broader than either NOS2 inhibitor or peroxynitrite scavenger. This is not surprising because as shown in Figure 13, AT38 caused a down-regulation of both NOS2 and ARG1 and, as shown in the first part of this thesis, inhibition of both enzymes is required to abrogate completely MDSC-mediated T cell suppression.

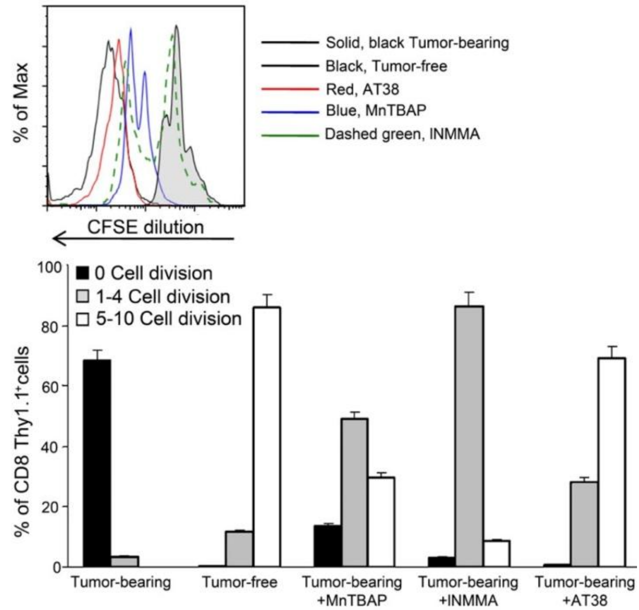
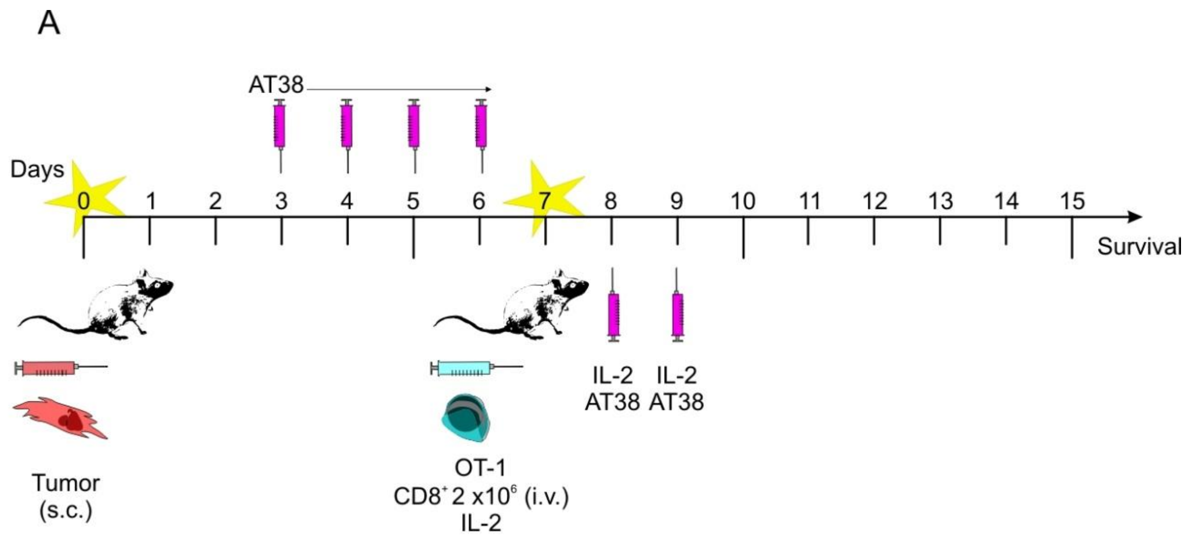


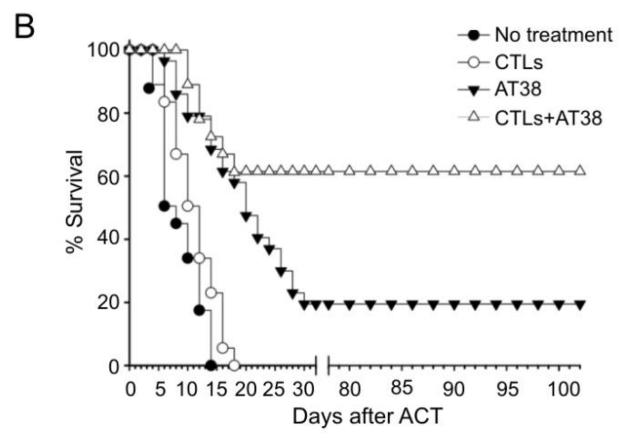
Figure 14. The proliferation of antigen-activated CD8$^+$ T lymphocytes depends on the levels of peroxynitrite.

