Altered differentiation of tumor-associated monocytes and macrophages following genetic ablation of disabled homolog 2 gene

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Summary

Tumor progression is usually associated with abnormal myelopoiesis and recruitment of several myeloid cell subsets into tissues. These cells heavily infiltrate the primary tumor and sustain its growth by providing local immune suppression and promoting angiogenesis. They also assist metastatic spreading by favoring the tumor invasion of adjacent tissues, and by supporting cancer cell seeding into distant sites. Recent advances have partially highlighted the mechanisms through which myeloid cells are recruited into the tumor mass and suppress the immune response against tumor cells, thus laying the basis for new antitumor immunotherapeutic approaches. However, very little is known about the molecular pathways which regulate myeloid cell differentiation and functions within the tumor microenvironment, especially in the context of the metastatic process.

To obtain starting cues about new relevant molecular pathways acting in tumor-infiltrating myeloid cells, we performed gene expression analysis in purified CD11b+ intratumoral myeloid cells isolated from different transplantable murine tumor models. Among the most upregulated genes, we got particularly interested in the disabled homolog 2, mitogen-responsive phosphoprotein (Dab2) gene. The DAB2 protein is a molecular adaptor which participates to endocytosis and signal transduction pathways. Its main function is to link membrane receptors with clathrin assemblies, allowing selective clathrin-mediated endocytosis of transmembrane proteins. The vesicular trafficking has the important function to distribute and organize the protein content of the plasma membrane, allowing the cell to spatially react to external stimuli. We thought that this process is of key-importance within the tumor microenvironment, where complex cell-to-cell interactions occur and oriented stimuli are released.

In this work we show that, once within the tumor, both monocytes and macrophages upregulated the DAB2 protein. Both the cytokines GM-CSF and M-CSF, which regulate the development of mononuclear phagocytes, were able to induce DAB2 expression by myeloid cells in vitro, through a mechanism requiring the transcription factor C/EBPβ. Conditional knockout of the Dab2 gene in the hematopoietic system resulted in a strong reduction of tumor-infiltrating monocytes and macrophages. In vivo tracking experiments showed that Dab2-knockout (Dab2−/−) monocytes were less efficient to generate tumor-
associated macrophages than wild type ones, and this was accompanied by reduced recovery of \( \text{Dab2}^{-/-} \) monocytes within the tumor mass. \textit{In vitro} differentiation of bone marrow-derived macrophages indicated that \( \text{Dab2}^{-/-} \) monocytes cannot efficiently carry out the autophagy process, suggesting a molecular mechanism that could explain their macrophage differentiation defects. Moreover, \( \text{Dab2}^{-/-} \) tumor-associated macrophages had increased expression of genes and membrane markers associated with the M2 macrophage polarization. Finally, we found that spontaneous generation of metastases was impaired in \( \text{Dab2}^{-/-} \) mice. These data strongly indicate that DAB2 is required for correct differentiation of tumor-associated macrophages, and suggest that this protein may be an optimal molecular target to obstruct myeloid cell-assisted dissemination of metastases in tumor-bearing hosts.
**Riassunto**

La progressione della malattia neoplastica è solitamente accompagnata da una mielopoiesi anormale, con il richiamo di diverse sottopopolazioni mieloidi nei tessuti. Queste sottopopolazioni infiltrano fortemente il tumore primario e sostengono la sua crescita fornendo immunosoppressione e stimoli pro-angiogenici. Queste cellule assistono anche il processo metastatico favorendo l’invasione tumorale dei tessuti adiacenti, e sostenendo l’atteggiamento delle cellule tumorali nei siti metastatici. Recenti scoperte hanno parzialmente compreso i meccanismi attraverso i quali le cellule mieloidi sono richiamate nella massa tumorale per sopprimere la risposta immunitaria contro le cellule tumorali, ponendo così le basi per nuovi approcci immunoterapeutici. Tuttavia, i meccanismi molecolari che regolano il differenziamento e le funzioni delle cellule mieloidi nel microambiente tumorale sono poco conosciuti, specialmente nel contesto del processo metastatico.

Per ottenere indizi iniziali riguardo nuove vie molecolari che agiscono nelle cellule mieloidi infiltranti il tumore, abbiamo effettuato un’analisi dell’espressione genica in cellule mieloidi CD11b⁺ intratumorali purificate da diversi modelli murini di tumore trapiantabile. Fra i geni più espressi, abbiamo trovato particolarmente interessante il gene **disabled homolog 2, mitogen-responsive phosphoprotein (Dab2)**. La proteina DAB2 è un adattatore molecolare che partecipa all’endocitosi e a diverse vie di trasduzione del segnale. La sua principale funzione è di collegare i recettori di membrana con i raggruppamenti di clatrina, permettendo l’endocitosi selettiva delle proteine di membrana mediata dalla clatrina. Il traffico vescicolare ha l’importante funzione di distribuire ed organizzare il contenuto proteico della membrana plasmatica, consentendo alla cellula di reagire spazialmente agli stimoli esterni. Riteniamo che questo processo sia di importanza chiave all’interno del microambiente tumorale, dove avvengono complesse interazioni fra cellule e sono rilasciati stimoli orientati.

In questo lavoro mostriamo che, raggiunto il tumore, sia i monociti che i macrofagi esprimono la proteina DAB2. Le citochine GM-CSF ed M-CSF, le quali regolano lo sviluppo dei fagociti mononucleati, inducono l’espressione di DAB2 nelle cellule mieloidi *in vitro*, attraverso un meccanismo che richiede il fattore di trascrizione C/EBPβ. Il knockout
condizionale del gene Dab2 nel sistema ematopoietico porta ad una forte riduzione dei monociti e macrofagi infiltranti il tumore. Esperimenti di tracciamento in vivo dei monociti Dab2-knockout (Dab2\(^{-/-}\)) mostrano che questi ultimi, rispetto ai monociti wild type, sono meno efficienti nel generare i macrofagi associati al tumore, e questo effetto si accompagna con un ridotto recupero dei monociti Dab2\(^{-/-}\) all’interno della massa tumorale. Il differenziamento in vitro di macrofagi ottenuti dal midollo osseo indica che i monociti Dab2\(^{-/-}\) non riescono a sostenere efficientemente il processo autofagico, suggerendo un meccanismo molecolare che può spiegare il loro difetto di differenziamento in macrofagi. Inoltre, i macrofagi Dab2\(^{-/-}\) associati al tumore hanno un’aumentata espressione di geni e marcatori di membrana correlati con la polarizzazione macrofagica M2. Infine, abbiamo scoperto che i topi Dab2\(^{-/-}\) sono resistenti alla formazione di metastasi. Questi dati indicano fortemente che DAB2 è necessario per il corretto differenziamento dei macrofagi intratumorali, e suggeriscono che questa proteina potrebbe essere un ottimo target molecolare per ostacolare la disseminazione di metastasi assistita dalle cellule mieloidi negli individui con tumore.
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Introduction

Cancer is a pathologic multistep process in which normal cells acquire the ability to proliferate without control and spread throughout the body, usually killing their host in this process. To become malignant, neoplastic cells need to acquire several traits, which are common to all types of cancer and have been defined as “the eight hallmarks of cancer” (Hanahan and Weinberg 2011):

1. Sustaining proliferative signaling;
2. Evading growth suppressors;
3. Resisting cell death;
4. Enabling replicative immortality;
5. Inducing angiogenesis;
6. Activating invasion and metastasis;
7. Deregulating cellular energetics;
8. Avoiding immune destruction.

Hallmarks 1-4 render neoplastic cells “masters of their own destiny”, allowing them to sustain chronic proliferation. This requires the subversion of the homeostatic control, which ensures normal tissue architecture and function. Hallmark 5 secures cell requirement for oxygen and nutrients, and give malignant cells an opportunity to enter the bloodstream and colonize distant tissues, which translates into hallmark 6. In order to sustain cell anabolism, hallmark 7 limits energy production largely to aerobic glycolysis (Lunt and Vander Heiden 2011). This ensures the availability of various glycolytic intermediates, which fuel both nucleoside and amino acid biosynthetic pathways. Finally, hallmark 8 allows cancer cells to avoid immune detection and escape immune surveillance (Schreiber, Old et al. 2011).

The earliest vision of tumor progression postulated that all these traits are acquired by tumor cells only through genetic/epigenetic mutations and genomic instability, thinking that tumor biology could be understand simply elucidating the cell-intrinsic properties of cancer cells. However, research in the past decade has recognized tumors as complex organs in which recruited normal cells, composing the tumor-associated stroma, are essential to achieve most if not all the eight hallmarks of cancer (Hanahan and Weinberg
2011). With this concept in mind, we can assume that transformed tumor cells are not self-sufficient in their needs and so they recruit normal cells to receive assistance. For this reason, research efforts aiming to oppose cancer disease have focused on understanding the single cell types that compose the tumor microenvironment and how they orchestrate to manage tumor progression.
Components of the tumor microenvironment

Cancer cells and cancer stem cells
Cancer cells are the drivers of the disease, carrying the oncogenic and tumor suppressor mutations that confer them malignancy. Traditionally, cancer cells were believed to have a relative stable homogeneity within the tumor mass, until continuous proliferation (combined with genomic instability) spawn distinct clonal subpopulations. This clonal heterogeneity makes the tumor mass a mosaic of different regions with various degrees of differentiation, proliferation, and invasiveness. Recently, a new dimension of heterogeneity has been added by the discovery of the cancer stem cells (CSCs). CSCs are a rare subpopulation of cancer cells defined by the ability to efficiently seed new tumors when injected in recipient host mice (Cho and Clarke 2008). CSCs also express markers shared with normal stem cells of their tissue of origin (Al-Hajj, Wicha et al. 2003). CSCs may account in major part for the regenerative ability of tumors, providing to their own self-renewal and, at the same time, generating more differentiated progenies, which constitute the great bulk of the tumor mass. It is believed that CSCs arise from genetic mutations that disrupt the proliferative and differentiation program of normal stem cells (Lobo, Shimono et al. 2007). The presence of CSCs has important implications in therapy, because these cells are more resistant to many commonly used chemotherapeutic treatments (Creighton, Li et al. 2009; Singh and Settleman 2010). This trait, combined with their cancer stemness ability, could explain the almost certain relapse which, sooner or later, occurs after successful chemotherapy. Moreover, several studies have highlighted that activation of the epithelial to mesenchymal transition (EMT) program by cancer cells, which is fundamental for invasion and metastatic spreading (Katsuno, Lamouille et al. 2013), confers many features of CSCs (Mani, Guo et al. 2008; Singh and Settleman 2010). This finding suggests that the same transcriptional program that allows cancer cells to disseminate into distant sites, also allows them to locally proliferate and establish new tumors.
Endothelial cells

Endothelial cells form the tumor-associated vasculature. Cancer cells need constant supply of oxygen and nutrients, and to fulfill this demand they induce the “angiogenic switch”, a cellular program that activates quiescent endothelial cells in order to construct new vessel (Hanahan and Folkman 1996). This process is regulated by a network of several signaling pathways, including the vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF), Notch, Neuropilin, Robo, and Eph-A/B signals (Carmeliet and Jain 2000; Ahmed and Bicknell 2009; Dejana, Orsenigo et al. 2009; Pasquale 2010). Recent researches have unveiled gene expression profiles of tumor endothelial cells, thus allowing to identify distinct luminal surface markers that are not expressed on normal vessels. These studies have provided the opportunity to target specifically the tumor neo-vasculature with novel therapeutic strategies (Nagy, Chang et al. 2010; Ruoslahti, Bhatia et al. 2010). A particular kind of endothelial cells forms actively growing lymphatic vessels at the periphery of tumors and adjacent tissues. These vessels provide cancer cells with channels to seed metastases in draining lymph nodes (Tammela and Alitalo 2010).

Pericytes

Pericytes are mesenchymal cells with finger-like projections that wrap around the endothelial tubing of blood vessels. They provide paracrine signals to the quiescent endothelium, for example secreting low levels of VEGF with trophic function in endothelial homeostasis (Bergers and Song 2005). They also collaborate with endothelial cells in synthetizing the vascular basement membrane that sustains the hydrostatic pressure within blood vessels. Pharmacological inhibition of the platelet-derived growth factor (PDGF) receptor-mediated signaling in tumor pericytes reduced their coverage of tumor vessels, resulting in compromised vascular integrity and function (Gaengel, Genove et al. 2009). Intriguingly, pericytes of normal vessels were immune to such pharmacological destruction, providing another example of the therapeutic opportunities springing from the molecular diversity between normal and tumor vessels.
Cancer-associated fibroblasts

Fibroblasts are often the major components of the tumor stroma. Within the tumor, two classes of fibroblasts can be found: (1) classic fibroblasts, which constitute the structural foundation supporting most epithelial tissues and (2) myofibroblasts, which are distinguished by expression of the α-smooth muscle actin (SMA) and can proliferate to physically sustain tissue repair (Hanahan and Weinberg 2011). Recruited myofibroblasts and reprogrammed variants of normal fibroblasts can enhance tumor proliferation, angiogenesis, invasion and metastasis. Fibroblasts can remodel the structure of the stroma through production of components of the extracellular matrix (ECM), and are able to recruit endothelial cells and pericytes through secretion of growth factors and chemokines. Their pro-tumoral activities have been defined by transplantation of cancer cells admixed with cancer-associated fibroblasts in recipient mice, or by genetic and pharmacological perturbation of their functions (Kalluri and Zeisberg 2006; Shimoda, Mellody et al. 2010).

Inflammatory leukocytes

Understanding the regulation of immune cells within the tumor microenvironment and their influence on cancer progression is the main purpose of this study, so the following sections will focus on the current knowledge about these topics.
Properties and functions of tumor-infiltrating inflammatory leukocytes

Histologic analysis of cancer biopsies indicate that virtually all tumors are infiltrated, although to various degrees, by leukocytes (Pages, Galon et al. 2010). These infiltrations are similar to those that arose in normal inflamed tissues, suggesting that an inflammatory reaction usually occurs in tumors. Historically, this was interpreted as an attempt of the immune system to eradicate neoplastic lesions, which is partially true. However, in the last ten years it has become increasing evident that inflammation within the tumor microenvironment is created and controlled by cancer cells, through the recruitment of inflammatory cells mainly belonging to the myeloid branch of the immune system. From this point of view, inflammation is the occurrence of two opposite actions of immune cells: one tempting to control neoplastic growth and eliminate transformed cells, while the other favoring all the processes that cancer cells need in order to proliferate and seed elsewhere.

The “good infiltrate”: theory of immune surveillance

The theory of immune surveillance proposes that cells of the immune system constantly monitor tissues for the occurrence of malignant transformation, eliminating them before the onset of a clinically evident tumor can occur. The logical consequence is that tumor cells need to escape immune recognition and elimination in order to become a life-threatening disease. This theory, initially proposed in the Fifties, received new attention thanks to the availability of transgenic mice that are deficient for various key components of the immune system (Schreiber, Old et al. 2011). Respect to immunocompetent littermates, these mice develop more frequently carcinogen-induced tumors, which grow more rapidly respect to controls. Moreover, cancer cells that arise in immunodeficient hosts fail to generate new tumors when transplanted in immunocompetent individuals, suggesting that tumor cells receive selective pressure from the immune system during their grow (Teng, Swann et al. 2008). The selective deficiency of particular immune cell types pinpointed a role for CD8$^+$ cytotoxic T lymphocytes (CTLs), CD4$^+$ T$\!$h$\!$1 helper T cells and natural killer (NK) cells in defending the host from tumor disease. Consistent with
these findings, colon and ovarian tumors, which are heavily infiltrated with CTLs and NK cells, have better prognosis than those lacking such infiltrates (Nelson 2008; Pages, Galon et al. 2010). Another clinical evidence comes from immunosuppressed organ transplant recipients who develop donor-derived cancers, suggesting that these cancer cells were kept in a dormant state by the functional immune system of the donor prior to transplantation (Strauss and Thomas 2010).

To justify the occurrence of tumors in light of the immune surveillance theory, the three phase-model of “immunoediting” has been proposed (Schreiber, Old et al. 2011). The first phase is called “elimination”: cancer cells are constantly eliminated by immune surveillance each time they arise. In the second phase of “equilibrium”, some cancer cells manage to survive to immune elimination, and a balance is established in which tumor cells constantly adapt and survive to the attempts of eradication by the immune system. In this phase immune killer cells (mainly CD8\(^+\) CTLs and NK cells) are still able to eliminate tumor clones that are excessively immunogenic or to deliver cytostatic signals that keep cancer cells dormant. It is believed that the equilibrium phase can persist even for decades, before cancer cells become fully competent in evading the immune system of the host. In the third phase, called “escape”, immune cells cannot eliminate tumor cells. Predictably, during the equilibrium phase there is a selective pressure for those tumor clones that somehow become less immunogenic (for example down-regulating the antigen presentation machinery); however it is increasingly evident that part of tumor-infiltrating leukocytes, together with the environmental conditions established within the tumor microenvironment, actively suppress the cell-mediated immune response against cancer cells.

The “bad infiltrate” and the “ugly outcome”: smoldering inflammation subverts anti-tumor immunity and promotes cancer disease

Chronic inflammation predisposes individuals to various types of cancer (Balkwill and Mantovani 2001). For example, inflammatory bowel disease (IBD) is associated with increased risk of developing colorectal cancer (Waldner and Neurath 2009). Conversely, chronic administration of non-steroidal anti-inflammatory agents reduces the incidence of several tumor types (Chan, Ogino et al. 2007). The positive relationship between cancer and inflammation seems counterintuitive, because inflammation activates the
immune system and facilitates the elimination of tissue-perturbing elements. Actually, the tumor microenvironment shows signs of “smoldering” inflammation, which is insufficient to trigger a fully operational cell-mediated immune response (Balkwill, Charles et al. 2005). Nonetheless, this condition provides the tumor with growth factors and anti-apoptotic signals, sustains angiogenesis, activates EMT, and produces ECM-remodeling enzymes, which facilitate invasion and extravasation into the bloodstream (Grivennikov, Greten et al. 2010). Importantly, it also establishes an immunosuppressive environment that actively suppresses the adaptive immune response against tumor cells. This is achieved by secreting anti-inflammatory cytokines that block the antitumor T\textsubscript{H}1-type immune response (TGF-\textbeta, IL-10, IL-4, IL-13, M-CSF, VEGF, IL-6); by inhibiting correct maturation and activation of professional antigen presenting cells (APCs), which are essential for correct priming of T lymphocytes (Gabrilovich 2004); and by either disabling or eliminating T lymphocytes that have successfully activated their tumoricidal functions, this task mainly being performed by particular classes of immune suppressive cells (Gabrilovich 2004). Recruitment of these cells also leads to their migration into secondary lymphoid organs and induction of immune tolerance to tumor antigens, further impairing the anti-tumor immune response (Nagaraj, Gupta et al. 2007; Ugel, Peranzoni et al. 2012). Tumor-induced immune suppression has important therapeutic implications, because it represents the main obstacle when anti-cancer immunotherapies are attempted (Zitvogel, Apetoh et al. 2008).

Chronic inflammation induced by infections or autoimmune diseases increases the risk of cancer and, once established, promotes its progression. However, inflammatory cells and mediators are present virtually in all tumors, also in those types for which there is no epidemiological basis for inflammation. An explanation for this is that oncogenes and tumor-suppressors, whose genetic modifications can induce neoplastic transformation, also coordinate inflammatory transcriptional programs. For example, in vitro constitutive activation of the protein tyrosine kinase RET in freshly isolated human thyrocytes, which is sufficient to induce papillary thyroid carcinoma in vivo, also induces several inflammation-related genes (Borrello, Alberti et al. 2005). These include colony-stimulating growth factors (CSFs), which recruit and promote the survival of leukocytes; IL-1\textbeta, one of the main pro-inflammatory cytokines; cyclooxygenase 2 (COX2), which synthetize prostaglandins (another class of proinflammatory molecules); ECM-degrading
enzymes; and various chemokines that recruit myeloid cells and promote angiogenesis (CCL2, CCL20, CXCL8). These proteins were also found in thyroid tumor specimens taken by biopsy. Another example is offered by activated oncogenic components of the RAS-RAF signaling pathway, which induces the production of tumor-promoting inflammatory chemokines and cytokines (Sparmann and Bar-Sagi 2004; Guerra, Schuhmacher et al. 2007).

Once inflammation is established, inflammatory cells are recruited in response to secreted chemokines, cytokines and prostaglandins (Mantovani, Allavena et al. 2008). These cells sustain inflammation itself and actively contribute to almost all tumor-promoting processes, including angiogenesis, metastatic spreading and immune suppression. Tumor-infiltrating inflammatory leukocytes are mainly composed of myeloid cells and include dendritic cells, granulocytes, myeloid derived suppressor cells, Tie2-expressing monocytes/macrophages and tumor-associated macrophages.

**Dendritic cells**

Dendritic cells (DCs) are terminally differentiated myeloid cells whose main property is to efficiently process and present antigens to T cells. They differentiate in tissues from bone marrow progenitors and, at least in part, from monocytes (Liu and Nussenzweig 2010). Two major subsets of DCs exist, with different morphology, phenotypes and functions (Liu and Nussenzweig 2010; Swiecki and Colonna 2010). Conventional DCs normally uptake antigens in peripheral tissues, but they do not functionally present them to T cells unless they are activated by “dangerous” stimuli. These stimuli include molecules associated with viruses, bacteria or damaged tissues and are commonly referred as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Both PAMPs and DAMPs are recognized by evolutionary conserved receptors called toll-like receptors (Rotta, Edwards et al. 2003). Their effect is to induce maturation of immature DCs, which involves both expression of co-stimulatory molecules and cytokines to promote T cell activation, and upregulation of chemokine receptors that drive migration of DCs to lymph nodes. The other subsets are plasmacytoid DCs, which have a morphology reminiscent of plasma cells and secrete high amounts of Interferon-α in response to viral nucleic acids or self DNA (Swiecki and Colonna 2010).
DCs in tumor-bearing hosts do not adequately stimulate immune responses. Tumor-secreted factors induce abnormal myelopoiesis that impair DC maturation, with three main results: decreased production of mature functionally competent DCs; accumulation of immature DCs at the tumor site; and increased production of immature myeloid cells (Gabrilovich 2004). Tumor-secreted factors that block DC maturation include macrophage colony-stimulating factor (M-CSF), IL-6 and VEGF, which mainly act through shifting DC precursors towards macrophage differentiation, and by activating the transcription factor signal transducer and activator of transcription 3 (STAT3). However, other factors within the tumor microenvironment alter DC differentiation. Hypoxia induces in DCs the transcription factor hypoxia-inducible factor 1α (HIF1α), which drives the expression of adenosine receptor A2B. Adenosine stimulation of DCs impairs their allostimulatory activity and causes them to drive the development of T<sub>H</sub>2 rather than T<sub>H</sub>1 helper T cells, thus mining the T<sub>H</sub>1-type antitumor immune response (Yang, Ma et al. 2010). Moreover, adenosine-treated DCs secrete VEGF, IL-6, IL-8, IL-10, COX2, transforming growth factor-β (TGFβ) and activate the enzyme indoleamine 2,3-dioxygenase (IDO), which all favor tumor progression in several ways (Novitskiy, Ryzhov et al. 2008). Lactic acid, which is abundant at the tumor site due to increased glycolytic catabolism, also induces dysfunction of DCs (Gottfried, Kunz-Schughart et al. 2006). Increased accumulation of lipids, which occurs in DCs isolated from tumor-bearing hosts, impairs DC ability to process soluble antigens and mount T cell responses (Herber, Cao et al. 2010). Not only tumor-infiltrating DCs (TIDCs) are dysfunctional, but also actively suppress CD8<sup>+</sup> T cell responses, through expression of the immune suppressive molecules arginase 1 (ARG1), IDO, IL-10 and TGFβ (Norian, Rodriguez et al. 2009; Watkins, Zhu et al. 2011). Plasmacytoid DCs exert their immune suppressive activity mainly through expression of IDO (Munn, Sharma et al. 2004). This enzyme limits T cell growth by consuming local L-tryptophan; it also enhances the regulatory activity of T<sub>reg</sub> cells, a particular subset of CD4<sup>+</sup> T helper cells which maintains peripheral tolerance in tissues (Baban, Chandler et al. 2009).

**Granulocytes**

Granulocytes are leukocytes characterized by the presence of cytoplasmic granules and multi-lobed shape of the nuclei. The most abundant type of granulocytes in the body are the neutrophils, which can be distinguished from eosinophils and basophils on the basis...
of their content by apposite histologic stainings. Neutrophils are phagocytic cells that rapidly respond to infections by destroying engulfed pathogens. They can be easily recognized in tissues by expression of the myeloid marker CD11b (also known as integrin αM) and LY6G, an isoform of the GR1 surface protein (Gabrilovich, Ostrand-Rosenberg et al. 2012).

The role of tumor-infiltrating granulocytes in cancer is controversial. Neutrophils assist angiogenesis and metastasis in several metastatic tumor models. Tumors resistant to antiangiogenic therapy secrete granulocyte colony-stimulating factor (G-CSF), which induces production of neutrophils in the bone marrow and their recruitment to the tumor. Once there, neutrophils secrete prokineticin 2 (PROK2), which stimulates angiogenesis and compensates for the therapeutic effects of anti-VEGF therapy (Shojaei, Wu et al. 2007; Shojaei, Wu et al. 2009). Moreover, G-CSF induces early migration of Ly6G<sup>+</sup>Ly6C<sup>+</sup> granulocytes in premetastatic lungs of tumor-bearing mice. Once in the premetastatic site, granulocytes secrete PROK2 that guides the arrival and seeding of metastatic cells through the activation of prokineticin receptor 1 (Kowanetz, Wu et al. 2010). Granulocytes also facilitate angiogenesis through secretion of matrix metalloproteinase 9 (MMP9), which augments VEGF bioavailability through its release from the ECM (Nozawa, Chiu et al. 2006). In contrast with these reports, however, neutrophils were showed to inhibit the formation of metastasis in a model of transplantable murine mammary carcinoma. This was mediated through cytotoxic effect against tumor cells by production of reactive oxygen species (Granot, Henke et al. 2011). This dualistic role of neutrophils in cancer could be explained by the finding that these cells can acquire two opposite behaviors in the context of tumor disease (Fridlender, Sun et al. 2009). Tumor-infiltrating neutrophils become “N1” after TGFβ blockade, resulting in antitumor cytotoxic effects and secretion of proinflammatory cytokines. Conversely, depletion of “N2” neutrophils in untreated tumor-bearing control mice resulted in reduced tumor growth and increased activation of CD8<sup>+</sup> CTLs. This “N2” state was characterized by expression of ARG1 and low levels of proinflammatory molecules.

**Myeloid-derived suppressor cells**

Tumor progression has two main consequences on myeloid cells. The first one is blockade of the differentiation program of several myeloid lineages, leading to the accumulation of
immature myeloid cells at the tumor site and secondary lymphoid organs. The second one is induction of powerful immune suppressive activity against CD8+ CTLs (Gabrilovich, Ostrand-Rosenberg et al. 2012). Highly immune suppressive immature myeloid cells are defined as myeloid-derived suppressor cells (MDSCs), which have further been divided in polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). Both these subsets have been described in cancer patients, although there are some differences in the hierarchy and the mechanisms of immune suppression between human and mouse species (Gabrilovich, Ostrand-Rosenberg et al. 2012).

PMN-MDSCs exert their suppressive activity through production of reactive oxygen species (ROS), which down-regulate the T cell receptor (TCR) ζ-chain of CD8+ T cells (Schmielau and Finn 2001). PMN-MDSCs can be distinguished from granulocytes for their suppressive activity and for higher levels of CD115 and CD244 but lower levels of CXC-chemokine receptor 1 (CXCR1) and CXCR2 than neutrophils (Youn, Nagaraj et al. 2008). M-MDSCs are mainly recognized for expression of CD11b and intermediate levels of GR1 (Dolcetti, Peranzoni et al. 2010). They also express varying levels of classic monocyte markers as CD115, F4/80 and CC-chemokine receptor 2 (CCR2) (Youn, Nagaraj et al. 2008; Dolcetti, Peranzoni et al. 2010). Although this phenotype is reminiscent of inflammatory monocytes (discussed later), M-MDSCs are highly immune suppressive (even higher than PMN-MDSCs) and can co-express the enzymes inducible nitric oxide synthase (iNOS) and ARG1 (Gallina, Dolcetti et al. 2006). In contrast, inflammatory monocytes do not have a coordinate regulation of these two enzymes and are not immune suppressive. While iNOS expression alone confers tumoricidal activity to macrophages, concomitant expression of this enzyme with ARG1 results in production of peroxynitrite, which undermine CD8+ T cell responsiveness by desensitizing the TCR and interfering with IL-2 receptor signaling (Bronte and Zanovello 2005; Nagaraj, Gupta et al. 2007). By nitrating CC-chemokine ligand 2 (CCL2), peroxynitrite also impair the ability of CD8+ CTLs to infiltrate the tumor mass, thus limiting their access to cancer cells for antigen-specific cell lysis (Molon, Ugel et al. 2011).

Synergistic role of ARG1 and iNOS in producing peroxynitrirites is due to their sharing of the substrate L-arginine. Biochemical studies indicate that when iNOS catalyzes the synthesis of nitric oxide (NO) under sub-optimal concentration of L-arginine, the main product of the reaction becomes the anion superoxide O2− (Xia, Roman et al. 1998). O2− is
highly reactive and instantly combines with other molecules, thus generating reactive nitrogen-oxide species that include peroxynitrite. ARG1 alone is also immune suppressive: by depleting local L-arginine, ARG1+ MDSCs deprive lymphocytes of an essential nutrient, resulting in down-regulation of the TCR ζ-chain and blockade of antigen-stimulated proliferation (Bronte and Zanovello 2005). ARG1 also allows MDSCs to convert naïve CD4+ T helper cells into induced Treg cells (Serafini, Mgebroff et al. 2008), thus exerting another (indirect) inhibitory effect on CD8+ CTLs. Other mechanisms of Treg expansion by MDSCs include CD40-CD40L interactions and production of soluble factors as IFNγ, IL-10, TGFβ, and retinoic acid (Huang, Pan et al. 2006; Pan, Ma et al. 2010; Hoechst, Gamrekelashvili et al. 2011).

MDSCs can migrate to secondary lymphoid organs (lymph nodes and spleen), where they exert their immune suppressive activity against CD8+ CTLs in an antigen-specific manner. CD11b+GR1+ cells were found in the lymph nodes of tumor-bearing mice and cancer patients, where they made CD8+ T lymphocytes unresponsive to antigen stimulation. Suppression required cell-to-cell contact between MDSCs and T cells and involved antigen-specific recognition between the TCR and peptide-major histocompatibility complex (MHC) class I dimers expressed by MDSCs (Nagaraj, Gupta et al. 2007). This allowed nitration of the TCR through short range-diffusion of peroxinitrite produced by MDSCs. TCR modification compromises its ability to recognize antigens exposed by MHC class I on APC cells, thus undermining T cell ability to mount antigen-specific responses (Nagaraj, Gupta et al. 2007). It is likely that the same mechanism also occurs in the spleen; however, we found that splenectomy is sufficient to restore completely CTL reactivity against tumor antigens (Ugel, Peranzoni et al. 2012). Further experiments indicated that CD11b+GR1int M-MDSCs have high proliferative potential and expand in the marginal zone of the spleen during tumor progression. In this site they closely associate with circulating CD8+ T lymphocytes, cross-presenting tumor antigens and inducing immune tolerance. Conversely, it is believed that tumor-infiltrating MDSCs inhibit nearby T cells without the need for cellular contact, resulting in antigen-unspecific suppression (Gallina, Dolcetti et al. 2006; Corzo, Condamine et al. 2010). Both ARG1 and iNOS are implicated in the process, probably through depletion of local L-arginine, production of NO, which interferes with IL-2 receptor signaling (Mazzoni, Bronte et al. 2002), and induction of Treg cells. Interestingly, IFNγ produced by activated CD8+ CTLs and Th1 cells is
required to induce the immune suppressive activity of MDSCs, suggesting that these cells might represent a “shut down” system aimed to counteract excessive immune responses (Bronte, Wang et al. 1998; Gabrilovich, Ostrand-Rosenberg et al. 2012). Although MDSCs have been mainly characterized for their immune suppressive properties, several studies reported a role for immature myeloid cells in sustaining other fundamental processes of tumor progression (Gabrilovich, Ostrand-Rosenberg et al. 2012). Examples are: support tumor invasion through secretion of MMP2 and MMP9 (Kitamura, Kometani et al. 2007); angiogenesis promotion through secretion of MMP9 and differentiation into endothelial-like cells (Yang, DeBusk et al. 2004); creation of a suitable environment for the arrival of cancer cells in future metastatic sites (Hiratsuka, Watanabe et al. 2006); induction of the EMT through TGFβ, epidermal grow factor (EGF) and hepatocyte grow factor (HGF) signaling pathways (Toh, Wang et al. 2011). Unfortunately, the majority of these studies did not verify whether these immature myeloid cells were immune suppressive or not, making improper to assert that they were indeed MDSCs. However, conditional deletion of the gene encoding p120 catenin, whose product is involved in cell-to-cell adhesion and signaling, caused the formation of invasive squamous cell cancer and desmoplasia in mice. Analysis of the tumor lesions showed production of several pro-inflammatory cytokines (GM-CSF, M-CSF, CCL2, TNFα) and accumulation of CD11b+GR1+CD124+ immune suppressive immature myeloid cells, which promoted tumor progression by converting normal fibroblasts in cancer-associated fibroblasts (Stairs, Bayne et al. 2011). It should be noted that immature myeloid cells are recruited early during tumor progression; acquisition of an immune suppressive program could occur later on, when consistent mounting of Th1-type adaptive immune response is sufficient to trigger MDSC functions (Gallina, Dolcetti et al. 2006; Marigo, Dolcetti et al. 2008; Gabrilovich, Ostrand-Rosenberg et al. 2012), thus preventing cancer cells from being harmed.

**Tie2-expressing monocytes/macrophages**

A fraction of blood resident monocytes weakly express the angiopoietin (ANG) receptor Tie2, which modulates endothelial cell biology and destabilizes blood vessels to facilitate angiogenesis (Augustin, Koh et al. 2009). Once within the tumor, these monocytes upregulate Tie2 expression and differentiate in perivascular macrophages (De Palma,
Venneri et al. 2005; Pucci, Venneri et al. 2009). Their proximity to blood vessels suggests that these cells provide paracrine signals to the angiogenic vasculature. Consistently with this hypothesis, TEM-specific depletion in tumor-bearing mice reduced tumor vasculature and impaired tumor growth (De Palma, Venneri et al. 2003; De Palma, Venneri et al. 2005). TEMs isolated from mammary tumors display a distinctive gene expression signature consistent with increased proangiogenic activity and reduced proinflammatory activity respect to Tie2-negative tumor macrophages (Pucci, Venneri et al. 2009). ANG2 stimulates leukocyte adhesion to vascular endothelium by enhancing expression of the adhesion protein intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) on endothelial cells (Fiedler, Reiss et al. 2006). In vitro experiments demonstrated that ANG2-stimulated TEMs up-regulate the proangiogenic genes thymidine phosphorylase and cathepsin-B, and secrete several proangiogenic factors (Coffelt, Tal et al. 2010), indicating that ANG2 acts as a TIE2 agonist on TEMs to enhance their proangiogenic functions.
Tumor-associated macrophages: functional versatility on demand

Macrophages are terminally differentiated myeloid cells that are specialized in engulfing pathogens and cellular debris through phagocytosis. Almost all tissues have a resident population of macrophages, whose markers and functions reflect their site of action. Their main functions in healthy individuals are to eliminate infectious agents, promote wound healing and regulate adaptive immunity. They also contribute to maintain tissue homeostasis by remodeling the ECM, clearing out apoptotic cells and monitoring immune activation. Macrophages are essential players during situations of intense tissue remodeling like embryogenesis, chronic inflammation, wound healing, fibrosis, and cancer (Mosser and Edwards 2008).

Macrophages can acquire opposite roles in the context of immune responses. Usually the term “macrophage polarization” is used to indicate the acquisition of particular functional states. “M1” or “classically activated” macrophages are induced by IFNγ and microbial products, secrete high levels of IL-12 and low levels of IL-10, and can efficiently kill engulfed bacteria and intracellular pathogens. Unlike DCs, macrophages are not professional APCs, but M1 macrophages upregulate MHC class II and the co-stimulatory molecule CD86, which allow them to present antigens to CD4+ T_H1 cells. This, combined with high secretion of IL-12, drives the induction of T_H1 cells and the initiation of a T_H1-type adaptive immune response (Biswas and Mantovani 2010). M1 macrophages upregulate iNOS and ROS-generating enzymes, allowing the production of reactive nitrogen and oxygen intermediates. This allows efficient destruction of engulfed pathogens but can trigger tissue-disruptive reactions (Gordon and Taylor 2005).