CFSE-labeled CD8$^+$ Thy1.1$^+$ T lymphocytes were admixed with splenocytes from either tumor-free or tumor-bearing mice and stimulated with the $HA_{512-520}$ peptide for 3 days. 500 µM L-NMMA, 500 µM MnTBAP, or 25 µM AT38 were added once at the beginning of the cultures. CFSE dilution was evaluated on gated CD8$^+$/Thy1.1$^+$ (top panel) and the number of cell divisions calculated with Flow-Jo Software (bottom panel).

Inhibition of RNS production boosts protocols of Adoptive Cells Transfer (ACT)

RNS can post-translationally modify proteins, as chemokines, cytokines and their receptors, interfering with the CTL recruitment to tumor sites and AT38 caused a reduction of RNS production within the tumor (Molon, Ugel et al. 2011). We thus tested whether AT38 could be used to increase the efficacy of passive immunotherapy in which antigen experienced T lymphocytes are transferred to tumor-bearing mice. To verify this hypothesis we used EG.7 tumor because specific antitumor CTLs can be obtained from OT-I transgenic mice; the $CD8^+$ T cells isolated from these mice express a clonal TCR specific for the SIINFEKL peptide of OVA. C57BL/6 (CD45.2) mice were injected s.c. with EG7-OVA cells and, when the tumor volume reached 150 mm^3, mice were treated with AT38 for 4 days. After this preconditioning, OVA-specific CTLs, obtained from OT-I mouse splenocytes (CD45.1) were adoptively transferred to the mice. Tumor growth and mice survival were monitored following this treatment. The schedule of the experiment is shown in Figure 15A. Mice treated with AT38 alone had a modest but significant increase of survival, resulting in a cure rate of 20%. However, ACT preconditioned with AT38 produced a significant overall prolongation of survival (Figure 15B, white triangles). Tumors treated only with CTLs or the combination CTLs + AT38 were also explanted and analyzed for the frequency and localization of CD3, CD8 and CD45.1 in order to measure the frequency of adoptively transferred T cells within the tumor. The pretreatment with AT38 strongly enhanced the recruitment of lymphocytes at the tumor mass, thus explaining the improved effect of ACT in presence of AT38 (Figure 15C).


A
AT38
Days
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Survival
IL-2
AT38
IL-2
AT38
Tumor
(s.c.)
OT-1
CD8+ 2 x10^6 (i.v.)
IL-2