Conversely, “M2” or “alternatively activated” macrophages are induced by T_H2 cell-derived IL-4 and IL-13, which are produced during helminth infection and allergy. M2 macrophages produce low levels of IL-12 and high levels of IL-10, thus precluding them from inducing T_H1 cells. Conversely, they produce a distinct set of chemokines including CCL17, CCL22 and CCL24, which attract T_H2 and T_reg cells, thus amplifying T_H2-type adaptive immune response and immune suppression (Biswas and Mantovani 2010). M2 macrophages upregulate ARG1, allowing production of polyamines, which sustain vascularization and fibrosis for wound healing (Bronte and Zanovello 2005). ARG1 also
suppress CD8⁺ CTL responses through local depletion of L-arginine, as discussed above. It should be noted that, contrary to MDSCs, macrophages do not co-express both iNOS and ARG1, whose alternate expression reflects either the M1 or M2 polarization, respectively. Actually, macrophages are believed to form in vivo a continuum of phenotypes, with the full M1 and M2 polarizations being the extremities. The M1/M2 nomenclature is a useful oversimplification to understand the many functions that macrophages can potentially exploit within tissues. M1 and M2 macrophages were initially outlined based on their immune functions, but the M2 classification has been progressively expanded to include many other functional states, which are not strictly connected to immune responses. Few years ago, Mosser and Edwards proposed that M2 macrophages should be further divided in two partially overlapping groups, named “wound-healing macrophages” and “regulatory macrophages” (Mosser and Edwards 2008). Like the “classic” M2 group, wound-healing macrophages are induced by IL-4 and IL-13 and contribute to the clearance of parasitic infections. However, this new classification stress their ability to facilitate tissue repair. Through ARG1, these macrophages furnish polyamines and L-proline, which are fundamental for the production of the ECM and nourishment of proliferating cells (Bronte and Zanovello 2005). From this point of view, the immune suppressive activity of ARG1 can be seen as another additional way to promote wound healing. Interestingly, the excessive fibrosis which is consequent to chronic schistosomiasis has been attributed to the uncontrolled activation of wound-healing macrophages (Hesse, Modolell et al. 2001).

Regulatory macrophages are induced by anti-inflammatory stimuli like IL-10 and glucocorticoioids (Mosser and Edwards 2008). In response to LPS, they produce high levels of IL-10 and low levels of IL-12, thus clearly contrasting with M1 proinflammatory macrophages. Interestingly, these regulatory macrophages can present antigens to T cells and also secrete TGFβ, suggesting that they can induce the development of antigen-specific CD4⁺ T_{reg} cells (Savage, de Boer et al. 2008). This macrophage subset has also been named “M2-like” macrophages (Biswas and Mantovani 2010). It must be pointed out that despite these differences, all those Authors agree that macrophages represent a circular continuum of phenotypes, with multiple overlapping functions, which can be individually attributed to discrete macrophage classification groups.
In the context of cancer, tumor-infiltrating M1 macrophages are cytotoxic to tumor cells and can sustain Th1-type antitumor immune response, while M2 and M2-like macrophages facilitate tumor progression (Gabrilovich, Ostrand-Rosenberg et al. 2012). Tumor-infiltrating macrophages with tumor-promoting functions are termed tumor-associated macrophages (TAMs). Their presence among human tumor-infiltrating leukocytes is usually associated with poor clinical outcome (Qian and Pollard 2010). TAMs represent a mixture of M2-like macrophage subsets, which resemble those those promoting tissue formation during development (Pollard 2009). They essentially create an advantageous environment for cancer cells, supporting their growth and spreading. The multiplicity of functions exploited by macrophages in normal tissues reflects the number of tumor processes where TAMs can intervene. With regard to this, Jeffrey Pollard proposed five main classes in which TAMs can be divided (Qian and Pollard 2010).

**Immune regulation of the tumor microenvironment by tumor-associated macrophages**

TAMs are not efficient APCs and when tumor-infiltrating macrophages are purified and stimulated *in vitro* with microbial products, high levels of IL-10 and low/absent levels of IL-12 are measured in culture supernatants (Sica, Saccani et al. 2000). Consistently, TAMs do not support the activation of NK cells and Th1 cells and hence fuel the cell-mediated antitumor cytotoxic responses. Conversely, the high production of IL-10 induces the development of Th2 cells, whose secretion of IL-4 maintains the M2-like phenotype in TAMs (DeNardo, Barreto et al. 2009). CCL22 secretion by TAMs recruit Treg cells (Curiel, Coukos et al. 2004); it is also possible that IL-10 produced by TAMs enhances Treg cell activity, thus further strengthening immune suppression within the tumor (Murai, Turovskaya et al. 2009). In addition to their ability to skew CD4⁺ Th cells toward an immune regulatory phenotype, TAMs contribute directly to immune suppression of CD8⁺ CTLs by secreting TGFβ and prostaglandin E2 (PGE2), by up-regulating ARG1, and by expressing PD1 ligand 1 (PD-L1) that binds to its receptor programmed cell death protein 1 (PD1) on activated T cells (Murai, Turovskaya et al. 2009). Besides Th2 cells, also Treg cells promote macrophage M2 polarization by (1) inhibiting macrophage responsiveness to lipopolysaccharide (LPS), a prototype inducer of M1 polarization; (2) increasing expression of the mannose receptor CD206 and scavenger receptor CD163 in macrophages. This is achieved by secretion of IL-10, IL-4 and IL-13 (Tiemessen, Jagger et al. 2007). Tumor cells
also induce the M2 phenotype by secreting TNFα, which upregulates macrophage scavenger receptor types I and II (Summers, Rankin et al. 2010).

Interesting, the composition of M1/M2 polarized intra-tumoral macrophages changes spatially and temporally during tumor progression. Through analysis of both Ly6C and MHC class II marker expression in tumor-infiltrating myeloid cells, Movahedi and colleagues were able to identify two distinct populations of TAMs (Movahedi, Laoui et al. 2010). Molecular comparison of these two populations indicated that Ly6C\textsuperscript{low}MHC II\textsuperscript{hi} macrophages were more M1-oriented respect to Ly6C\textsuperscript{low}MHC II\textsuperscript{low} macrophages, which had a prominent M2-like phenotype. During progression of either lung or mammary carcinoma, the percentage of Ly6C\textsuperscript{low}MHC II\textsuperscript{low} macrophages progressively increased with respect to Ly6C\textsuperscript{low}MHC II\textsuperscript{hi} ones, indicating a progressive shift of tumor-infiltrating macrophages toward the M2 polarization. Interestingly, O'Sullivan and colleagues found that, in the absence of adaptive immunity, M1 macrophages become the main leukocyte population responsible for the immunoediting. In this study M1 polarization was found to be induced by NK cell-secreted IFNγ at the tumor site (O'Sullivan, Saddawi-Konefka et al. 2012). It could be speculated that tumor-infiltrating macrophages, continuously recruited by tumor inflammatory stimuli, can acquire an M1-oriented phenotype in the presence of IFNγ secreted by NK cells and/or IFNγ-secreting T lymphocytes. However, during tumor progression, there is a shift toward a progressive accumulation of M2-oriented macrophages, possibly due to depletion of IFNγ-producing cells and interaction of macrophages with the regulatory elements of the adaptive immune system. Polarization of macrophages is controlled at the transcriptional level by nuclear factor κB (NF-κB), which is the central transducer of signals that cause inflammation downstream of TLR activation (Karin and Greten 2005). NF-κB activity induces the expression of IL-12, TNFα and iNOS, which are hallmarks of M1 polarization. NF-κB signaling in tumor-infiltrating macrophages is inhibited by massive overexpression of the p50 NF-κB inhibitory subunit. p50 homodimers translocate to the nucleus and negatively regulate NF-κB activity; as a consequence, macrophages display the M2-like phenotype (Saccani, Schioppa et al. 2006).

Another class of transcription factors that regulates macrophage polarization is the signal transducer and activator of transcription (STAT) family, in particular the STAT1, STAT3 and STAT6 members. STAT1 is activated in response to M1-orienting signals; however its role
in the context of immune surveillance is controversial. Experiments with mice lacking functional STAT1 have shown increased resistance to chemical induction of tumors (Kaplan, Shankaran et al. 1998), and these results were confirmed using preclinical tumor models (Zhou, Wang et al. 2001; Lesinski, Anghelina et al. 2003). However, other studies have shown a protumoral role for STAT1. For example, STAT1-activation in TAMs was necessary for suppression of T cell responses in transplantable models of fibrosarcoma and colon-carcinoma (Kusmartsev and Gabrilovich 2005). Moreover, in a murine squamous cell carcinoma, STAT1 deficiency enhanced IL-12-mediated tumor rejection by a T cell-dependent mechanism (Torrero, Xia et al. 2006). These discrepancies could be attributed to the heterogeneity of examined tumor models. However, it should be noted that activation of IFNγ-dependent signaling, which requires STAT1, is necessary for the induction of immune suppressive program in MDSCs (Gallina, Dolcetti et al. 2006). In this sense, abrogation of STAT1 activity could have a dualistic effect within the tumor-bearing host (abrogation of both M1 macrophage-mediated antitumor activity and MDSC-mediated immune suppressive activity), and the net result on tumor growth could be dependent on the specific tumor model examined.

STAT3 and STAT6 are associated with M2 macrophage polarization. STAT3 constitutive activation in TAMs leads to inhibition of proinflammatory cytokine production and release of factors which block DC maturation (Kortylewski, Kujawski et al. 2005). STAT3 has also a profound role in the cancer-associated block of immature myeloid cell maturation and DC development, as described later. TAMs from Stat6−/− mice display an M1 phenotype, with low levels of ARG1 and high iNOS-mediated production of NO, which promotes tumor cell death (Sinha, Clements et al. 2005).

**Proangiogenic TAMs**

TAMs greatly contribute to tumor angiogenesis. Depletion of these cells using the null mutation of the Csf1 gene, which encodes for the macrophage growth factor CSF-1, strongly impairs the angiogenic switch in mammary tumors, and this effect is reversed by re-expression of CSF-1 in the mammary epithelium (Lin, Li et al. 2006). Conversely, overexpression of CSF-1 in wild type mice results in premature accumulation of macrophages in hyperplastic lesions, with concomitant early appearance of the angiogenic switch and progression to malignancy (Lin and Pollard 2007). Similar results
were obtained in an osteosarcoma model and with different strategies of macrophage depletion (Zeisberger, Odermatt et al. 2006; Gazzaniga, Bravo et al. 2007; Kubota, Takubo et al. 2009).

TEMs probably account for most of TAM contribution to angiogenesis. Transcriptional profiling of *Ets2*-deficient TAMs showed that the transcriptional factor ETS2 regulates the expression of many proangiogenic molecules, whose mRNA transcripts are enriched in TIE2⁺ TEMs with respect to TIE2⁻ TAMs (Ojalvo, King et al. 2009; Zabuawala, Taffany et al. 2010). Consistently, conditional ablation of the *Ets2* gene *in vivo* inhibits angiogenesis (Zabuawala, Taffany et al. 2010). TEM transcriptional signature is predictive of poor survival when compared with available clinical databases (Ojalvo, King et al. 2009; Zabuawala, Taffany et al. 2010), which is in accord with the clinical observations in breast cancer correlating macrophage density with increased microvessel density and poor prognosis (Leek and Harris 2002).

Macrophages constitutively express the transcription factor HIF1α (Murdoch, Muthana et al. 2008), which modulates their CCL2⁻ and endothelin-mediated recruitment to tumor hypoxic areas. HIF1α also regulates the transcription of several genes associated with angiogenesis, including VEGF (Lewis and Hughes 2007). Macrophages not only can produce VEGF, but also make it available through the production of MMP9, which releases VEGF from extracellular depots (Giraudo, Inoue et al. 2004). CCL2 is generally required for recruitment of macrophages; however, in many tumor models CCL2 is dispensable because of the compensatory recruitment of MMP9-producing neutrophils (Pahler, Tazzyman et al. 2008).

**Macrophages assist cancer cells in all phases of the metastatic process**

Metastatic disease account for as much as 90% of cancer-associated mortality (Chaffer and Weinberg 2011). Although the majority of cancer clinical cases eventually end up with the occurrence of metastases, this process is very inefficient and requires multiple steps in order to be completed (Joyce and Pollard 2009). Supposing that a connection to blood vessels has been reached, metastatic cells need to invade the surrounding stroma, intravasate into the blood stream, and extravasate in a distant site. Once there, metastatic cells must seed and proliferate *in situ*, which represents the most limiting step
Myeloid cells, in particular macrophages, play essential roles in all the process. A particular subset of invasive TAMs lead the way to cancer cells across the stroma. These macrophages are transcriptionally similar to fetal macrophages that promote tissue formation during embryogenesis (Ojalvo, Whittaker et al. 2010). They are enriched in developmental pathway genes, in particular the Wnt signaling pathway. It has been shown in vitro that co-culture of tumor cells with macrophages induces the expression of Wnt5a in the latter. This regulates tumor cell migration through activation of the Wnt non-canonical pathway, while in macrophages it determines expression of MMP7 that increases their ECM-remodeling ability (Pukrop, Klemm et al. 2006).

Macrophage assistance to breast tumor invasion requires a strict interplay with cancer cells. These cells, in fact, synthetize M-CSF that stimulates macrophages to move and produce EGF, a factor that in turn activates migration of the tumor cells (Wyckoff, Wang et al. 2004). Both cell types require each other continuous assistance to progress into tissues by this mechanism, and inhibition of either M-CSF or EGF signaling completely blocks the entire process. Several in vitro experiments have demonstrated that macrophages and tumor cells are sufficient for this paracrine interaction (DeNardo, Barreto et al. 2009; Green, Liu et al. 2009). M-CSF production by mammary tumor cells is regulated by steroid hormone receptor coactivator-1 (SRC-1), and SRC-1 deficiency in vivo impairs macrophage recruitment and inhibits tumor intravasation without affecting tumor growth (Wang, Yuan et al. 2009). IL-4 produced by either CD4+ T_{H2} cells or tumor cells is also required to induce the invasive phenotype in macrophages (DeNardo, Barreto et al. 2009). Several cytokines, like heregulin and CXCL12, can initiate the co-migration of tumor cells and macrophages, but the M-CSF/EGF paracrine loop is always required to sustain it (Hernandez, Smirnova et al. 2009). Importantly, M-CSF is abundant at the invasive edge of human tumors, a site abundantly populated by macrophages (Lin, Leu et al. 2011).

The ECM plays a major role in cancer invasiveness. Macrophages secrete osteonectin, which is important for deposition of collagen IV. Interestingly, osteonectin is necessary for spontaneous metastases from the primary tumor (Sangaletti, Di Carlo et al. 2008). Macrophages and tumor cells move approximately ten times faster on collagen structures than through the stroma itself. These collagenous fibrils anchor blood vessels, thus
leading metastatic cells toward the blood stream (Condeelis and Segall 2003). Once close to the abluminal side of the vessel, macrophages cluster on its surface to assist cancer cells in the intravasation process (Wyckoff, Wang et al. 2007). This localized movement near to vessels has been confirmed by intravital imaging of xenografted tumors (Gligorijevic, Kedrin et al. 2009).

Tumor cell migration requires proteolytic destruction of the ECM to allow detachment of cancer cells from the basement membrane, and for migration through the stroma. Macrophages are strong producers of many proteases, such cathepsins, MMPs, and serine proteases (Egeblad and Werb 2002). Loss of cathepsin B, S and urokinase in macrophages inhibits metastasis formation (Almholt, Lund et al. 2005; Gocheva, Wang et al. 2010).

Cancer is a systemic disease, and neoplastic cells in the primary tumor can influence metastatic outcome at distant sites. Once in the bloodstream, circulating cancer cells need to extravasate and seed to a new site. This steps are the most limiting in all the metastatic process, and tumor cells require the support of myeloid cells, which are mobilized and recruited in future metastatic tissues by soluble factors released by the primary tumor. The colonization of anatomic sites by the arrival of myeloid cells is referred to as the formation of the “premetastatic niche”, and interfering with its establishment impairs the metastatic process. Kaplan et colleagues described for the first time the presence of clusters formed by bone-marrow derived cells in the lungs, prior to the arrival of metastatic cells (Kaplan, Riba et al. 2005). These clusters accommodate incoming cancer cells and sustain their growth in situ. The niche-forming cells are CD11b+ and VEGFR1+, indicative of mononuclear phagocytic cells (Kaplan, Riba et al. 2005; Hiratsuka, Watanabe et al. 2006). It is not clear what is the exact function of these niches: it is believed that they provide sites for tumor cells to adhere and prosper, supplying nourishment and possibly protection from antitumor immune responses. The choice of the organ for the formation of the premetastatic niches is determined by tumor-released soluble factors, postulating that the spectrum of molecules secreted by a particular tumor will influence the tissues in which metastases will occur (Kaplan, Riba et al. 2005). The myeloid chemoattractants S100A8 and S100A9 are among the tumor-produced factors required for the premetastatic niche formation (Hiratsuka, Watanabe et al. 2006). Tumor cells can induce the recruitment of myeloid cells in premetastatic sites by also remodeling the ECM in distant tissues. By releasing Lysyl oxidase (LOX), breast tumor cells can
crosslink collagen IV in the premetastatic sites, inducing the recruitment of CD11b⁺ myeloid cells. Adherence of these cells to cross-linked collagen IV induce their secretion of MMP2, which cleaves collagen and enhances the extravasation of circulating tumor cells (Erler, Bennewith et al. 2009).

It is not clear whether macrophages participate to the premetastatic niche formation. However, a population of CCR2⁺ metastases-associated macrophages (MAMs) were shown to facilitate extravasation of circulating tumor cells, and to sustain their in situ growth (Qian, Deng et al. 2009). MAMs origin from circulating CCR2⁺ inflammatory monocytes, which are recruited through CCL2 secreted by tumor cells and the stroma. Blocking of the CCL2-CCR2 chemokine axis reduces the recruitment of MAMs and impairs metastatic seeding (Qian, Li et al. 2011). Physical interactions have been observed between MAMs and tumor cells at the metastatic site, suggesting short-range transmission of growth and survival signals (Qian, Deng et al. 2009).
Developmental origin of TAMs and MDSCs

Bone marrow is the source of circulating blood leukocytes. It is also where most terminally differentiated myeloid cells originate. Myeloid cells have a common origin in bone marrow-resident hematopoietic stem cells (HSCs) and develop through the process of myelopoiesis, along several myeloid progenitors and differentiation pathways. This process is inherently governed by interaction of myeloid progenitors with the bone marrow stroma, which creates an organized microenvironment where trophic and differentiation stimuli are strictly regulated (Kiel and Morrison 2008). However, the bone marrow dynamically changes its myeloid output in response to external stimuli, like those generated by infections and other tissue homeostasis-perturbing situations. Inflamed tissues release soluble factors which reach the bone marrow through the blood stream and alter the rate and composition of myeloid cell production. Considering the myeloid nature of several components of the tumor microenvironment, it is not unexpected that tumor cells can influence myelopoiesis. The following part will first focus on the hematopoietic development of the mononuclear phagocyte system under steady state. Then the focus will shift to discuss how tumors manage to obtain the two most essential tumor-infiltrating myeloid populations, that is TAMs and MDSCs.

Differentiation of monocytes, macrophages and dendritic cells

Current models propose that mononuclear phagocytic cells origin from HSC-progenitors with myeloid-restricted differentiation potential. Successive commitment steps include common myeloid progenitors (CMPs), granulocyte-macrophage precursors (GMPs), and macrophage/DC progenitors (MDPs) (Geissmann, Manz et al. 2010). MDPs are proliferating cells which give rise to many subsets of macrophages and DCs but cannot generate granulocytes, thus representing the step in which the mononuclear phagocytic commitment occurs in myelopoiesis (Fogg, Sibon et al. 2006). Still within the bone marrow, MDPs differentiate to monocytes and to common DC precursors (CDPs). CDPs are proliferating cells which differentiate into plasmacytoid DCs and the precursors of classical DCs (pre-cDCs), but have lost the potential to generate monocytes (Liu, Victora et al. 2009). Pre-cDCs migrate through the blood into lymphoid tissues, where they acquire a
mature DC phenotype and morphology. Monocytes enter the blood and migrate into tissues under inflammatory conditions. Once there, they differentiate into subsets of macrophages and inflammatory DCs, which share many functions with classical DCs like the ability to process and present antigens to T cells (Geissmann, Jung et al. 2003; Auffray, Fogg et al. 2009; Murray and Wynn 2011).

**Monocytes consist of two distinct subsets**

Monocytes are immune effector cells that circulate in the blood, spleen and bone marrow and do not proliferate under steady state (Auffray, Sieweke et al. 2009). They can produce inflammatory cytokines and engulf cellular and toxic molecules. They are also equipped with chemokine receptors and adhesion molecules that allow them to migrate to inflamed tissues, where they differentiate into inflammatory DCs or macrophages (Serbina, Jia et al. 2008). Migration and differentiation of monocytes are likely regulated by the inflammatory milieu and microbial products (PAMPs).

Monocytes can be recognized by expression of the myeloid marker CD11b and the monocyte/macrophage marker CSF-1 receptor (CSF-1R, also known as CD115), whose agonist is the macrophage colony stimulating factor (M-CSF, also known as CSF-1). M-CSF regulates the proliferation, survival, differentiation and motility of both monocytes and macrophages (Pixley and Stanley 2004). Circulating monocytes can further be divided in two distinct subsets. Inflammatory monocytes have high expression of the LY6C marker. They are termed as such because they migrate to inflamed tissues and differentiate into inflammatory DCs or macrophages (Geissmann, Manz et al. 2010). For example, during infection with *Listeria monocytogenes*, LY6C\textsuperscript{high} monocytes differentiate into DCs that produce inflammatory mediators as TNF\(\alpha\), NO and ROS (Narni-Mancinelli, Campisi et al. 2007). These cells are called TNF\(\alpha\) and iNOS-producing dendritic cells or TipDCs; however, some Authors assert they can be considered just a variant of M1 macrophages (Murray and Wynn 2011). LY6C\textsuperscript{high} monocytes are also required against infection with *Toxoplasma gondii*, but in this case they differentiate into mucosal macrophages (Dunay, Damatta et al. 2008). The spleen harbors large numbers of LY6C\textsuperscript{high} monocytes in the subcapsular red pulp that can rapidly emigrate to inflammatory sites (Swirski, Nahrendorf et al. 2009). Inflammatory monocytes express high levels of CCR2 and L-selectin, and respond to CCL2 to exit the bone marrow and enter inflamed tissues (Kim, Kamada et al. 2011; Shi, Jia et
Interestingly, inflammatory monocytes are phenotypically similar to LY6C<sup>high</sup>CCR2<sup>+</sup> M-MDSCs, which migrate within tumors in response to CCL2 (Ugel, Peranzoni et al. 2012).

A population of LY6C<sup>neg</sup> monocytes is also found in the spleen, blood and bone marrow. These monocytes lack expression of CCR2 but have high quantities of CX<sub>3</sub>C chemokine receptor 1 (CX<sub>3</sub>CR1) and integrin LFA-1 on their surface. They are termed resident monocytes because of their longer half-life <i>in vivo</i> and their localization to both resting and inflamed tissues after adoptive transfer (Auffray, Fogg et al. 2007). Resident monocytes patrol the luminal side of the vascular endothelium and ideally survey endothelial cells and surrounding tissues for damage or infection. They also accumulate in the ischemic myocardium at a late phase, to promote healing through VEGF-induced angiogenesis and deposition of collagen (Nahrendorf, Swirski et al. 2007). The orphan nuclear receptor NR4A1 is required for the development of resident monocytes (Hanna, Carlin et al. 2011). Inflammatory monocytes have been seen to shuttle between the blood and bone marrow and lose their LY6C expression (Varol, Landsman et al. 2007); however, the hypothesis of a developmental relationship between the two monocyte subsets still waits a formal proof.

**Differentiation potential of monocyte subsets**

It is classically accepted that monocytes are plastic cells with multiple differentiation options, allowing them to generate macrophages and DC-like cells with several possible phenotypes. This concept of plasticity is largely based on the effects of cytokines on monocytes <i>in vitro</i>. The main cytokines which drive myeloid differentiation are the CSFs (Burgess and Metcalf 1980), molecules termed in this way according to their ability to drive <i>in vitro</i> formation of colonies comprising mature myeloid cells from bone marrow precursors. G-CSF and M-CSF generate granulocytic and monocyte/macrophage colonies respectively, while GM-CSF generates a mixture of both colonies. Monocytes exposed <i>in vitro</i> to a combination of GM-CSF and IL-4 generate DCs, while exposure to M-CSF induces monocyte differentiation into macrophages (Sallusto and Lanzavecchia 1994; Zhang, Goncalves et al. 2008). The latter can further be stimulated <i>in vitro</i> with either IFN<sub>γ</sub> and LPS or IL-4 and IL-13, in order to obtain M1 and M2 macrophages, respectively (Mosser and Zhang 2008). GM-CSF alone is able to induce macrophage differentiation, however
these macrophages are not similar to those obtain with M-CSF. GM-CSF-exposed macrophages secrete the proinflammatory cytokines TNFα, IL-6, IL-12p70 and IL-23 following stimulation with microbial products; these macrophages have antigen-presenting and other DC-like capabilities. Conversely, M-CSF-exposed macrophages produce IL-10 and CCL2, have phagocytic functions and a more macrophage-like morphology (Hamilton 2008). Although reductive, it could be summarized that GM-CSF-induced macrophages are primed toward a proinflammatory “M1-like” phenotype, while M-CSF-induced macrophages have an anti-inflammatory behavior which resemble M2 polarization. To explain these observations, it has been proposed that tissue-resident macrophages are maintained in an anti-inflammatory status by the continuous exposure to M-CSF, which circulates at detectable levels under steady-state. M-CSF could have the function to prevent inappropriate activation of macrophages by proinflammatory signals, in order to avoid unwanted inflammation and tissue damage. Conversely, GM-CSF is detected in the blood only after perturbation of the steady-state, for example in the case of infections. Exposure of macrophages to increasing levels of GM-CSF and other proinflammatory stimuli could induce a sort of “M-CSF resistance”, where M-CSF signaling is reduced or cut out (Hamilton 2008). When proinflammatory stimuli gradually decrease, the continuous presence of M-CSF in tissues should facilitate the macrophage-mediated resolution of inflammation.

Both monocyte subsets have the same differentiation behavior when stimulated in vitro. However, when adoptively transferred to mice carrying L. monocytogenes infection, resident monocytes initiate a macrophage differentiation program that resembles the one described for M2 macrophages (Auffray, Fogg et al. 2007). Conversely, inflammatory monocytes that enter the site of infection initiate a differentiation program that mimics the one leading to either TipDCs or M1 macrophage skewing (Narni-Mancinelli, Campisi et al. 2007). These evidences seemingly contradict the notion of a general plasticity of monocytes and macrophages, rather suggesting a programmed evolution from different precursors; in vitro studies may not, however, fully recapitulate in vivo differentiation of mononuclear phagocytes (Geissmann, Manz et al. 2010). This stresses the need for in vivo studies to rigorously understand, both spatially and temporally, the differentiation and cell fate decisions of monocytes and macrophages, both under steady-state and inflammatory conditions.
Hematopoietic stem cell-independent origin of macrophages

Recent findings indicate that macrophages can sometimes undergo self-renewal in tissues without the contribution of bone marrow-derived precursors. During helminth infection, macrophages were shown to proliferate in situ in response to IL-4 produced by T\textsubscript{h}2 cells (Jenkins, Ruckerl et al. 2011). This resulted in increased numbers of M2 effector macrophages, which contributed to expel the worms. The mechanism used by IL-4-signaling to induce proliferation is not clear at the moment, but may be related to the regulation of the transcription factors macrophage-activating factor (MAF) and MAFB, which suppress proliferation in mature monocytes and macrophages (Aziz, Soucie et al. 2009).

A recent paper by the group of F. Geissmann has shown that the transcription factor myeloblastosis oncogene (MYB), which is essential for the development of the hematopoietic system, is dispensable for the generation of yolk sac-derived macrophages found in several tissues (Schulz, Gomez Perdiguero et al. 2012). These macrophages comprehend liver Kupffer cells, epidermal Langerhans cells and microglia, which can persist in adult mice independently of HSCs. The HSC-independent generation of macrophages requires the transcription factor PU.1, which operates also the bone marrow-dependent macrophage development.

Transcriptional regulation of mononuclear phagocyte lineage commitments

Lineage potentials are progressively restricted during differentiation of mononuclear phagocytes; this requires the selection of specific gene expression programs. \textit{In vivo} knockout murine models have shown a role for several transcription factors, but their deficiency often causes broad effects in multiple cell types (Auffray, Sieweke et al. 2009). However, some exceptions have been found, like the Kruppel-like factor 4 (KLF4), whose depletion affects \textit{in vivo} the generation of inflammatory monocytes (Alder, Georgantas et al. 2008), or the role of NR4A1 in the development of resident monocytes (Hanna, Carlin et al. 2011). Nevertheless, important functions in specific commitment events have been found for various transcription factors, despite their knockouts cause broad effects on many myeloid lineages \textit{in vivo}. The transcription factor PU.1 is necessary for the early steps of myeloid lineage commitment of HSCs and its absence results in general myeloid deficiency (McKercher, Torbett et al. 1996). However, it has key functions during specific
commitment choices of the myeloid lineage diversification, in particular the macrophage-versus-DC choice of monocytes. Generally, the balance between antagonistic transcription factors drives the commitment fate of myeloid progenitors. In line with this concept, high expression of PU.1 is required to induce DC fate in monocytes and to antagonize the macrophage differentiation-inducing transcription factors MAF and MAFB (Bakri, Sarrazin et al. 2005). Gain-of-function experiments have been used to determine the potential of transcription factors in defining the differentiation fate of myeloid progenitor cells. Monocyte/macrophage commitment in early myeloid progenitors is driven by ectopic expression of the transcription factors MAFB, MAF, early growth response 1 (EGR1), interferon regulatory factor 8 (IRF8), KLF4 and PU.1, while DC differentiation is induced by PU.1 and viral reticuloendotheliosis (v-rel) oncogene homolog B (RelB) (Geissmann, Manz et al. 2010). It is important to note that lineage commitments ground on the coupling of these transcription factors with cytokine receptor signaling. In the case of the M-CSF-dependent signaling, which is pivotal for monocyte/macrophage commitment and differentiation, it has been established that MAFB limits M-CSF signals and inhibits PU.1 activation in HSCs (Sarrazin, Mossadegh-Keller et al. 2009), while MAFB and MAF together inhibit M-CSF-instructed proliferative signals in mature monocytes and macrophages, thus assuring their withdrawal from cell cycle (Aziz, Soucie et al. 2009).

**Monocyte/macrophage differentiation mainly depends on the M-CSF-induced signaling pathways and requires autophagy**

M-CSF-signaling is the main regulator of monocytes and macrophages developments. Mice that are homozygous for a null allele of the *Csf1* gene are termed osteopetrotic (*Csf1<sup>op/op</sup>), due to the negative effects on the bone reabsorption consequent to a strong decrease in osteoclasts (Wiktor-Jedrzejczak, Bartocci et al. 1990). These mice also have developmental defects in many tissues, due to a reduction of resident macrophages. Although GM-CSF induces macrophage differentiation, GM-CSF-deficient mice have only an isolated lung lesion reminiscent of pulmonary alveolar proteinosis, index of impaired scavenger activity by alveolar macrophages (Dranoff, Crawford et al. 1994). The apparent GM-CSF-independency of most macrophages for their development *in vivo* highlights the importance of M-CSF as the main growth factor for monocytes and macrophages. It
should be noted that monocytes secrete M-CSF when they are stimulated with GM-CSF in vitro, thus suggesting that GM-CSF-induced macrophage differentiation could actually be M-CSF-dependent (Chomarat, Banchereau et al. 2000).

CSF-1R (CD115) is a lineage specific marker and is expressed on MDPs, monocytes and macrophages (Geissmann, Manz et al. 2010). It is a receptor tyrosine kinase, whose stimulation by M-CSF induces strong tyrosine phosphorylation of intracellular proteins (Pixley and Stanley 2004). CSF-1R activation also results in increased size and number of complexes containing phosphotyrosine proteins, which include cytoskeletal and cytoskeleton-interacting molecules (Yeung and Stanley 2003). Through its receptor, M-CSF controls the survival, proliferation, differentiation and motility of macrophages. The CSF-1R downstream pathways are, however, poorly understood, in particular those controlling cell growth and differentiation programs (Pixley and Stanley 2004). Moreover, we have a poor knowledge of the cellular processes that sustain macrophage differentiation and how these processes are governed by the CSF-1R downstream signals. Interestingly, the M-CSF-dependent differentiation of macrophages was recently linked to the process of autophagy (Jacquel, Obba et al. 2012; Zhang, Morgan et al. 2012).

Autophagy allows cells to degrade cytoplasmic material in the lysosome, in order to produce new building blocks and energy for cellular renovation and homeostasis. Autophagy is classically seen as an adaptive metabolic response to cell starvation: by recycling their amino acids, autophagic cells can build new proteins and readapt their proteome to new environmental conditions (Mizushima and Komatsu 2011). Nitrogen starvation induces the highest levels of autophagy in yeasts and mammalian cells; when local levels of amino acids are restored, the serine/threonine protein kinase mammalian target of rapamycin complex 1 (mTORC1) is activated and this determines termination of autophagy (Yu, McPhee et al. 2010). During starvation, recycled amino acids are mainly used to synthetize vacuolar/lysosomal enzymes, respiratory chain proteins, antioxidant enzymes, and proteins involved in pathways of amino acid biosynthesis (Suzuki, Onodera et al. 2011). These proteins have the main purpose to maintain the functionality of mitochondria: autophagy-deficient yeast cells are not able to sustain their respiratory functions, and as a result, high levels of ROS accumulate, compromising mitochondrial DNA integrity and cell survival (Suzuki, Onodera et al. 2011). Fresh amino acids are also converted in intermediates of the tricarboxylic acid cycle, thus fueling ATP production and
protein synthesis in spite of nutrient starvation (Guo, Chen et al. 2011). Nevertheless, different types of cells also display basal autophagic activity under nutrient-rich conditions, indicating other roles for autophagy that are not strictly related to metabolic needs. These include the quality check of organelles and intracellular proteins, and the recycling of unnecessary material during development and renovation of tissues (Mizushima and Komatsu 2011). These functions are based on the ability of cells to selectively target specific proteins and organelles to the autophagic machinery, thus exploiting “selective autophagy”.

Two independent research groups reported that monocytes activate autophagy after either M-CSF or GM-CSF in vitro stimulation. This was required to undergo macrophage differentiation, as assessed by genetic and pharmacological approaches (Jacquel, Obba et al. 2012; Zhang, Morgan et al. 2012). In the study by Zhang et colleagues, blocking autophagy had a detrimental effect on monocyte survival; however, the group of Jacquel reported that autophagy inhibition impaired the maturation of macrophages without evident effects on monocyte viability. This discrepancy could be explained by the use of different stimuli (either GM-CSF or M-CSF) to trigger macrophage differentiation, and by the choice of different approaches to inhibit autophagy. Interestingly, both reports indicate that autophagy impairment also compromise macrophage functionality, as assessed by measurement of cytokine production and phagocytic functions. Zhang et colleagues reported that induction of autophagy by GM-CSF required the enzymatic activity of c-jun N-terminal kinase 1 (JNK1). Activated JNK1 induces phosphorylation of B-cell lymphoma 2 (Bcl2), allowing dissociation of the Bcl2-Beclin 1 complex and consequent Beclin 1-mediated triggering of autophagy (Wei, Pattingre et al. 2008). Interestingly, Bcl2 has anti-apoptotic functions, which are exploited by physical interaction with the pro-apoptotic protein BCL2-associated X protein Bax (Pope 2002). Dissociation from Beclin 1 could allow Bcl2 to interact with Bax and inhibit loss of mitochondrial transmembrane potential, thus explaining the anti-apoptotic protective effect of autophagy on monocytes.

**Description of the autophagy process**

The autophagy process is based on the function of a short-lived organelle called the “autophagosome”. An isolation membrane, termed “phagophore”, sequesters a small
portion of the cytoplasm together with its content, to form the autophagosome. Autophagosomes fuse with late endosomes or mature lysosomes in order to degrade their cargo (Mizushima and Komatsu 2011). A major concern in our understanding of autophagy is where the autophagosome membrane comes from. So far, the main candidate has been the endoplasmic reticulum (ER), because autophagosomes are usually generated in close proximity to it (Mizushima, Yoshimori et al. 2011). However, several studies have suggested that the Golgi complex, the mitochondria, and the plasma membrane also contribute to autophagosome formation (Hailey, Rambold et al. 2010; Ravikumar, Moreau et al. 2010; Tooze and Yoshimori 2010).