B
No treatment
CTLs
AT38
CTLs+AT38
% Survival
Days after ACT

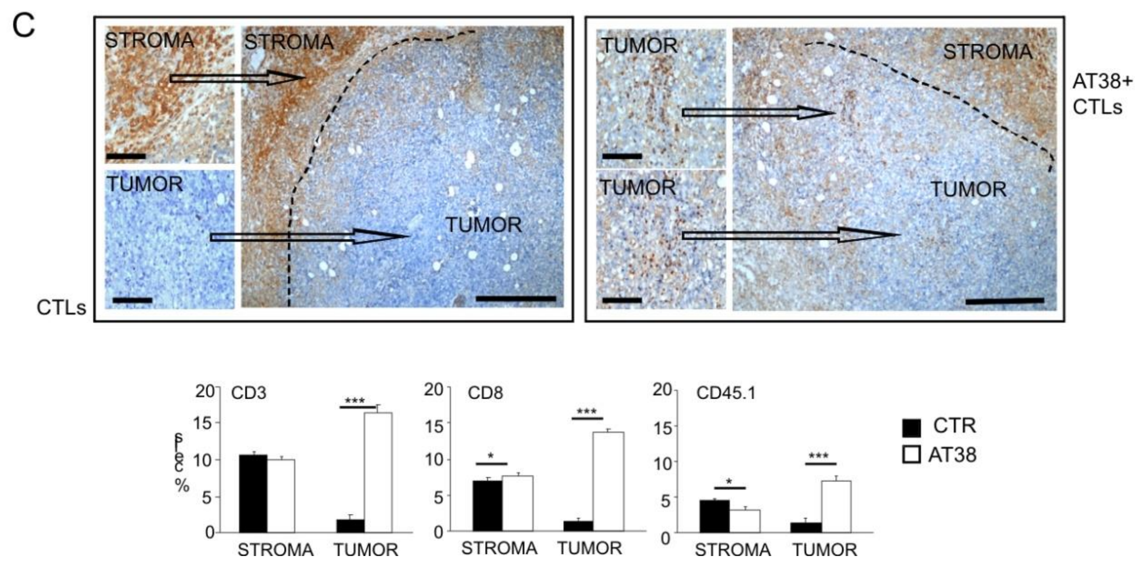

C
STROMA
STROMA
TUMOR
TUMOR
CTLs
TUMOR
TUMOR
STROMA
TUMOR
AT38+
CTLs
CD3
CD8
CD45.1
STROMA
TUMOR
CTR
AT38

Figure 15. ***Inhibition of RNS production promotes tumor infiltration and therapeutic effectiveness of adoptively transferred, tumor-specific CTLs.***

(A) Experimental procedure for ACT and AT38 treatment. Mice were injected with EG.7 cells. The day corresponding to ACT is indicated as day 0. AT38 was administrated 4 days before and 3 days after ACT.

(B) EG7-OVA tumor-bearing mice (n = 18) were either untreated or treated with AT38 for 7 days (30 mg/kg/d; AT38 only) with adoptive transfer of 2×10^6 CTLs (when the tumor volume was ∼300 mm^3; CTLs only) or with a combination of CTLs and AT38 (4 days before and 3 days after ACT; CTLs+AT38). Tumors were measured using digital calipers. Mice were euthanized when the tumor area reached 1,000 mm^3. Mantel-Haenszel statistics: CTLs versus AT38, P = 0.004; CTLs versus AT38+CTLs, P = 0.00003; AT38 versus AT38+CTLs, P = 0.029.

(C) Mice were euthanized and tumors removed to analyze the frequency and localization of $CD3^+$, $CD8^+$ and $CD45.1^+$ T cells within the tumors. Immunohistochemical images are shown for $CD3^+$ T cells only, whereas the graphs represent the quantification of immunoreactive $CD3^+$, $CD8^+$ and $CD45.1^+$ T cells within the tumor and in the tumor-surrounding stroma. Data are expressed as the means ± SE. (Student's *t* test, *, P ≤ 0.05; **, P ≤ 0.01.***, P < 0.001; n = 20). Bars, 50 µm.

Discussion

MDSCs are one of the most important immunoregulatory populations involved in the generation of a permissive environment allowing tumor escape, progression and spreading. MDSCs are present in the organism under physiologic conditions, where they act to limit exacerbated immune responses by effector cells, thus avoiding tissue damage. During tumor growth, cancer cells produce TDSFs, which induce MDSCs accumulation and acquisition of a persistent suppressive activity. There are several known mechanisms exploited by MDSCs to exert their suppressive function, including the production of NO, triggering of apoptosis in antigen-activated T cells, depletion of L-arginine and L-cysteine (Apolloni, Bronte et al. 2000; Mazzoni, Bronte et al. 2002; Kusmartsev, Nefedova et al. 2004; Dolcetti, Marigo et al. 2008; Rodriguez and Ochoa 2008; Srivastava, Sinha et al.). In this regard, L-arginine metabolism seems to be fundamental for the alteration of the tumor microenvironment and for the generation of T effector cell dysfunctions.

The starting point of this work was based on previous observations showing that *Arg1* and *Nos2* transcriptional levels were highly enriched in MDSCs isolated from C26GM, 4T1 and MCA203 tumor masses, when CD11b$^+$ isolated from the spleen of healthy mice were compared to the same cells present in tumor infiltrate. First, we addressed the localization of the cells expressing these two enzymes by confocal microscopy. Next we assayed the relative amounts of ARG1 and NOS2 in different organs and tumors by western blot. We have shown that in the EG.7 tumor, the model chosen for this work, these two enzymes are indeed expressed in tumor-derived MDSCs. Conversely, ARG1 and NOS2 are present only in negligible amounts in the spleen of tumor-bearing mice, indicating that the expression of these enzymes is a peculiar trait of tumor-infiltrating MDSCs. These data support recent work demonstrating that contemporaneous ARG1 and NOS2 enzymatic activity can only be found in MDSCs isolated from tumors and inflammatory sites, while this activity is very low in the

spleen of tumor-bearing mice and completely absent in the spleen of healthy mice (Haverkamp, Crist et al.).