Mechanically, the autophagy process is conducted by the protein products of several genes originally identified in yeast, which have been called “autophagy-related” (ATG) genes (Nakatogawa, Suzuki et al. 2009). Among the 35 Atg proteins thus far identified in yeast, Atg1-10, 12-14, and 18 are the “core Atg proteins”. These proteins, together with Atg17, 29, and 31, are necessary for autophagosome formation (Nakatogawa, Suzuki et al. 2009), and are highly conserved in other eukaryotes. In mammals, autophagy is initiated by the Atg1/Unc-51-like Kinase (Atg1/ULK) complex, which comprises ULK1, mAtg13, focal adhesion kinase family interacting protein of 200 kDa (FIP200) and Atg101 (Mizushima, Yoshimori et al. 2011). This complex is present in the cytosol, but it relocates to some domains of the ER upon autophagy induction, possibly forming pre-autophagosomal (PAS) structures (Itakura and Mizushima 2010). The function of the ULK1 complex is to organize PAS by ULK1-mediated phosphorylation of several downstream autophagy-related proteins. The main target of its kinase activity is the class III phosphatidylinositol (PtdIns) 3-kinase (PI3K) complex, which includes Beclin 1, Atg14(L)/Barkor, vacuolar protein sorting 15 (Vps15), Vps34, and autophagy/beclin 1 regulator 1 (AMBRA1). The PI3K complex is localized to the ER membrane thanks to Atg14(L), and this requires phosphorylation of the latter by the ULK1 complex (Matsunaga, Morita et al. 2010). The PI3K complex produces PtdIns(3)P on the membrane of the ER; this allows the recruitment of FYVE-containing protein 1 (DFCP1) through its PtdIns(3)-binding FYVE domain (Axe, Walker et al. 2008). During autophagy, DFCP1 concentrates in spots on the ER; these spots provide a platform for the expansion of the phagophore (Axe, Walker et al. 2008). “Omegasome” is the name of the Ω-like shaped PtdIns(3)P-enriched ER region which is specific to autophagosome biogenesis (Mizushima, Yoshimori et al. 2011).
Two ubiquitin-like conjugation systems are required for the expansion and closure of the isolation membrane. The two conjugates, Atg12-Atg5 and microtubule-associated protein 1 light chain 3-phosphatidyl ethanolamine (LC3-PE; also known as LC3-II) are good markers for the detection of autophagy-related membrane structures and autophagosomes (Suzuki, Kubota et al. 2007). Atg12-Atg5 interacts with Atg16L1 to create a complex that is necessary for LC3-PE formation (Hanada, Noda et al. 2007). The LC3 precursor (LC3-I) is cleaved by the cysteine protease Atg4; the resulting C-terminal glycine-exposed form of LC3 is activated by the E1 enzyme, transferred to the E2 enzyme, and finally covalently linked to an amino group of PE (Ichimura, Kirisako et al. 2000). LC3-PE has membrane tethering and hemifusion activities (Nakatogawa, Ichimura et al. 2007), which are required for expansion of the phagophore. Another important function of LC3 is to serve as receptor for selective autophagy. LC3 recognizes the WXXL-like sequence in substrate proteins or adaptors like sequestosome 1 (SQSTM1/p62), which is selectively incorporated into autophagosomes (Johansen and Lamark 2011). The protein p62 has an ubiquitin-associated domain through which it can mediate sequestration of ubiquitinated proteins into autophagosomes. Impairment of autophagy leads to intracellular accumulation of p62 and the formation of large p62-ubiquitin aggregates (Komatsu, Kurokawa et al. 2010).

Abnormal myelopoiesis in cancer: development of MDSCs

Immature myeloid cells accumulate in the blood and lymphoid organs of tumor-bearing hosts (Bronte, Chappell et al. 1999; Almand, Resser et al. 2000; Bronte, Apolloni et al. 2000; Gabrilovich, Velders et al. 2001). As discussed above, these cells initiate highly immune suppressive programs when triggered by activation of a cell-mediated immune response, thus becoming MDSCs. They also accumulate in patients with chronic infections, trauma, and autoimmune diseases, suggesting that MDSCs represent an emergency system that hampers excessive immune activation (Gabrilovich, Ostrand-Rosenberg et al. 2012). Cancer cells induce the development of MDSCs by releasing several tumor-derived soluble factors (TSFs) that directly act on the HSC (Gabrilovich, Ostrand-Rosenberg et al. 2012). TSFs not only augment the production of immature myeloid cells in the bone marrow, but also determine a blockade of their maturation skills (Bronte, Chappell et al. 1999; Bronte, Apolloni et al. 2000; Bronte, Serafini et al. 2001).
GM-CSF, G-CSF, M-CSF, stem cell factor (SCF), VEGF and IL-3 are well-known TSFs which alter normal myelopoiesis during cancer (Bronte, Serafini et al. 2001; Gabrilovich 2004; Serafini, Carbley et al. 2004).

So far the GM-CSF has been shown to have a fundamental action on MDSC development. GM-CSF is one of the most common cytokines secreted by tumors, and its chronic production impairs the antigen-specific responsiveness of CD8+ CTLs (Bronte, Chappell et al. 1999). Knockdown of GM-CSF in tumor cells inhibits in vivo the recruitment of M-MDSCs, thus resulting in the interruption of tumor-induced tolerance (Dolcetti, Peranzoni et al. 2010). Recently, the prevalent role of GM-CSF in inducing tumor-associated immune dysfunction has been confirmed using spontaneous models of pancreatic carcinoma. In early stage neoplastic lesions, the oncogene Kras(G12D) activates GM-CSF secretion in pancreatic ductal epithelial cells (Pylayev-Gupta, Lee et al. 2012). The suppression of GM-CSF production in tumor cells has a negative impact on the recruitment of CD11b+GR1+ MDSCs, resulting in CD8+ CTL-dependent inhibition of tumor growth (Bayne, Beatty et al. 2012; Pylayeva-Gupta, Lee et al. 2012). Importantly, the Authors found that human pancreatic carcinomas abundantly secrete GM-CSF in vivo. GM-CSF is often used as an adjuvant in antitumor immunotherapies because it stimulates DC differentiation; however, excessive administration of this cytokine exacerbates immune suppression in vivo, thus mining the therapeutic outcome of antitumor vaccination (Serafini, Carbley et al. 2004).

The transcription factor STAT3 has been implicated as the main mediator of TSF-induced arrest of myeloid maturation. The normal differentiation of early myeloid progenitor cells require STAT3 activation, which progressively decreases during the late stages of DC differentiation (Smithgall, Briggs et al. 2000; Nefedova, Huang et al. 2004). However, STAT3 is found constitutively activated in MDSCs (Nefedova, Huang et al. 2004), and its activation blocks the differentiation of CD11c+CD86+ mature DCs (Wang, Niu et al. 2004). Persistent STAT3 activation is induced in myeloid progenitor cells by in vitro treatment with TSFs, and this activation prevents DC maturation induced by appropriate stimuli (Nefedova, Huang et al. 2004). Similar results are obtained when immature myeloid cells from tumor-bearing mice are adoptively transferred in tumor-free versus tumor-bearing littermates (Kusmartsev and Gabrilovich 2003). STAT3 induces cell proliferation and prevents apoptosis by upregulating the antiapoptotic proteins BCL-XL and survivin, and
the proproliferative proteins MYC and cyclin D1, thus sustaining the expansion of MDSCs (Yu, Pardoll et al. 2009). Moreover, STAT3 inhibits DC differentiation by upregulating the calcium-binding proinflammatory proteins S100A8 and S100A9, and by downregulating protein kinase Cβ isoform II (Cheng, Corzo et al. 2008; Farren, Carlson et al. 2010). The transcription factor CAAT-enhancer-binding protein-β (C/EBPβ) is also required for generation of M-MDSCs and acquisition of their immune suppressive functions (Marigo, Bosio et al. 2010). The C/EBP family of transcription factors is tightly regulated during macrophage and granulocyte differentiation (Rosenbauer and Tenen 2007). In normal individuals, C/EBPβ controls emergency granulopoiesis (Hirai, Zhang et al. 2006). C/EBPβ mediates the generation of M-MDSCs induced by GM-CSF and IL-6, both of which are required for complete acquisition of the MDSC immune suppressive program (Marigo, Bosio et al. 2010). C/EBPβ expression is under the control of STAT3 (Zhang, Nguyen-Jackson et al. 2010), and individual knockouts of these transcription factors have similar beneficial results on the accumulation of mature myeloid cells in tumor-bearing mice (Nefedova, Huang et al. 2004; Wang, Niu et al. 2004; Marigo, Bosio et al. 2010). Interestingly, both STAT3 and C/EBPβ co-regulate MYC expression by modulating, at the promoter level, the dissociation of the Myc repressor C/EBPα (Zhang, Nguyen-Jackson et al. 2010). Considering that C/EBPα regulates granulopoiesis under steady-state conditions, it seems that both STAT3 and C/EBPβ cooperate to promote myeloid lineage commitments required during stress-responses, including cancer.

**Origin of TAMs**

Understanding the mechanisms governing TAM differentiation is of great importance for developing new therapeutic strategies. Oddly, there are only few reports which have focused on the origin and in situ differentiation of tumor-infiltrating macrophages. A relatively recent report by the group of Ginderachter showed that, in two different transplantable models of lung and mammary carcinomas, TAMs originated by circulating Ly6C<sup>high</sup> inflammatory monocytes (Movahedi, Laoui et al. 2010). Monocytes were shown to undergo progressive phenotypic changes during intratumoral differentiation, finally becoming Ly6C<sup>low/neg</sup>Ly6G<sup>neg</sup>F4/80<sup>high</sup> macrophages. In the same report, Ly6C<sup>neg</sup> resident monocytes were not shown to migrate to primary tumors, thus excluding their contribution to TAM replenishment (Movahedi, Laoui et al. 2010). However, this is in
contrast with a recent paper by Qian and colleagues showing that in a spontaneous model of metastatic mammary carcinoma, resident monocytes preferentially accumulate into the primary tumor, while inflammatory monocytes are found to migrate to metastatic sites (Qian, Li et al. 2011). This discrepancy could be ascribed to the use of different kind of tumor models (transplantable versus spontaneous one); however, the exact contribution of both monocyte subsets to TAM replenishment need to be further clarified.

Another subject of discussion is the exact relationship between MDSCs and TAMs. It has been proposed that MDSCs could be the precursors of TAMs (Sica, Schioppa et al. 2006); a direct proof was given by Corzo and colleagues, showing that purified splenic CD11b^+GR1^+ MDSCs differentiate in CD11b^+GR1^-F4/80^- macrophages when injected in ascites induced by the EL4 tumor (Corzo, Condamine et al. 2010). The authors found that the same differentiation process could be induced by culturing MDSCs under hypoxic conditions and that the transcription factor HIF1α was responsible for it. Interestingly, hypoxia was able to induce the HIF1α-mediated upregulation of both iNOS and ARG1 in MDSC-derived macrophages without the need for macrophage polarization stimuli (Corzo, Condamine et al. 2010). This evidence reflects the MDSC ability to co-express these two enzymes, which are mutually exclusive when induced in macrophages (Gabrilovich, Ostrand-Rosenberg et al. 2012).

It should be noted that Corzo and co-workers used the CD11b and GR1 markers to isolate splenic MDSCs; unfortunately, these markers alone are not sufficient to discriminate these cells from other related myeloid subsets, like inflammatory monocytes. This issue raises the question whether the true source of macrophage precursors were transferred MDSCs or other cellular contaminants during in vivo differentiation experiments. Nevertheless, this paper highlights the regulatory potential of hypoxia in shaping the differentiation and behavior of TAMs. Consistent with this notion, Movahedi and colleagues found that M2 TAMs were enriched in hypoxic tumor areas (Movahedi, Laoui et al. 2010). HIF1α is constitutively expressed in macrophages, and its transcriptional activity modulates CCL2- and endothelin-mediated recruitment of macrophages to hypoxic regions of the tumor (Murdoch, Muthana et al. 2008). In a mouse model of spontaneous mammary carcinoma, HIF1α increased the expression of iNOS and ARG1 when macrophages were stimulated with either Th1 or Th2 cytokines, and HIF1α
deficiency impaired the suppressive ability of TAMs (Doedens, Stockmann et al. 2010). This transcription factor also regulates the expression of a large panel of genes associated with angiogenesis, including VEGF (Murdoch, Muthana et al. 2008). All these findings highlight the importance of local oxygen tension in regulating macrophage differentiation and behavior. Proteins of the ECM have also been implicated in controlling macrophage protumoral activities. For example, the proteoglycan versican, which is secreted by Lewis lung carcinoma cells, stimulates metastasis through TLR2 signaling in macrophages (Kim, Takahashi et al. 2009). It is likely that both environmental factors and structural components of the tumor stroma, in concert with cytokines that promote macrophage differentiation (like M-CSF and IL-6), altogether regulate TAM development and functions; however, the underlying mechanisms still remain largely unresolved.
Aim of the present study

At first sight, cancer seems a chaotic environment in which neoplastic cells proliferate without control, generating a disorganized mass that compromises nearby tissues. However, the arguments discussed so far indicate that tumors are highly organized systems, where cancer cells and normal cells strictly cooperate to sustain tumor growth and subvert host defenses. Tumor-infiltrating myeloid cells are of capital importance in this scenario, and for this reason tumor-myeloid cell interactions are the focus of intense research efforts. To obtain starting cues about new relevant molecular pathways acting in tumor-infiltrating myeloid cells, we performed gene expression analysis in purified CD11b⁺ intratumoral myeloid cells isolated from different transplantable mouse tumor models. The choice of these models was made in order to cover different genetic backgrounds and different tumor histologies. Using Affimex® high-density gene expression arrays, we analyzed the transcriptome of CD11b⁺ tumor-infiltrating myeloid cells, comparing them to splenic CD11b⁺ myeloid cells purified from healthy mice as baseline control. The comparison was operated matching the relative genetic backgrounds. Among the most upregulated genes, we were particularly interested in the disabled homolog 2, mitogen-responsive phosphoprotein (Dab2) gene. Fold-change results for this gene are shown in table 1.

<table>
<thead>
<tr>
<th>tumor model</th>
<th>histology</th>
<th>genetic background</th>
<th>relative fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>4T1</td>
<td>mammary carcinoma</td>
<td>BALB/c</td>
<td>63.83</td>
</tr>
<tr>
<td>C26GM</td>
<td>colon carcinoma</td>
<td>BALB/c</td>
<td>53.82</td>
</tr>
<tr>
<td>MCA203</td>
<td>fibrosarcoma</td>
<td>C57Bl/6</td>
<td>50.22</td>
</tr>
<tr>
<td>EL4</td>
<td>thymoma</td>
<td>C57Bl/6</td>
<td>33.02</td>
</tr>
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Table 1. Relative fold-changes of Dab2 gene expression in tumor-infiltrating CD11b⁺ myeloid cells isolated from different tumor models.

The DAB2 protein is a molecular adaptor which participates to endocytosis and signal transduction pathways. Its main function is to link membrane receptors with clathrin
assemblies, allowing selective clathrin-mediated endocytosis of transmembrane proteins (Maurer and Cooper 2006). The vesicular trafficking has the important function to distribute and organize the protein content of the plasma membrane, allowing the cell to spatially react to external stimuli. We think that this process is of key-importance within the tumor microenvironment, where complex cell-to-cell interactions occur and oriented stimuli are released. Many of the processes we described above require cells to obtain spatial organization of their surface receptors and intracellular complexes. Interestingly, DAB2 regulates macrophage adhesion and cell spreading, and it is phosphorylated following M-CSF stimulation (Xu, Yang et al. 1995; Rosenbauer, Kallies et al. 2002). In the present study, we have characterized DAB2 expression in myeloid cells during tumor progression, and we have unclosed a novel function for this protein in the development and protumoral activities of myeloid cells.

The disabled homolog 2, mitogen-responsive phosphoprotein

The DAB2 protein was initially discovered in Drosophila Melanogaster, but human and mouse homologs have been characterized. It is a phosphoprotein with an actin-binding, N-terminal domain, a central domain with a high degree of similarity to the Drosophila disabled gene product, and a proline/serine-rich C-terminal domain with binding sites for SH3-domains (Xu, Yang et al. 1995). Two isoforms of DAB2 exist, with a molecular weight of 96 and 67 kilodaltons; these isoforms are generated through alternative splicing. DAB2 was initially characterized in a macrophage cell line for its phosphorylation following exposure to the M-CSF cytokine (Xu, Yang et al. 1995). The protein has shown to have several functions, most of which are summarized in its role as “surface positioning gene” (Yang, Cai et al. 2007). DAB2 acts as an adaptor/linker protein between the plasma membrane receptors and the machinery responsible for the formation of the clathrin-coated pits; this allows the clathrin-dependent endocytosis and the consequent internalization of several surface receptors. Combined with directional mechanisms of esocytosis, it also "concentrate" the proteins into specific sides of the cell membrane, thus contrasting their spontaneous diffusion in the phospholipidic bilayer (Spudich, Chibalina et al. 2007; Chetrit, Ziv et al. 2009). These functions allow DAB2 to participate in processes like the spatial organization of epithelial cells in tissues (Yang, Cai et al. 2007), the contact-dependent inhibition of the cellular growth in tumor cells (Fazili, Sun
et al. 1999), the cellular adhesion to the extracellular matrix, and the cellular migration and spreading (Rosenbauer, Kallies et al. 2002; Teckchandani, Toida et al. 2009). Moreover, DAB2 can act as an adaptor protein in several signal transduction pathways, in particular the WNT and TGF-β ones (Hocevar EMBO J 2003; Prunier J Biol Chem 2005).

What we know about the endocytic properties of DAB2 are mainly based on the studies about the p96 isoform. The phosphotyrosine-binding (PTB) domain binds peptides containing the sequence FXN-PXY (Morris and Cooper 2001). This core sequence is found in the intracellular domains of several membrane receptors, and is important for their internalization. The low-density lipoprotein receptor (LDLR) family is a well-known DAB2-interacting protein (Morris and Cooper 2001; Maurer and Cooper 2006). DAB2 transiently co-localizes with LDLR in clathrin-coated pits, but it is not present in endosomes and lysosomes (Morris and Cooper 2001). DAB2 induces clathrin assemblies at the plasma membrane, whose formation recruits additional endocytic proteins like adaptor protein 2 (AP2). To achieve this effect, DAB2 requires a functional PTB domain, the ability to bind phospholipids, and a protein sequence that is present in p96, but not p67 (Chetrit, Ziv et al. 2009). This sequence contains both clathrin- and adaptor protein 2 (AP2)-binding motifs. Interaction of DAB2 with phospholipids is mediated by an evolutionarily conserved poly-lysine stretch, which precedes the PTB domain and interacts with negatively charged phosphoinositides. This interaction, which allows recruitment of DAB2 at the inner leaflet of the plasma membrane, can be regulated by phosphorylation of Ser24 near the poly-lysine stretch (Huang, Cheng et al. 2004; Chetrit, Ziv et al. 2009). This phosphorylation has either positive or negative effects on DAB2 function depending on the cellular context (Rosenbauer, Kallies et al. 2002; Chetrit, Ziv et al. 2009; Chetrit, Barzilay et al. 2010).

Intracellular transport of clathrin-coated vesicles requires myosin VI; this protein is the only known molecular entity that moves toward the minus ends of actin filaments (Morris, Arden et al. 2002). The cargo-binding domain of myosin VI interacts with the C-terminus region of DAB2 (Morris, Arden et al. 2002; Yu, Feng et al. 2009). By this way, DAB2 acts as a molecular adaptor between myosin VI and clathrin, allowing the trafficking of clathrin-coated vesicles away from the plasma membrane into the cell (Spudich, Chibalina et al. 2007).
DAB2 has been shown to interact with several integrins (Huang, Cheng et al. 2004; Chao and Kunz 2009; Teckchandani, Toida et al. 2009). Cell migration is based on the forming of new focal adhesion contacts at the front of the cell. Integrins are cell surface receptors for various ECM components, with different combinations of integrin α and β subunits conferring ECM ligand specificity (Hynes 1992). Binding to both the ECM and cytoskeleton induces the clustering of active integrins into structures known as focal complexes or adhesions, from which signals are generated to regulate cellular responses. Conversely, unbound integrins are free to diffuse in the plane of the membrane (Carman and Springer 2003). Cell migration requires active focal adhesion disassembly and integrin recycling, in order to allow new contacts to form near the front of the cell (Jones, Caswell et al. 2006). DAB2-mediated endocytosis of inactive integrins mediates their transport toward the leading edge of the cell movement, allowing the formation of new focal adhesion contacts (Teckchandani, Toida et al. 2009).

Little is known about the functions of the p67 isoform. DAB2 is expressed in the visceral endoderm of mice embryos, and is required for uptake and internalization of megalin, its co-receptor cubilin, and a cubilin ligand, transferrin (Maurer and Cooper 2005). Prior to placental formation, transport across the visceral endoderm is the only way by which maternal proteins are transferred to the developing embryo. Dab2 knockout embryos arrest their development and they die prior to gastrulation (Morris, Tallquist et al. 2002). p96 expression is sufficient to fully rescue endocytosis in the visceral endoderm, thus allowing embryo development. Conversely, p67 only partially rescues endocytosis, and half of p67 knockin embryos are lost between E10.5 and P1 (Maurer and Cooper 2005). As discussed before, p67 lacks the exon encoding for the AP2- and clathrin-binding region, which conversely is present in p96. Nevertheless, it has been reported that p67 shows nuclear localization following treatment of fibroblast F9 cells with retinoic acid (Cho, Jeon et al. 2000). Once in the nucleus, p67 interacts with mDab2-interacting protein (mDIP), and can act as a transcriptional activator. However, the significance of this transcriptional activity in physiologic contexts has not been explored.

There is only one report about DAB2 functions in myeloid cells. Rosenbauer and colleagues found that DAB2 is expressed in bone marrow-derived macrophages and required for cell adhesion and spreading (Rosenbauer, Kallies et al. 2002). DAB2 was found to regulate macrophage adhesion to laminin and collagen IV, two components of
the ECM. Cell adhesion to the substrate induced DAB2 phosphorylation and its translocation from the cytosol to the cytoskeletal fraction. Moreover, the Dab2 promoter was found to be recognized by the transcription factors IRF8 and PU.1; activation of IRF8 by stimulation with IFNγ resulted in strong reduction of DAB2 expression.
Materials and methods

Mice
Eight-week-old C57BL/6 (H-2b), BALB/c (H-2d) and congenic CD45.1 (Ly5.1+) mice were purchased from Charles River Laboratories. OT-1 transgenic mice in C57BL/6 background (C57Bl/6-Tg(TCRαTCRβ))1100mjb), which bear a Vα2Vβ5.1-5.2 H2Kb restricted-TCR specific for ovalbumine peptide OVA257-264 on CD8+ T lymphocytes, were purchased from Charles River Laboratories. Tie2cre+/+ and Dab2floxflox mice were a gift from P.J. Murray (Department of Immunology, St. Jude Children’s Research Hospital, Memphis, Tennessee). For simplicity, in this thesis Dab2floxflox;Tie2cre+/+ mice were named Dab2-/ mice. 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 using a digital caliper.

Cell lines
MBL-2 lymphoma, MCA203 fibrosarcoma, EL4 thymoma and Lewis Lung carcinoma (LLC) cell lines are derived from C57BL/6 mice (aplotype H-2b). 4T1 mammary carcinoma and C26 colon carcinoma cell lines are derived from BALB/c mice (aplotype H-2d). C26-GM cell line 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 genetricin antibiotic at concentration of 0.8 mg/ml. MCA-MN, a C57Bl/6 primary cell line of fibrosarcoma 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.
To generate the GFP-expressing LLC/F4 cell line, LLC cells were plated in 24 well-plates and allowed to grow until 80% of confluence. Cells were then exposed overnight to supernatants from 293T cells transfected with the GFP-reporter lentiviral vector, and finally cloned by limiting dilution.
MCA203 and EL4 cells were injected s.c. at the dose of $1 \times 10^6$ and $0.5 \times 10^6$ cells/mouse, respectively. C26-GM cells were injected in the inguinal fold at the dose of $0.5 \times 10^6$ cells/mouse. 4T1 and LLC-GFP cells were injected s.c. at the dose of $0.5 \times 10^6$ and $2 \times 10^6$ cells/mouse, respectively. MCA-MN were injected intra-muscle at the dose of $1 \times 10^5$ cells/mouse.

Cytokines and synthetic peptides
Recombinant murine GM-CSF (100 ng/ml final concentration) and M-CSF (100 ng/ml final concentration) were purchased from Peprotech Inc. OVA$_{257-264}$ (SIINFEKL) aiplotype H-2b-restricted peptide was synthetized by JPT. The lyophilized peptide was resuspended in DMSO (Sigma-Aldrich) and stored at $-20^\circ$C until used.

Organ cryoconservation and slice preparation
Mice were euthanized and organs were explanted. Immediately after removal, organs were fixed in 3.7% formaldehyde for 3 hours at 4°C. After fixation, organs were dehydrated by solutions with increasing sucrose concentration for some days (PBS 20% sucrose and PBS 30% sucrose). Organs were included when they sunk to the bottom of the tubes. After dehydration, organs were included in optimal cutting medium (OCT), frozen on liquid nitrogen vapors and stored at $-80^\circ$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
biosciences) and rabbit anti-mouse DAB2 H-110 (Santa Cruz Biotechnology, Inc.). Secondary antibodies were all purchased from Jackson Immune Research.

**Immunoblot**
Cells were collected and rinsed once in PBS, then immediately frozen in liquid nitrogen. Samples were dissolved in Laemmli buffer and denatured for 10 min at 100°C. Samples were electrophoretically separated on a 8-15% SDS-PAGE gel and transferred onto Immobilon P membranes (Millipore). Rabbit anti-mouse ARG1, anti-mouse NOS2 and anti-mouse DAB2 H-110 antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-mouse p62/SQSTM1 and anti-mouse LC3 antibodies were purchased from Sigma-Aldrich. Secondary HRP-conjugated anti-rabbit antibody was purchased from GE Healthcare.

**Total RNA purification and Real-time PCR**
Total RNA was extracted by TRIzol (Invitrogen) from CD11b+Ly6G/C F4/80+ sorted macrophages, according to the manufacturer’s instructions with minor modifications. The quality and quantity of RNA samples were determined by Agilent RNA 6000 Nano Chip (Agilent Technologies). cDNA from purified total RNA was produced by SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Five nanograms of template cDNA was used in TaqMan real-time PCR (TaqMan Gene Expression Assay; 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, 1 minute at 60°C, for 45 cycles) performed on an ABI PRISM 7700 (Applied Biosystems). Inventoried Taqman probes for genes of interest were purchased by Applied Biosystems.

**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 1X, 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 2 ml sorting buffer and eluted with LS columns according to manufacturer instructions (Miltenyi Biotec).

**Cytofluorimetric staining and fluorescence-activated cell sorting**

After hypotonic lysis of red blood cells (when necessary), 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 washed in PBS and resuspended in 300 μl of PBS for cytofluorimetric analysis. Anti-CD11b-PerCPCy5.5, anti-GR1-APC, anti-GR1-Pacific Blue, anti-CD115-PE, anti-CD115-APC, anti-F4/80-APCe780, anti-CD86-biotin, anti CD45.1-PE, anti-c-Kit-APCe780, and anti-FLT3-PE were from eBioscience; anti-I^A/I^E (MHC-II) and anti-LY6G-FITC were from BD Biosciences; anti-CD206-Alexa647, anti-LY6C-FITC, anti-LY6C-APC, and anti-LY6G-Pacific Blue were from Biolegend. Anti-CCR2-APC was from R&D systems. To analyze cell viability, cells were stained with live/dead aqua dye for 20 minutes at 4°C and then washed with PBS. Samples were acquired with a FACS LSR-II cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star, Inc.) software. To perform fluorescence-activated cell sorting, cells were stained as described above, resuspended in FACS buffer (PBS 1X, EDTA 0.5 mM, FBS 3%, 150 U/ml streptomycin, 200 U/ml penicillin) and then sorted with a FACS Aria (BD Biosciences).

**In vivo tracking of monocytes**

Tibiae and femurs of C57BL/6 and Dab2^−/− healthy mice were removed with sterile techniques and bone marrow cells were flushed with medium. CD11b^+ cells were enriched by immunomagnetic sorting and then stained with anti-LY6G-FITC and anti-
CD115-PE as described above. Ly6G^CD115^ monocytes were sorted with a FACS Aria (BD Biosciences). Purified monocytes 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. CFSE-labeled monocytes from C57Bl/6 and Dab2^-/- were admixed 1:1 and 0.2x10^6 cells/mice cells were injected i.v. in tumor-bearing Ly5.1 mice.

**Evaluation of CTL response (immune suppression assay)**

Mixed lymphocyte peptide culture (MLPC) on C57BL/6 background was prepared by mixing γ-irradiated C57BL/6 splenocytes with OT-1 splenocytes, in order to obtain 1% OVA-specific CFSE labeled lymphocytes in the final culture (typical ratio 20:1). After 5 days, cultures were tested for ability to lyse specific target (MBL-2 loaded with the OVA257-264 peptide) in a 5-hour ^51^Cr-release assay, using 2x10^3 target cells previously labeled with 100 μCi of Na^2^Na^51^CrO_4 for 60 minutes. The percentage of specific lysis was calculated from triplicate samples as follows: (experimental cpm x spontaneous cpm)/(maximal cpm x spontaneous cpm)/100. Lytic units (L.U.) were calculated as the number of cells giving 30% specific lysis of 2x10^3 specific target cells per 10^6 effector cells (L.U.30/10^6 cells). When present, the percent nonspecific lysis of unloaded MBL-2 control targets was subtracted from that obtained with MBL-2 target cells. The number of L.U.30/10^6 cells was then used to calculate L.U.30 per culture from the number of viable cells recovered in the cultures. The percentage of L.U.30 was calculated as follows: L.U.30 of experimental group/L.U.30 of control group x 100 (Dolcetti, Peranzoni et al. 2010).

**Generation of bone marrow-derived macrophages**

Tibiae and femurs of C57BL/6 and Dab2^-/- mice were removed with sterile techniques and bone marrow cells were flushed with medium. Red blood cells were lysed with hypotonic solution. To obtain macrophages from bone marrow cultures, 2.5x10^5 cells were plated in 33 mm petri dishes (Falcon, Becton Dickinson) in 1.5 ml of RPMI medium supplemented with either GM-CSF (100 ng/ml) or M-CSF (100 ng/ml). Cultures were incubated for 7 days at 37°C with 5% CO_2_. Cells from both the non-adherent and adherent fraction were removed by rinsing the dishes with PBS 2mM EDTA.
Metastasis induction

In order to induce lung metastasis, C57BL/6 mice and Dab2\textsuperscript{-/-} transgenic mice were injected i.m. with 1x10\textsuperscript{5} MCA-MN cells. After 26 days, when tumors exceeded 200 mm\textsuperscript{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.

Statistical analysis

Values are reported as mean ± standard error (SE). 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

Expression of DAB2 protein in CD11b⁺ myeloid cells

According to our Affimetrix® transcriptional profiling, the Dab2 gene was overexpressed in intratumoral CD11b⁺ myeloid cells regardless of the examined tumor model and genetic background. To confirm these data, we analyzed DAB2 protein expression by immunoblot. Tumor single-cell suspensions were obtained after enzymatic digestion of tumor tissues, and CD11b⁺ myeloid cells were purified by immunomagnetic sorting. In accordance with the Affimetrix® profiling experiment, we used purified, splenic CD11b⁺ myeloid cells from healthy mice as control, matching for the tumor genetic background. Detection with a specific α-DAB2 antibody showed that both isoforms of DAB2 were expressed in intratumoral myeloid cells, but not in control splenic myeloid cells (Figure 1A and 1B). DAB2 was detected regardless of the tumor model, although we noticed a tumor-specific relative expression of the two DAB2 isoforms, p96 and p67. Upregulation of the DAB2 protein by myeloid cells was specific for the tumor microenvironment, because analysis of CD11b⁺ myeloid cells purified from the spleen (Figure 1A and 1B) and bone marrow (Figure 1C) of tumor-bearing mice showed no expression of the protein. Only injection of the C26GM tumor cell line induced a slight upregulation of the p96 isoform in splenic myeloid cells, however this was not reproduced in other examined tumor models (Figure 1B). We concluded that induction of DAB2 expression in CD11b⁺ myeloid cells is a general feature of the tumor microenvironment, regardless of both the histologic origin of the tumor and the genetic background of the organism.
Figure 1. Expression of DAB2 protein in CD11b⁺ myeloid cells.

Immunoblot analysis for the expression of DAB2 isoforms (p96 and p67) in CD11b⁺ cells isolated from the spleen and tumor of mice injected with different tumor cells both in (A) C57Bl/6 (EL4 and MCA203) and (B) BALB/c (C26GM and 4T1) backgrounds. Splenic CD11b⁺ cells from healthy mice matching genetic backgrounds were used as negative controls (ctrl). (C) Immunoblot analysis for DAB2 expression in CD11b⁺ cells isolated from the bone marrow and tumor of mice injected with MCA203 cells. Actin was used as loading control.

Knockout of DAB2 affects accumulation of intratumoral monocytes and macrophages

The tumor-specific induction of DAB2 in myeloid cells prompted us to speculate that this protein could have a specific function within the tumor microenvironment. To establish the consequences of DAB2 deficiency in intratumoral myeloid cells, we generated a conditional knockout mouse strain based on the flox/cre deletion system. The $\text{Dab2}^{\text{flox/flox};\text{Tie2Cre}^+/-}$ (Dab2⁻/⁻) mice express the CRE recombinase under the control of the Tie2 gene promoter, allowing a specific deletion of the floxed Dab2 gene in the hematopoietic precursors (Marigo, Bosio et al. 2010).

As a model of tumor disease we chose the MCA203 fibrosarcoma cell line, which has been extensively used to study the altered myelopoiesis and immune tolerance induced by neoplastic cells (Marigo, Bosio et al. 2010; Ugel, Peranzoni et al. 2012). Subcutaneous
injection of MCA203 cells in Dab2⁻/⁻ mice resulted in a modest delay of the tumor growth respect to wild type C57Bl/6 mice, as determined by daily measurement of the tumor area (Figure 2).

We supposed that a modification of the tumor microenvironment occurs in Dab2⁻/⁻ mice, thus resulting in a diminished growth of neoplastic cells. To understand better the nature of this modification, we decided to check the composition of the myeloid tumor infiltrate, using a multiparameter cytofluorimetric analysis of tumor-derived, single-cell suspensions. The analysis was performed two weeks after injection of MCA203 cells, when the tumor growth in Dab2⁻/⁻ mice started to significantly diverge from the C57Bl/6 group. The gating strategy for this analysis is reported in Appendix A. Strikingly, we found a significant reduction of intratumoral CD11b⁺Ly6G⁻Ly6CʰʰF4/80⁺ monocytes and

Figure 2. Tumor growth is delayed in Dab2⁻/⁻ mice.
Tumor growth curve of MCA203 in C57Bl/6 and Dab2⁻/⁻ mice. Animals were injected subcutaneously with 1x10⁶ MCA203 tumor cells. Tumors were measured blindly every 2 days with a digital caliper (N = 12, * = p<0.05; ** = p<0.01).
CD11b⁺Ly6G⁻Ly6C<sub>low/neg</sub>F4/80⁺CD115⁺ macrophages in Dab2<sup>−/−</sup> mice with respect to control. Conversely, the percentage of CD11b⁺Ly6G⁺ granulocytes was augmented within the tumor of Dab2<sup>−/−</sup> versus C57Bl/6 mice (Figure 3A). We also found a significant increment in a population of CD11b⁺Ly6G⁺F4/80⁻CD115⁻ myeloid cells (either positive or negative for Ly6C, Fig. 3A), however we were not able to find additional markers for this subset, thus precluding its further characterization.
Figure 3. Knockout of DAB2 affects accumulation of intratumoral monocytes and macrophages.

(A) Multicolor cytofluorimetric analysis of intratumoral myeloid cells two weeks after injection of MCA203 cells in either C57Bl/6 or Dab2−/− mice. Analysis was performed gating on CD11b+ living cells. Populations were defined as described in Appendix A (Gran: granulocytes; Mono: monocytes; Mφ: macrophages). All graphs are representative of three independent experiments with 3-4 mice per group (* = p<0.05; ** = p<0.001). (B) Immunoblot analysis for DAB2 expression in intratumoral myeloid subsets, two weeks after injection of MCA203 cells in C57Bl/6 mice. Cells were purified by FACS; myeloid subsets were defined as described in Appendix A (CD11b+: total myeloid cells; Gran: granulocytes; Mono: monocytes; Mφ: macrophages). (C) Thin cryosections of MCA203 tumors showing distribution of the DAB2+CD11b+ myeloid infiltrate. Samples were stained with α-DAB2 (red), α-CD11b (blue) and counterstained with DAPI (white).

Analysis of the myeloid tumor infiltrate showed that DAB2 deficiency affects myeloid cells in a cell type-specific manner. For this reason we decided to better characterize DAB2 expression in intratumoral myeloid cells. Wild type intratumoral myeloid subsets as defined in Figure 3A were sorted using Fluorescence Activated Cell Sorting (FACS), and protein lysates were analyzed for DAB2 expression by immunoblot. Interestingly, granulocytes had no expression of DAB2 isoforms, while both p96 and p67 were expressed in monocytes, macrophages and CD11b+Ly6G−F4/80− cells (Figure 3B). Relative expression of DAB2 isoforms was not constant in these subsets. To characterize DAB2 expression in regard to cell position within the tumor mass, we performed confocal analysis of thin MCA203 tumor microsections. We found that DAB2 was preferentially expressed in CD11b+ myeloid cells localized to the border zone of the tumor (Figure 3C). All these data demonstrate that DAB2 is mainly expressed in cells of the myelomonocytic lineage localized to the border of the tumor mass, and its depletion affects accumulation of intratumoral monocytes and macrophages.