To analyze the *in vivo* role of ARG1/NOS2 for the function of MDSCs, we used knock-out mice for each enzyme and a double KO mouse lacking both. Complete KO of *Arg1* gene is not viable as newborn mice die after few days. For this reason we employed conditional KO of *Arg1* that was generated by one of our collaborators, professor Peter Murray (St. Jude Children's Research Hospital, Memphis). These mice do not express ARG1 in the myeloid lineage and in all the cells expressing the TIE2 molecule. Given the complexity of tumor microenvironment and the variability of cell populations involved locally and systemically, conditional KOs represent the best models to study the function of a specific protein in a particular cell type or environment. However, it is important to specify that NosKO mice used in this work are whole KO, since a conditional KO for *Nos2* gene in the myeloid lineage has not been generated so far and many groups are currently working on this issue. So it is important to keep in mind that while the data obtained for ArgKO are linked to the depletion of this enzyme almost exclusively in the myeloid compartment, we cannot rule out that data derived from NosKO mice could be due to unanticipated effects, due to its depletion in other cell types.

MDSCs isolated from tumors of ArgKO, NosKO and Arg/NosKO mice were tested for their ability to suppress T lymphocyte proliferation and function. The absence of the sole ARG1 had no effect on the proliferation nor the cytotoxic activity of T lymphocytes, whereas NOS2 deletion resulted in an evident decrease of the suppressive activity of MDSCs. Interestingly, while NosKO-derived MDSCs maintain an immunoregulatory activity when added to the culture in high percentage (24%), Arg/NosKO derived MDSCs completely lost this function. This data indicate a possible collaboration between ARG1 and NOS2 and the necessity of both functions to completely abrogate MDSC suppressive potential. In other words, NOS2 effect is exacerbated by ARG1 absence and the effect of ARG1 deletion is detectable only when NOS2 is missing. It is intriguing to notice that the functionality of splenic MDSCs is comparable to that of tumor-derived MDSCs, even though both enzymes can only be

detected in tumor-infiltrating MDSCs. This observation can have different explanations. ARG1 and NOS2 enzymes are up-regulated in splenic MDSCs when they are cultured *in vitro* (data not shown): their expression and activation can thus be conditioned by the culture conditions (see below for further comments). On the other hand, we cannot exclude phenomena of recirculation of MDSCs within the organism: it is possible that tumor-educated MDSCs expressing ARG1/NOS2 reach the spleen in a different moment. ARG1/NOS2 expression might be driven by specific stimuli and, once functionally differentiated, the expression of these two enzymes is no longer necessary, therefore these suppressive MDSCs could reach the spleen in a second phase. In this scenario, activation of ARG1 and NOS2 might be fundamental to induce the suppressive properties of MDSCs but their continue expression could not be necessary. Moreover, splenic myeloid cells can be converted into functional MDSCs by exposure to activated T cells producing IFN-γ, which stimulates NOS2 production.

A possible synergy between ARG1 and NOS2 was already advanced in our previous works. Indeed, our group had already hypothesized, few years ago, an important role for INF-γ in the activation of the suppressive program of MDSCs. INF-γ production by activated T lymphocytes and by MDSCs allows prolonged expression and signaling of IL-4Rα, after engagement of this receptor by IL-13 released by MDSCs in an autocrine manner. Depletion of both IL-13 and IFN-γ, via specific antibodies for these two cytokines, induced a stronger recovery of T lymphocyte cytotoxic function compared to the blockade of single cytokines (Gallina, Dolcetti et al. 2006). We speculated that these cytokines sustain the continuous activation of ARG1 and NOS2 enzymes, that than mediates the suppressive effect of MDSCs (Gallina, Dolcetti et al. 2006). Through the use of mice that are KO for both ARG1 and NOS2 we now confirm the synergistic effect of these enzymes in suppressive activity of MDSCs.

In the spleen, but not in the tumor mass, of tumor-bearing Arg/NosKO mice we found a reduced number of MDSCs compared to WT mice, with all MDSCs subsets being under-represented. We wondered whether these differences could be due to a defective proliferative ability of MDSC bone marrow progenitors in Arg/NosKO animal. We developed a protocol for *in vitro* differentiation of MDSCs from bone marrow of

healthy mice. These cells are defined as BM-MDSCs and are similar to tumor induced MDSCs in terms of phenotype and function (Marigo, Bosio et al. 2010). We analyzed the proliferation potential of MDSC subsets during BM differentiation by measuring CFSE dilution. This experiment did not show any significant difference between KO and control mice in all strains. Regardless depletion of ARG1 and/or NOS2, the Gr-1^{low} and Gr-1^{int} subsets displayed a higher proliferative potential than Gr-1^{hi} cells. Therefore, it seems that at least under these conditions, ARG1/NOS2 do not influence either differentiation or proliferation of myeloid precursors during MDSC generation.