Dab2−/− monocytes cannot efficiently generate intratumoral macrophages

The reduction of intratumoral monocytes and macrophages in Dab2−/− mice could be the consequence of a defective myelomonocytic lineage development. This hypothesis is supported by the fact that DAB2 is phosphorylated following activation of the M-CSF signaling pathway, the main cytokine driving monocyte and macrophage differentiation (Cecchini, Dominguez et al. 1994; Xu, Yang et al. 1995). Moreover, the Dab2 promoter is transactivated in vitro by the transcription factor PU.1, which is a master regulator of
We decided to monitor myelopoiesis during tumor progression in $Dab2^{-/-}$ mice, by performing multiparameter cytfluorimetric analysis of ex vivo bone marrow cells. The gating strategy for this analysis is reported in Appendix B. Healthy $Dab2^{-/-}$ mice showed a significant reduction in the percentage of bone marrow $CD11b^+Ly6G^-CD115^+Ly6C_{high}CCR2^+$ inflammatory monocytes, $CD11b^+Ly6G^-CD115^+Ly6C_{low/neg}$ resident monocytes, and $CD11b^+Ly6C/G^F4/80_{high}$ macrophages respect to C57Bl/6 mice, while the percentage of $CD11b^+Ly6G^+$ granulocytes was not affected (Figure 4A, left panel). However, the same analysis performed two weeks after injection of MCA203 cells showed a reduction only in the percentage of bone marrow macrophages in $Dab2^{-/-}$ mice (Figure 4A, right panel). To understand the reason for the constitutive lack of bone marrow monocytes in healthy $Dab2^{-/-}$ mice, we monitored the percentage of Macrophage/Dendritic Cell Progenitors (MDPs) (which generate monocytes) and Common Dendritic Cell Precursors (CDP) (which originate from MDPs). Results revealed no differences between $Dab2^{-/-}$ and C57Bl/6 mice, both under steady-state conditions and during tumor-induced myelopoiesis (Figure 4B).
Figure 4. Healthy Dab2−/− mice have reduced percentage of monocytes in lymphoid organs.

(A) Multicolor cytofluorimetric analysis of bone marrow myeloid cells in C57Bl/6 versus Dab2−/− mice, both in healthy (left graph) and tumor-bearing (right graph) mice. Analysis was performed gating on CD11b+ living cells. Populations were defined as described in Appendix B and C (Gran: granulocytes; Ly6C^high: inflammatory monocytes; Ly6C^low/neg: resident monocytes; Mφ: macrophages). (B) Analysis of MDPs (Lin− CD115−FLT3−c-Kit^high cells) and CDPs (Lin CD115−FLT3−c-Kit^low cells) in C57Bl/6 versus Dab2−/− mice, following
injection of MCA203 tumor cells. (C) Multicolor cytofluorimetric analysis of splenic myeloid cells in C57Bl/6 versus Dab2−/− mice, both in healthy (left graph) and tumor-bearing (right graph) mice. Analysis was performed gating on CD11b+ living cells. All graphs are representative of three independent experiments with 3-4 mice per group (* = p<0.05; ** = p<0.01; *** = p<0.001).

It was recently reported that during tumor progression, inflammatory monocytes are mobilized from the bone marrow and colonize a specialized niche in the spleen, where they induce peripheral tolerance to tumor antigens and form a pool of precursors able to replenish TAMs (Cortez-Retamozo, Etzrodt et al. 2012; Ugel, Peranzoni et al. 2012). Interestingly, in our analysis the percentage of bone marrow inflammatory monocytes, but not resident monocytes, was greatly diminished in wild type tumor-bearing mice with respect to healthy littermates (1.119±0.421% versus 5.172±0.525% respectively; Figure 4A). We supposed that lack of bone marrow monocytes in Dab2−/− mice was masked during tumor progression by splenic extramedullary myelopoiesis, and that an altered accumulation of monocytes in this organ could explain the reduction of intratumoral macrophages in Dab2−/− mice. Cytofluorimetric analysis of the spleen revealed that healthy Dab2−/− mice had a reduced percentage of inflammatory monocytes and macrophages compared to C57Bl/6 mice, with a concomitant increase in the percentage of granulocytes (Figure 4C, left panel). However, these differences were lost two weeks post-injection of MCA203 cells (Figure 4C, right panel). All together, these data show that Dab2−/− mice have a defect in the accumulation of monocytes in lymphoid organs; however, this defect is lost under conditions of tumor-driven myelopoiesis, thus not reflecting phenotypic landscape of the tumor infiltrate.

To understand better the reason for the lack of intratumoral monocytes and macrophages in Dab2−/− mice, we set up in vivo tracking of bone marrow monocytes in tumor-bearing mice. We purified monocytes from the bone marrow of healthy congenic CD45.1 wild type and CD45.2 Dab2−/− mice, using FACS. Purified cells were stained with the fluorescent dye CFSE and mixed 1:1, then injected in tumor bearing CD45.2 wild type mice (Figure 5A). Four days post-adoptive transfer of monocytes, we checked for the presence of CFSE+ cells in the blood and tumor of CD45.2 host mice, using cytofluorimetric analysis (Figure 5B). While the ratio of CFSE+ CD45.1+ wild type and CD45.2+Dab2−/− monocytes was 1:1 in the blood, thus demonstrating their equal access to
the bloodstream, this ratio dramatically changed in favor of CD45.1+ wild type monocytes within the tumor (Figure 5C, left). Moreover, the remaining CFSE+ CD45.2+ Dab2/− monocytes seemed less prone to differentiate into macrophages with respect to CD45.1+ wild type monocytes (Figure 5C, right). Altogether, these data suggest that alteration of the tumor myeloid infiltrate in Dab2/− mice is tumor-intrinsic and imputable to a defective ability of monocytes to differentiate to macrophages.
Figure 5. *Dab2*−/− monocytes cannot efficiently generate intra-tumoral macrophages

(A) Schematic representation of the monocyte *in vivo* tracking experiment. (B) Gating strategy to monitor monocyte differentiation to macrophages. (C) The percentage of wild type versus *Dab2*−/− adoptively transferred, CFSE+ monocytes in the blood and tumor of host mice is reported on the left graph. The
differentiation of monocytes in macrophages was determined as the ratio between Gr-1^- and Gr-1^+ cells among CFSE^+ transferred monocytes detected within the tumor (right graph; N=4 mice per group in two independent experiments).

**GM-CSF and M-CSF induce DAB2 expression in myeloid cells through a mechanism requiring the C/EBPβ transcription factor**

Having demonstrated that monocytes require DAB2 to differentiate in intratumoral macrophages, we exploited the signals required to upregulate the protein within the tumor. We speculated that the same stimuli, which initiate macrophage differentiation, were also responsible for expression of DAB2. GM-CSF and M-CSF are two cytokines which are abundantly secreted in the tumor microenvironment (Gabrilovich 2004); both of them are known to drive the differentiation of monocytes and macrophages (Hamilton 2008). To test whether DAB2 could be upregulated in response to these stimuli, we purified CD11b^+ cells from the spleen of wild type tumor-bearing mice using immunomagnetic sorting. Purified myeloid cells were exposed *in vitro* for 24 hours to GM-CSF and M-CSF alone or in combination (see Figure 6A for schematic representation of the experiment). Cells were harvested and protein lysates were analyzed for DAB2 expression by immunoblot. Fresh splenic and intratumoral CD11b^+ cells were used as negative and positive controls, respectively. We found that after exposure to either GM-CSF, M-CSF or a combination of both, cells upregulated both DAB2 isoforms compared to untreated cultured cells (Figure 6B). Conversely, cells exposed to G-CSF, which drives the differentiation of granulocytes, did not upregulate DAB2 proteins (Figure 6C).
Figure 6. GM-CSF and M-CSF induce expression of DAB2 in myeloid cells.

(A) CD11b⁺ myeloid cells were isolated by immunomagnetic sorting from the spleen of tumor-bearing mice. Purified cells were either frozen for subsequent analysis or cultured for 24 hours with or without GM-CSF and M-CSF cytokines. Total cell extracts were analyzed for the expression of DAB2 isoforms by immunoblot (B). Intratumoral CD11b⁺ cells from the same mice were used as positive control for DAB2 expression. t₀ = splenic CD11b⁺ cells; - = splenic CD11b⁺ cells in culture without cytokines; GM = as before, with GM-CSF; M = as before, with M-CSF; GM + M = as before, with both GM-CSF and M-CSF; + = intratumoral CD11b⁺ cells. (C) Immunoblot showing DAB2 expression in splenic CD11b⁺ cells after in vitro stimulation with G-CSF. t₀ = splenic CD11b⁺ cells; - = splenic CD11b⁺ cells in culture without cytokines; G = as before, with G-CSF; GM = as before, with GM-CSF; GM + G = as before, with both GM-CSF and G-CSF; + = intratumoral CD11b⁺ cells. Actin was used as loading control in all the experiments.

We previously demonstrated that the C/EBPβ transcription factor is required for the in vivo homeostasis of CD11b⁺Gr-1int monocytic MDSCs, likely acting as downstream transcription factor for GM-CSF and IL-6 signaling pathways (Marigo, Bosio et al. 2010). In fact, conditional knockout of this protein in the hematopoietic system resulted in a strong reduction of both splenic and intratumoral monocytic MDSCs; simultaneously, the tumor microenvironment was radically changed, with loss of myeloid immune suppressive activity and reduced metastatic spreading of tumor cells (Marigo, Bosio et al. 2010). Having seen a relationship between DAB2 and GM-CSF, we speculated that C/EBPβ could
be required for expression of DAB2 in intratumoral myeloid cells. First of all, we verified the effects of c/EBPβ deficiency on splenic inflammatory monocytes, which are considered to be a fraction of monocytic MDSCs (Geissmann, Manz et al. 2010; Ugel, Peranzoni et al. 2012). As expected, conditional knockout of the Cebpb gene in the hematopoietic lineage resulted in a strong reduction of splenic CD11b^+Gr-1^int^CD115^+ inflammatory monocytes in tumor-bearing mice (Figure 7A). Intratumoral CD11b^+ myeloid cells isolated from the same mice showed a dramatic depletion of the DAB2 protein with respect to wild type C57Bl/6 controls (Figure 7B). Altogether, these data were consistent with our finding that DAB2 is mainly expressed by cells of the myelomonocytic lineage within the tumor. Finally, we verified whether splenic Cebpb^−/− CD11b^+ myeloid cells could upregulate DAB2 in vitro. Stimulation of these cells with GM-CSF and M-CSF failed to induce DAB2 expression, thus supporting a role for C/EBPβ in regulating this pathway (Figure 7C).

Figure 7. C/EBPβ is required for expression of DAB2 in myeloid cells.

(A) Cytofluorimetric analysis showing accumulation of CD115^Gr-1^int^ monocytes in the spleen of either C57Bl/6 or Cebpb^−/− mice, two weeks after injection of MCA203 cells. Analysis was performed gating on CD11b^+ cells. (B) Immunoblot showing expression of DAB2 in CD11b^+ myeloid cells purified from tumors of
either C57Bl/6 or Cebpb<sup>−/−</sup> mice. Actin was used as loading control. (C) Splenic CD11b<sup>+</sup> myeloid cells from either C57Bl/6 or Cebpb<sup>−/−</sup> tumor-bearing mice were purified and in vitro stimulated with both GM-CSF and M-CSF for 24 hours. Cells were harvested and protein lysates were analyzed for DAB2 expression by immunoblot (t 0 = splenic CD11b<sup>+</sup> cells; - = splenic CD11b<sup>+</sup> cells in culture without cytokines; GM+M = splenic CD11b<sup>+</sup> cells in culture with both GM-CSF and M-CSF). Actin was used as loading control.

In summary, we suggest that CD11b<sup>+</sup> intratumoral myeloid cells upregulate DAB2 due to exposure to locally secreted cytokines such as GM-CSF and M-CSF. Moreover, abrogation of C/EBPβ transcriptional activity can impair this process.

**Knockout of Dab2 alters M1/M2 macrophage balance and immune suppressive activity within the tumor**

DAB2 depletion in myeloid cells resulted in a strong reduction of intratumoral monocytes and macrophages. Both cell types have main roles in the regulation of the tumor microenvironment: monocytes suppress antitumor adaptive immunity and can promote angiogenesis (De Palma, Venneri et al. 2005; Gabrilovich and Nagaraj 2009), while macrophages virtually control all processes which influence tumor growth and tissue remodeling (Qian and Pollard 2010). Having observed a reduction in the tumor growth in Dab2<sup>−/−</sup> mice with respect to wild type controls, we decided to measure the immune suppressive activity of Dab2<sup>−/−</sup> intratumoral myeloid cells. CD11b<sup>+</sup> cells were purified with anti-CD11b microbeads from the tumor of Dab2<sup>−/−</sup> versus wild type mice and co-cultured with ovoalbumin-specific CD8<sup>+</sup> T cells in a Mixed Lymphocyte-Peptide Culture (MLPC). After five days, cytotoxic activity of CD8<sup>+</sup> T cells was evaluated with <sup>51</sup>Chromium release assay. Surprisingly, we found that Dab2<sup>−/−</sup> CD11b<sup>+</sup> myeloid cells had increased suppressive activity with compared to wild type ones (Figure 8A). To explain this, we checked the expression of immune suppressive enzymes ARG1 and iNOS in Dab2<sup>−/−</sup> intratumoral CD11b<sup>+</sup> cells(Bronte and Zanovello 2005). Immunoblot revealed an increased expression of both enzymes in Dab2<sup>−/−</sup> samples with respect to wild type ones, which might explain their increased suppressive activity (Figure 8B).
Figure 8. Increased suppressive activity of \( Dab2^{-/} \) intratumoral CD11b\(^+\) myeloid cells.

(A) Ovoalbumin (OVA) antigen specific T lymphocytes were stimulated with the OVA immunodominant peptide in presence of CD11b\(^+\) cells isolated from MCA203 tumors growing in either C57Bl/6 or Dab\(2^{-/}\)-mice. After 5 days of culture, the cytotoxic activity of T cells was evaluated in a \(^{51}\)Cr release assay. Values are indicated as lytic unit 30\% (L.U.30), as a measure of the lytic potential of T lymphocytes. Graph shows results from three independent experiments (N=3). (B) CD11b\(^+\) cells were purified from MCA203 tumors of either C57Bl/6 or \( Dab2^{-/}\) mice. Protein lysates were analyzed for expression of ARG1 and iNOS by immunoblot. Actin was used as loading control.

Regulation of ARG1 and iNOS expression in macrophages is classically considered antithetical and a reflex of their M1/M2 polarization (Gordon and Taylor 2005), whose balance controls the immune response within the tumor microenvironment (Qian and Pollard 2010). Having seen a reduction of macrophages and an altered expression of ARG1 and iNOS in \( Dab2^{-/}\) myeloid cells, we wondered whether DAB2 deficiency could influence the M1/M2 polarization. Using quantitative PCR, we measured the relative expression of a panel of genes associated with either M1 or M2 macrophages (Murray and Wynn 2011). Analysis was performed on FACS-purified, intratumoral macrophages from either \( Dab2^{-/}\) or wild type tumor-bearing mice. Interestingly, \( Dab2^{-/}\) macrophages had an increased M2 signature respect to those isolated from wild type mice (Figure 9A). Notably, \( Nos2 \) gene expression was also increased, reflecting protein expression data obtained by immunoblot. To confirm further these results, we analyzed expression of several M1/M2 macrophage surface markers in tumor myeloid infiltrates, using
multiparameter cytofluorimetric analysis. In accordance with gene expression data, \(Dab2^{-/-}\) mice had a reduced percentage of MHCII\^{\text{high}}\ CD86\^{+} M1 macrophages in their tumor infiltrates respect to C57Bl/6 controls (Figure 8B); moreover, CD206 expression in \(Dab2^{-/-}\) macrophages was increased respect to wild type ones, suggesting a more M2-oriented phenotype (Figure 8C; (Mosser and Zhang 2008). Concluding, our data suggest that DAB2 deficiency shift the balance of macrophage polarization towards the M2 status, thus increasing immune suppression within the tumor.
Figure A: Log2 Fold Change with respect to C57Bl/6

Figure B: Flow cytometry analysis of MHC-II^high Cd86^cells and CD206 expression in C57Bl/6 and Dab2/− mice.
Figure 9. Knockout of Dab2 alters the M1/M2 balance of intratumoral macrophages.

(A) Quantitative PCR evaluation of genes known to be associated with either M1 or M2 macrophage polarization. Data are expressed, on a base 2 logarithmic scale, as the fold change of mRNA abundance in CD11b^Ly6C/G F4/80^- macrophages purified by FACS from MCA203 tumors of Dab2^-/- mice, normalized to the expression of S18 housekeeping gene and compared to abundance in wild type C57BL/6 macrophages purified in the same way (N=4 mice per group in one experiment). (B) Top, cytofluorimetric analysis of tumor cell suspensions from either MCA203 tumor-bearing C57Bl/6 or Dab2^-/- mice, showing the percentage of MHCII^high CD86^+ M1 macrophages. Analysis was performed gating on CD11b^-Gr-1^-F4/80^- macrophages. Bottom, the same gating strategy was used to analyze CD206 expression, reported as Mean Fluorescence Intensity (MFI; N=4 mice per group in one experiment).

Dab2^-/- mice have a metastasis resistant phenotype

Metastatic spreading is a multistep process that begins in the primary tumor. Knowing that macrophages have a basic role in assisting all these steps, we wondered whether DAB2 depletion could limit the metastatic potential of tumor cells. To establish this, we used the MN/MCA1 sarcoma metastatic model, which induces macroscopic lung metastases four weeks after tumor injection (Sica, Saccani et al. 2000). In this model, we did not observe significant changes in the primary tumor growth between Dab2^-/- and C57Bl/6 mice; however, the number of macrometastasis was dramatically reduced in Dab2^-/- mice with respect to control group (Figure 10A). To confirm this result, we used another metastatic tumor model, the Lewis Lung Carcinoma (LLC) cell line (Kaplan, Riba et al. 2005). To allow direct observation of each single step of the metastatic process, we generated a LLC clone stably expressing the Green Fluorescent Protein (GFP). GFP expression allows accurate tracking of single fluorescent metastatic cells by a combination of in vivo imaging, confocal microscopy and quantitative-PCR of the gfp gene. The GFP^+ LLC clone (LLC/F4) grew equally well and caused a similar composition of splenic, tumor and lung myeloid infiltrates with respect to its parental cell line in vivo. LLC/F4 growth in Dab2^-/- mice was similar to its wild type counterparts (Figure 10B); however, even in this model we found a strong reduction of intratumoral macrophages, with a concomitant increase in the percentage of granulocytes (Figure 10C). To monitor the magnitude of the metastatic spreading, lungs were collected from tumor-bearing mice and total mRNA was extracted for subsequent cDNA synthesis. Presence of the gfp mRNA transcript was evaluated by PCR using specific primers. By this way, we could
detect GFP⁺ cells in the lungs starting from two weeks after subcutaneous injection of LLC/F4 cells. At this time point, the percentage of mice with GFP-positive lungs was reduced in the Dab2⁻/⁻ group respect to wild type one (40% versus 80%, respectively). Three weeks after tumor injection we still found the same reduction, thus confirming an impairment of the metastatic spreading in Dab2⁻/⁻ mice. In the present study we did not perform further experiments to explain these findings, which will be the aim of future research activity. However the LLC/F4 clone represents a tool to finely dissecting which steps of the metastatic process are regulated by DAB2⁺ myeloid cells, and how this regulation is accomplished.
Figure 10. Dab2\textsuperscript{-/-} mice have a metastasis resistant phenotype.