However, it is worth mentioning that BM-MDSCs are not fully identical to tumor-induced MDSCs: indeed NosKO is sufficient in BM-MDSCs to completely abolish their suppressive activity, while tumor infiltrating MDSCs require both ARG1 and NOS2 to efficiently suppress T cell proliferation. From these data it is clear that the two enzymes involved in L-arginine metabolism play fundamental roles in MDSC function but do not interfere with the proliferation of precursor cells in the bone marrow. In particular the role of NOS2 appears crucial for the function of these cells, while the involvement of ARG1 and the mechanism by which ARG1 collaborate with NOS2 to improve the suppressive function of MDSCs remains to be better defined.

With respect to ArgKO mice, ARG1 deficiency could be rescued by other mechanisms, such as a compensatory effect of NOS2. This hypothesis is supported by the observation that macrophages isolated from ArgKO mice produce more NO in response to LPS stimulation or Mycobacterium bovis infection; besides increased NO activity has also been found in liver granulomas of ArgKO mice infected with *M. tuberculosis* (El Kasmi, Qualls et al. 2008).

MDSC functions and requirements are strictly dependent on the signals triggering MDSC accumulation and on the anatomical compartment considered. We have shown a differential expression of ARG1/NOS2 depending on whether MDSCs were tumor- or spleen-infiltrating. This suggest the existence of a tumor-restricted stimulus inducing up-regulation of ARG1 in the tumor environment. One candidate is the hypoxic nature of the tumor micro-environment, which was already shown to up-regulate directly both *Arg1* and *Nos2* via the transcription factor HIF-1α (Corzo, Condamine et al.). Since

we have also shown that ARG1 expression is not stable once the trigger-signals ceased (Gallina, Dolcetti et al. 2006), it is possible that splenic MDSCs express ARG1 only transiently when they make contacts with T cells in order to induce their suppression. Another simple possibility is that in our T cell suppression assays, ARG1 expression could be induced directly by T cell activation and proliferation, thus during the 3-5 day time lapse of our experimental setup MDSCs could undergo functional changes. We have preliminary indication that this is in fact the case: when we setup the *in vitro* T cell suppression assays using MDSCs from YARG reporter mice, we detected a moderate up-regulation of ARG1 expression towards day 3-4 of our assays (data not shown). Further investigation is required to clarify these points.

Cells strongly expressing ARG1 and NOS2 in the tumor could represent a distinct subset from those inducing T cell tolerance, which could also be found in the spleen. A possible scenario would be that MDSCs responsible for T cell tolerance only transiently express ARG1/NOS2, while MDSC-like TAMs (Lewis and Pollard 2006), which express high levels of these enzymes, are more important for the control of tumor spreading. In support of this hypothesis, in this work, we show that metastatic spread is strongly reduced also by the single deletion of ARG1 without affecting the growth of the primary tumor.

Another important aspect in MDSC biology and suppressive activity is the participation of ARG1 and NOS2 in RNS production. As mentioned earlier, within tumor microenvironment, the ARG1-driven depletion of L-arginine induces NOS2 to produce O_2^-; O_2^- reacts with other molecule to generate RNS. RNS are important factors preventing T cell infiltration within tumor mass. Successful localization of tumor-specific CTLs is widely recognized as a crucial determinant for tumor immunity. The presence of tumor-infiltrating lymphocytes (TILs) within the tumor mass is often a favorable prognostic value. In 2006 Galon and colleagues showed that the type and positioning of immune cell infiltrate within the tumor correlated with improved patient survival in human colorectal cancer (Galon, Costes et al. 2006). The presence of RNS can induce protein nitration/nitrosylation, which might alter both protein functionality or the protein-protein interaction. It was recently documented how nitrative stress

directly affects T-cell signaling molecules, leading to T-lymphocyte dysfunctions (Nagaraj, Schrum et al.; Kasic, Colombo et al.). Indeed, by examining the presence of nitrotyrosines in various human tumors, it was possible to unveil an opposite correlation between nitrotyrosine levels and T lymphocyte presence within tumor mass. T cell tumor infiltration can be impaired by the alteration of chemokines regulating leukocytes recruitment. Chemokine activity is controlled at multiple levels, through a direct regulation of their expression but also through scavenger receptors and post-translational modifications, including proteolytic processing, glycosylation, deamination or citrullination (Loos, Mortier et al. 2009). In a recent work, our group demonstrated that CCL2, an inflammatory chemokine fostering CTL and myeloid cell recruitment to tumors, could be modified by RNS through nitration/nitrosylation (Molon, Ugel et al.). The presence of nitrated/nitrosylated-CCL2 (N-CCL2) within human prostate and colon cancer inversely correlated with the presence of T lymphocytes in the periphery of tumors, indicating that N-CCL2 cannot recruit T cells inside the tumor lesions. On the contrary, myeloid cells retained their ability to respond even to N-CCL2. This can be due to a differential threshold of response between T cells and MDSCs. T cells express lower levels of CCL2 receptor (CCR2) compared to myeloid cells: N-CCL2 displays a reduced affinity for its receptor, thus lower expression of CCR2 could become a limiting factor for migration towards the modified chemokine. Based on these findings, our group designed and developed AT38 drug; AT38 was shown to interfere efficiently with RNS generation by modulating ARG1 and NOS2 expression. In this thesis, we presented part of the data relative to the *in vivo* administration of this compound. Here, we show that the treatment with AT38 in tumor-bearing mice causes a reduction in nitrotyrosine formation, leading to the subsequent unmasking of T lymphocytes chemoattractant signals. We tested AT38 in combination with adoptive cell therapy (ACT). Current ACT protocols are limited by a number of variables: immunosuppression of T $CD8^+$ cell activity at the tumor site, lack of strong antigen recognition, and insufficient recruitment of the transferred T cells to the tumor site. In mice injected with EG.7 tumor, the combination of AT38 with ACT

protocols allowed the adoptively transferred CTLs to migrate properly to the tumor core and initiate and effective tumor rejection (Molon, Ugel et al.).
As previously indicated, AT38 modulates the activity of ARG1 and NOS2. Here we also verified its direct effect using MSC-2 cells, a myeloid-derived cell line that up-regulates ARG1 and NOS2 expression upon stimulation with IL-4 or IFN-γ, respectively (Apolloni, Bronte et al. 2000). We demonstrated *in vitro* that AT38 decreases the expression levels of these two enzymes. Moreover, we showed by confocal microscopy and cytofluorimetric analysis that in NosKO mice, MDSCs present in the tumor mass display greatly reduced levels of nitrotyrosines.
On these bases, it will be very interesting to investigate whether the absence of ARG1 and NOS2 in tumor-bearing mice can lead to the enhancement of ACT protocol resulting in extended survival or tumor regression in Arg/NosKO mice, similarly to the effects of adjuvant therapy.
Suppression is probably not the only pro-tumoral feature of MDSCs, since it is known that myeloid cells can contribute to angiogenesis and metastasis formation (Chioda, Peranzoni et al.). In particular, the first evidence of the involvement of myeloid cells in the metastatic site formation was provided in 2005 by Kaplan and colleagues. These authors proposed a model in which a receptive microenvironment (pre-metastatic niche), distinct from the primary tumor, can subsequently evolve in order to promote the engraftment (micrometastasis), and the proliferation of cancer cells at secondary sites (macrometastasis) (Kaplan, Riba et al. 2005). To investigate a possible role of ARG1 and NOS2 in tumor spreading and metastasis formation, we injected the spontaneous metastatic fibrosarcoma MCA-MN in WT and KO mice (Sica, Saccani et al. 2000). From our data it is clear that the absence of ARG1, NOS2 or both causes a significant decrease in lung metastasis in mice with comparable primary tumor volumes. These data are in agreement with results recently published by our group describing a reduction of lung metastasis in response to C/EBPβ depletion (Marigo, Bosio et al.). C/EBPβ is a transcription factor known to regulate the expression of both *Arg1* and *Nos2* (Gupta and Kone 1999; Gray, Poljakovic et al. 2005; Marigo, Bosio et al. 2010). Besides a direct transcriptional regulation on *Arg1* and *Nos2*, C/EBPβ controls a

series of genes involved in MDSC differentiation; indeed, based on our previous work, metastasis reduction could be explained by the absence of a strongly suppressive MDSC population characterized by an intermediate intensity of Gr-1 expression. It is tempting to speculate that the suppressive MDSC population missing in C/EBPβ KO mice is the one either comprising or generating ARG1 and/or NOS2 expressing MDSCs.

In order to better understand the role of ARG1 in tumor biology, we tried to characterize phenotypically ARG1-expressing cells. For this purpose, we took advantage of ARG1 reporter mice (YARG mice) that produce YFP protein along with ARG1 expression (Reese, Liang et al. 2007). We performed a phenotypic characterization of $ARG1^+$ cells, which resulted $CD11b^+$/$Gr\text{-}1^{int/low}$/$F4/80^+$ and were positive for CD115 in nearly 50% of cells, thus classifying them as elements belonging to monocytic/macrophagic lineage.

$ARG1^+$ cells, sharing markers with monocytes and macrophages, can probably be associated with TAMs with a M2 phenotype, of which ARG1 activation was considered to be a distinctive characteristic (Mantovani, Sozzani et al. 2002). The expression of TIE2 is of particular interest. The angiopoietin 2 (ANG2) receptor is present in a small subset of $ARG1^+$ cells. TIE2 is a marker that identifies TEMs, a population known for its angiogenic properties (De Palma, Murdoch et al. 2007; Coffelt, Tal et al.). Moreover, when monocytes are exposed to ANG2, three markers of M2 macrophages, IL-10, mannose receptor (MRC1) and CCL17, are up-regulated (Coffelt, Tal et al.), indicating that these cells could represent a continuum in the differentiation program of TEMs.

The fact that only a portion of $ARG1^+$ cells express TIE2, could be indicative of a population shifting between TEM and TAM-M2 phenotype, suggesting that these two populations could represent different functional states of the same kind of MDSCs. It would be interesting in this sense to expose monocytes and macrophages isolated from ArgKO mice to tumor-conditioned medium in order to test whether ARG1 is responsible for TAM and/or TEM differentiation.

A still unresolved question about ARG1 and NOS2 is whether these enzymes can be co-expressed by the same cell at the same time. Some molecular evidence about a direct interaction between ARG1 and NOS2, therefore implying their co-expression in the

single cell, exists: in a model of age-induced vascular dysfunction, in fact, Dunn and co-workers showed that NOS2 S-nitrosates ARG1 by directly binding to the enzyme; this modification activates ARG1 by stabilizing its homo-trimeric structure (Dunn, Gutbrod et al.).

The analysis of EG.7 tumor thin sections prepared from C57BL/6 mice confirms the presence of a small cell population co-expressesing both ARG1 and NOS2; interestingly in these cells the signal intensity for both enzymes is lower compared to cells expressing either enzyme alone. This double ARG1/NOS2 population could represent a transition phase resembling the switch from M1 to M2 state of activated macrophages or vice versa. It is known that tumor microenvironment can re-educate cytotoxic macrophages expressing NOS2 (M1) to become TAMs expressing ARG1, with a phenotype similar to alternatively activated M2 (Mantovani, Sozzani et al. 2002).

This work highlights the *in vivo* requirements for ARG1 and NOS2 in the biology of MDSCs during tumor development. MDSCs are known for their phenotypic and functional complexity and even though the amount of knowledge about them is rapidly increasing, it is clear that many factors can shape their identity and function. Here we show that tumor microenvironment can “educate” them progressively. We show that ARG1 and NOS2 are fundamental *in vivo* for the MDSC-mediated T cell suppression but also for the expansion of the suppressive subsets of MDSCs. Metabolism of L-arginine mediated by these enzymes is heavily involved in tumor spreading and metastasis formation, as depletion of the single enzymes produces a reduction of 50-75% in the number of secondary metastasis. Finally, we show that the pharmacologic effects of the novel drug AT38 are mediated by the ARG1/NOS2 reduction and sufficient to improve ACT therapeutic approaches. Data produced here represent a body of evidence that could be exploited for improving the efficiency of cancer therapies, and identify ARG1 and NOS2 as key targets for restoring T cell-mediated tumor responses.

Abbreviations

μCi .... Microcurie

2β-ME 2-Mercaptoethanol

7-AAD .... 7-Aminoactinomycin D

Ab .... Antibody

ACT .... Adoptive cell therapy

AKT .... Protein kinase B

ANG .... Angiopoietin

APC .... Antigen presenting cell

APC .... Allophycocyanin

ARG .... Arginase

ATRA .... All-trans retinoic acid

Bcl2 .... B-cell lymphoma 2

BH_4 .... Tetrahydrobiopterin

BM .... Bone Marrow

C/EBP .... CCAAT-enhancer-binding proteins

cAMP .... Cyclic adenosine monophosphate

CD .... Cluster of differentiation

CMC .... Carboxymethyl cellulose

COX .... Cyclooxygenase

CPM .... Carboxy peptidase M

CSFE .... Carboxyfluorescein diacetate succinimidyl ester

CTL .... Cytotoxic T lymphocytes

CTLA4 .... Cytotoxic T-Lymphocyte Antigen 4

DAB 3,3-diaminobenzidine

DAPI .... 4',6-diamidino-2-phenylindole

DC .... Dendritic cell

DMEM.. Dulbecco's Modified Eagle Medium

DMSO .... Dimethyl sulfoxide

EAE...Experimental autoimmune encephalomyelitis

EDTA .... Ethylenediaminetetraacetic acid

EGF...Epidermal growth factor

eIF .... Eukaryotic initiation factor

EMT.. Epithelial-mesenchymal transition

eNOS...Endothelial Nitric Oxide Synthase

ERK... Extracellular signal-regulated kinases

FAD ....Flavin adenine dinucleotide

FBS .... Fetal bovine serum

FITC .... Fluorescein isothiocyanate

FMN .... Flavin mononucleotide

Foxp3 .... Forkhead box P3

GCN2..General control non-repressible 2

G-CSF ....Granulocyte colony-stimulating factor

GITR ....Glucocorticoid-induced TNFR-related protein

GM-CSF...Granulocyte-macrophage colony-stimulating factor

H_2O_2 .... Hydrogen peroxide

HEPES .... 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF.. Hypoxia-inducible factors

HLA ....Human leukocyte antigen

HRP .... Horseradish peroxidase

i.p. .... Intraperitoneal

iDC .... Immature dendritic cell

IDO .... Indoleamine 2,3-dioxygenase

IL .... Interleukin

iMC .... Immature myeloid cell

INF .... Interferon

iNOS .... Inducible Nitric oxide synthase

IU .... International Unit

JAK .... Janus kinase

KO .... Knock-Out

L.U. .... Lytic Unit

L-NMMA .... L-N^{G}-monomethyl Arginine citrate

LPS .... Lipopolysaccharide

mAb .... Monoclonal Antibody

MAPK .... Mitogen-activated protein kinase

MCA .... Methylcholanthrene

M-CSF .... Macrophage colony-stimulating factor

MDSC .... Myeloid derived suppressor cell

MHC .... Major histocompatibility complex

MLPC .... Mixed lymphocyte-peptide culture

MMP .... Matrix metalloproteinase

Mn-TBAP .... Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride

MO-MDSC .... Monocytic-Myeloid derived suppressor cell

MP .... Multipotent precursor

mtNOS .... Mitochondrial Nitric oxide synthase

MUC-1 .... Mucin 1

NADPH ... Nicotinamide adenine dinucleotide phosphate

NF-κB ... Nuclear factor kappa-light-chain-enhancer of activated B cells

NK ... Natural killer cell

NKT ... Natural killer t cell

nNOS ... Neuronal Nitric oxide synthase

NO ... Nitric oxide

NOD mice ... Non-obese diabetic mice

NOS ... Nitric oxide synthase

O.N. ... Over night

O_2^- ... Superoxide anion

OCT ... Optimal cutting temperature compound

OVA ... Ovalbumin

PBS ... Phosphate buffered saline

pDC ... Plasmacytoid dendritic cell

PDE5 ... Phosphodiesterase 5

PDGF ... Platelet-derived growth factor

PE ... Phycoerythrin

PerCPCy5.5 ... Peridinin-chlorophyll-protein complex cyanin5.5

PGE2 ... Prostaglandin E2

PLP ... Periodate-lysine-paraformaldehyde

PMN-MDSC ... Polymorphonuclear myeloid derived suppressor cell

RNS ... Reactive nitrogen species

ROS ... Reactive Oxygen Species

RT ... Room temperature

s.c. ... Subcutaneous

SCF ... Stem cell factor

SDS-PAGE .... Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SE .... Standard error

SHIP SH2-containing inositol-5-phosphatase

SNAP .... S-Nitroso-N-Acetyl-D,L-Penicillamine

STAT .... Signal transducer and activator of transcription

TAA .... Tumor associated antigen

TAM .... Tumor associated macrophage

TCR .... T cell receptor

TDO .... Tryptophan dioxygenase

TDSF .... Tumor derived soluble factor

TEM .... Tie2 expressing monocyte

TGF .... Tumor growth factor

Th .... T helper lymphocyte

TIL .... Tumor infiltrating lymphocyte

TLR .... Toll-like receptor

TNF .... Tumor necrosis factor

Treg .... T regulatory lymphocyte

VEGF .... Vascular endothelial growth factor

YFP .... Yellow fluorescent protein

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