(A) 0.1x10\textsuperscript{6} MN/MCA\textsubscript{3} cells were intramuscularly injected in either Dab2\textsuperscript{-/-} or C57Bl/6 mice. When the primary tumor reached 200 mm\textsuperscript{2}, lungs were collected and superficial macrometastases were counted with Bouin staining. Number of metastases and tumor area were normalized to the C57Bl/6 group (N=22 mice per group, *** p<0.001).

(B) Tumor growth curve of LLC/F4 in C57Bl/6 and Dab2\textsuperscript{-/-} mice. Animals were injected subcutaneously with 2x10\textsuperscript{6} LLC/F4 tumor cells. Tumors were measured blindly every 2 days with a digital caliper (N=8).

(C) Multicolor cytofluorimetric analysis of intratumoral myeloid cells two weeks after injection of LLC/F4 cells in either C57Bl/6 or Dab2\textsuperscript{-/-} mice. Analysis was performed gating on CD11b\textsuperscript{+} living
cells. Populations were defined as described in Appendix A (Gran: granulocytes; Mono: monocytes; Mφ: macrophages). Graph shows results from two independent experiments with 3-4 mice per group (N=8, * = p<0.05). (D) 2×10⁶ LLC/F4 cells were injected subcutaneously in either C57Bl/6 or Dab2⁻/⁻ mice. Mice were euthanized 2 (black) and 3 (red) weeks post-tumor injection and total RNA was extracted from lungs for subsequent cDNA synthesis. Presence of the gfp transcript was evaluated by PCR. Results are reported in tables on the right as percentage of mice with gfp⁺ lungs.

**Reduced number of monocytes during in vitro cultures of Dab2⁻/⁻ bone marrow cells**

To describe the mechanism by which DAB2 influences the generation of intratumoral macrophages, we stimulated bone marrow cells with either GM-CSF or M-CSF, which represents a simplified in vitro model of macrophage differentiation (Fleetwood, Lawrence et al. 2007). Both cytokines induce expansion of bone marrow monocytes and their subsequent differentiation in macrophages, although with different extent and phenotypic traits (Hamilton 2008). As expected, treatment with either GM-CSF or M-CSF induced a progressive upregulation of the DAB2 protein, with a peak on day 6 (Figure 11A). When we put in culture Dab2⁻/⁻ bone marrow cells, we measured a reduced number of monocytes compared to wild type control, indicating a defect in the proliferation and/or survival of these cells if DAB2 is depleted (Figure 11B). Unexpectedly, Dab2⁻/⁻ macrophages grew regularly during the culture, thus not reflecting the differentiation defect observed in vivo. These data cannot be easily reconciled with the literature and could indicate a DAB2- and monocyte-independent origin of the in vitro bone marrow-derived macrophages. Further studies will be performed to address this issue.
Figure 11. Reduced number of monocytes during in vitro cultures of \textit{Dab2}^{-/-} bone marrow cells

(A) Bone marrow cells collected from healthy C57Bl/6 mice were treated \textit{in vitro} for 7 days with either GM-CSF or M-CSF. Cells were collected every day and protein lysates were analyzed for DAB2 expression by immunoblot. Actin was used as loading control. (B) Number of monocytes (upper graphs) and macrophages (lower graphs) during \textit{in vitro} GM-CSF/M-CSF-induced differentiation of bone marrow cells collected from either C57Bl/6 or \textit{Dab2}^{-/-} mice. Values were obtained multiplying the number of retrieved cells by the percentage of either monocytes or macrophages, as assessed by cytofluorimetric analysis.
Defects in the induction of autophagy during differentiation of Dab2^/- bone marrow-derived macrophages

It was recently published that autophagy is required for correct monocyte-macrophage differentiation (Jacquel, Obba et al. 2012; Zhang, Morgan et al. 2012). Knockout of the Atg7 gene, which impairs autophagy, caused a blockage of the differentiation of monocytes to macrophages. Moreover, inhibition of autophagy can lead to increased apoptosis (Moscat and Diaz-Meco 2009). Supposing that DAB2 deficiency induced cell death in monocytes, we wondered whether there could be a link between DAB2 and autophagy. To test this, we collected protein lysates from Dab2^/- and wild type bone marrow cells during M-CSF-induced macrophage differentiation. By immunoblot, we checked for p62 and LC3-II, two markers of autophagosome formation and degradation in the lysosome. Expression of p62 gradually incremented during M-CSF treatment; however, we measured a drastic reduction of the protein between day 6 and 7, suggestive of the autophagy process being activated. Interestingly, Dab2^/- bone marrow-derived macrophages degraded p62 at a lesser extent with respect to wild type (Figure 12A). Moreover, autophagosome formation seemed to be inhibited in Dab2^/- bone marrow-derived macrophages, because we detected reduced quantities of LC3-II respect to wild type controls, both in terms of LC3-II/LC3-I ratio and LC3-II expression per se (Figure 12B and 12C). Altogether, these data suggest that autophagy is inhibited when DAB2 is not available, and suggest a potential molecular mechanism explaining the in vivo and in vitro defects, which we observed in monocytes following DAB2 depletion.
Figure 12. Defects in the induction of autophagy during differentiation of Dab2−/− BM-derived macrophages.

(A) Bone marrow cells isolated from healthy C57Bl/6 versus Dab2−/− mice were cultured with 100 ng/ml of M-CSF for 7 days. Protein lysates were collected at different time points and resolved on a SDS-page for detection of p62 protein by immunoblot. Actin was used as loading control. (B) The ratio between LC3-II and LC3-I in wild type versus Dab2−/− mice bone marrow-derived macrophages was quantified with ImageJ® after detection of the proteins by immunoblot. (C) Expression of LC3-II at day 7 in Dab2−/− bone marrow-derived macrophages was normalized with respect to wild type control and expressed as Arbitrary Units (A.U.). Actin was used as loading control.
Discussion

Macrophages are common members of the tumor microenvironment. They are found infiltrating many types of cancer, and their abundance usually correlates with poor patient prognosis (Bingle, Brown et al. 2002; Chen, Lin et al. 2005; Ryder, Ghossein et al. 2008; Zhu, Zhang et al. 2008). This is probably due to their active contribution to almost all processes that sustain cancer progression (Qian and Pollard 2010). The present study highlights a new possible mechanism that regulates differentiation of TAMs and opening to additional therapeutic options for cancer patients. Based on our Affimetrix® data, the Dab2 gene show a strong expression in the myeloid infiltrate regardless of the histologic origin of the tumor; this was confirmed at protein level (Figure 1). This finding, in addition to our data showing a connection between the DAB2 protein and TAMs, suggests that artificial modulation of DAB2 expression and functions could have a beneficial therapeutic effect in many cancer diseases.

According to our study, the main effect of Dab2 targeted deletion in the hematopoietic system is a strong reduction in monocyte and macrophage presence within the tumor. Cytolfluorimetric analysis of the tumor infiltrate, together with in vivo monocyte tracking experiments strongly suggest that DAB2 is required for differentiation of TAMs (Figure 3A and 5); blockage of this process by DAB2 depletion also resulted in reduced accumulation of monocytes within the tumor, likely dependent on the negative impact on cell availability. At the moment we cannot exclude that DAB2 is required for the entry of monocytes into the tumor, a hypothesis which is supported by the notion that DAB2 regulates integrin trafficking (Rosenbauer, Kallies et al. 2002; Chetrit, Ziv et al. 2009; Teckchandani, Toida et al. 2009). However our conclusion is sustained by the finding that in vitro macrophage differentiation of Dab2−/− bone marrow cells generated less monocytes compared to wild type cells (Figure 11). Interestingly, caspase activation and apoptosis are finely regulated during in vitro differentiation of monocytes to macrophages, and interfering with these pathways can lead to increased cell death (Meinhardt, Roth et al. 2000; Sordet, Rebe et al. 2002; Lin, Leu et al. 2011).

The link between DAB2, macrophage differentiation and monocyte survival is further strengthened by our finding that both GM-CSF and M-CSF upregulate expression of the
protein in splenic CD11b⁺ myeloid cells and bone marrow cells (Figure 6 and 11A). Both cytokines control the proliferation, survival and differentiation of macrophages, and the balance between them influences the macrophage versus dendritict cell differentiation choice of monocytes (Menetrier-Caux, Montmain et al. 1998; Hamilton 2008). In this work we did not verify whether the deletion of Dab2 had consequences on the dendritic cell compartment too, an issue which will be addressed in future studies. If we assume that DAB2 expression is mandatory for macrophage differentiation, for what we know we can formulate at least three hypotheses regarding DAB2 function, which are not mutually exclusive.

The first hypothesis consider the role of DAB2 as regulator of the integrin endocytic trafficking (Chetrit, Ziv et al. 2009; Teckchandani, Toida et al. 2009). It has been demonstrated that DAB2 regulates adhesion and spreading of macrophages in vitro (Rosenbauer, Kallies et al. 2002). DAB2 was shown to be phosphorylated and translocate from the cytosol to the cytoskeletal/membrane fraction following macrophage adhesion to the extracellular matrix. Forced overexpression of DAB2 leaded to increased adherence and spreading, suggesting that DAB2 is implicated in integrin signaling and cytoskeleton reorganization in macrophages (Rosenbauer, Kallies et al. 2002). Both phenomena are strictly interconnected to stemness and cell differentiation (Streuli 2009). Little is known about involvement of integrin signaling in macrophage differentiation; however, it was demonstrated that clustering of the β₂ integrin Mac-1 (CD11b) can induce Csf1r expression through downregulation of the transcriptional repressor FOXP1, thus allowing monocyte maturation into macrophages (Shi, Zhang et al. 2004; Shi, Sakuma et al. 2008). Conversely, the M-CSF signaling pathway modulates integrin and cytoskeleton reorganization (Pixley and Stanley 2004). Considering our data regarding the tumor-specific expression of DAB2 in myeloid cells and the in vivo behavior of adoptively transferred Dab2⁻/⁻ monocytes (Figure 1 and 5), we can suppose that, once within the tumor, monocytes upregulate DAB2 in response to increased levels of tumor-secreted GM-CSF and M-CSF. This allows to re-organize integrins and respond to extracellular matrix and cell-to-cell contact stimuli, promoting intratumoral macrophage differentiation. Our confocal analysis of DAB2 distribution in tumor-infiltrated myeloid cells is in accordance with this model. We found that expression of the protein is mainly
localized in CD11b+ cells within the periphery of the tumor mass (Figure 3C), possibly indicating that DAB2 upregulation is an early event when cells infiltrate the tissue.

The second hypothesis is based on the finding that the p67 isoform of DAB2 can translocate into the nucleus and function as a transcriptional activator in retinoic acid-treated F9 cells (Cho, Jeon et al. 2000). This would add further complexity to the transcriptional changes orchestrated by GM-CSF and M-CSF signaling. Most important, this hypothesis would stress the necessity of determining the relative expression of p96 and p67 within cells. Interestingly, we noticed a difference in the expression of DAB2 isoforms between intratumoral monocytes and macrophages (Figure 3B). Cellular localization of p96 is dependent on a region of the protein which allows binding to the alpha-adaptin subunit of the clathrin-adaptor protein AP2 (Morris and Cooper 2001). This region is absent in p67, suggesting not-overlapping functions with p96. In our confocal studies we could not detect a nuclear distribution of the DAB2 protein in myeloid cells, neither in vivo nor in vitro. Nonetheless, changes in the relative expression of DAB2 isoforms during myeloid development are a major point which needs to be addressed.

The third hypothesis is based on our data regarding a possible involvement for DAB2 in autophagy. During differentiation of Dab2−/− bone marrow-derived macrophages we found an impairment of the autophagic process, as assessed by measurement of LC3-II intracellular levels and p62 degradation (Figure 12). These data can explain the negative impact of DAB2 depletion on macrophage differentiation, as autophagy is essential for the latter (Jacquel, Obba et al. 2012; Zhang, Morgan et al. 2012). Moreover, the reduced accumulation of transferred Dab2−/− monocytes within the tumor could be an effect of increased cell death caused by inefficient autophagy (Moscat and Diaz-Meco 2009). Although this hypothesis requires further experiments to be confirmed, evidences from the literature support it. Research activity in the field of autophagy has recently focused on where autophagosomes assume their membrane from. Presently it is believed that organelles such as the endoplasmic reticulum, mitochondria and the Golgi complex are membrane sources for autophagosome formation (Mizushima and Komatsu 2011). However, a recent paper showed that also the plasma membrane contributes to the biogenesis of pre-autophagosomal structures, the so-called “phagophores” (Ravikumar, Moreau et al. 2010). The Authors demonstrated that ATG16L1-positive early autophagosome structures are generated from the plasma membrane through clathrin-
mediated endocytosis; this requires the interaction between clathrin and ATG16L1 through the adaptor AP2. Interestingly, knockdown of AP2 did not completely block the process, suggesting that other adaptor proteins could be implicated. DAB2 could be one of these adaptors, having already been demonstrated to substitute AP2 in endocytosis of LDLR (Maurer and Cooper 2006; Mulkearns and Cooper 2012). Another possibility is that DAB2 participates to the autophagic process through interaction of its PTB domain with phosphatidylinositol 4,5-bisphosphate, which promotes autophagosome biogenesis by influencing endocytic uptake of plasma membrane into autophagosome precursors (Howell, Lanier et al. 1999; Moreau, Ravikumar et al. 2012). These considerations make the relationship between DAB2 and autophagy quite conceivable and will be further explored in future studies.

A major issue of our study is the exact origin of DAB2+ intratumoral myeloid cells, especially if we consider that an HSC-independent lineage of macrophages exists in tissues (Jenkins, Ruckerl et al. 2011; Schulz, Gomez Perdiguer et al. 2012). According to our immunoblot analysis, both monocytes and macrophages express DAB2 within the tumor (Figure 3B). At least a fraction of these DAB2+ cells probably originate from the blood stream, because adoptively transferred bone marrow monocytes efficiently generated macrophages within the tumor and splenic myeloid cells upregulated DAB2 when properly stimulated with cytokines which induce macrophage differentiation (Figure 5 and 6). At the moment we cannot establish whether one or both monocyte subsets are the source of DAB2+ macrophages, and we do not know the anatomical site where they are stored (spleen or bone marrow). However, conditional knockout of the transcription factor c/EBPβ, which affected accumulation of splenic inflammatory monocytes, strongly impaired the expression of DAB2 in intratumoral CD11b+ cells, while splenic Cebpβ−/− CD11b+ cells failed to upregulate DAB2 after in vitro stimulation with GM-CSF and M-CSF (Figure 7). This indirectly supports the notion that inflammatory monocytes could, at least in part, be the source of intratumoral DAB2+ macrophages. Nevertheless, a population of intratumoral CD11b+Ly6G−F4/80− cells, which we were unable to classify, also expressed high levels of DAB2 (Figure 3). The presence of these cells in the myeloid tumor infiltrate increased in Dab2−/− mice with respect to wild type littermates, at least in the MCA203 tumor model. We can make several hypotheses regarding the nature of this population. If we assume that CD11b+Ly6G−F4/80− cells are
developmentally connected to CD11b^Ly6G^-F4/80^ mature macrophages, they could represent an immature/intermediate precursor of the monocyte differentiation into macrophages. In this case, their accumulation within the tumor of Dab2^-/- mice could be the consequence of the defective differentiation process. Otherwise, they could represent a distinct myeloid lineage, either originating from the bone marrow or reflecting HSC-independent local proliferation of macrophages (Jenkins, Ruckerl et al. 2011; Schulz, Gomez Perdiguero et al. 2012). If part of the DAB2^+ tumor myeloid infiltrate does not originate from the bone marrow, we cannot be sure that all intratumoral myeloid cells are targeted by conditional Dab2 deletion in Dab2^floxfloxflox;Tie2Cre^v/v^- mice, thus urging the need for alternative conditional knockout strategies. Finally, we cannot exclude the possibility that an impairment of the DAB2-regulated endocytic trafficking could alter the expression of surface markers like F4/80 and CD115, thus “masking” the true identity of cells when performing cytofluorimetric analysis. If this is the case, morphologic and confocal studies will surely be helpful in characterizing intratumoral CD11b^Ly6G^-F4/80^- cells. Noteworthy, a population of immature myeloid cells with similar phenotypic traits (CD11b^+CD34^-F4/80^-Gr-1^-) was shown to promote invasion of colorectal cancer cells by secreting MMP2 and MMP9 at the invasive front (Kitamura, Kometani et al. 2007).

The concept of a partial monocyte-independent origin of macrophages comes in help when discussing the behavior of Dab2^-/- bone marrow cells in vitro stimulated with GM-CSF/M-CSF. Although the number of generated monocytes was diminished during the culture, thus reflecting our in vivo data, macrophage numbers were not affected and grew normally, indicating that proliferation of these cells could occur independently of monocyte fate (Figure 11B). Alternatively, considering that DAB2 availability is limiting for monocyte survival, a competition between monocyte clones could be established in Dab2^-/- bone marrow cultures, in which those clones that stochastically do not delete the Dab2 gene shall prevail and replenish macrophage numbers. This possibility must be considered in light of the deleting efficiency of Tie2 promoter, which is not able to drive 100% deletion in all examined mice during laboratory practice (P. Murray, personal communication). Finally, another technical issue concerns the inefficiency of in vitro differentiation systems at reproducing the complex signaling network of in vivo microenvironments. From this point of view, lacking of particular differentiation signals in
vitro could result in the discrepancies we observed during culture of Dab2\(^{-/-}\) bone marrow cells. Based on the concept we discussed above regarding the role of integrins in regulating macrophage differentiation, we are planning to test if the use of components of the extracellular matrix can improve our in vitro differentiation systems.

Although DAB2 deficiency mainly affected the differentiation of myeloid cells within the tumor, also the spleen and bone marrow of tumor-free mice showed reduced percentages of monocytes and macrophages (Figure 4). Unexpectedly, monocyte levels returned normal after tumor implantation, suggesting some rescue mechanisms activated by tumor-driven myelopoiesis. We were not able to detect DAB2 protein in CD11b\(^+\) myeloid cells isolated from the spleen and bone marrow, so we supposed that in these organs DAB2 could be expressed in Lineage\(^-\) precursors of the myelomonocytic lineage, thus influencing the generation of monocytes. However, when we monitored the percentage of MDPs and CDPs during tumor progression, we did not find differences between Dab2\(^{-/-}\) and wild type mice, thus excluding a defect in the generation/expansion of direct monocyte precursors. Noteworthy, macrophage percentages in lymphoid organs of Dab2\(^{-/-}\) tumor-bearing mice were reduced despite normal monocyte levels, supporting the notion that DAB2 acts during macrophage maturation.

The immunosuppressive activity of Dab2\(^{-/-}\) intratumoral CD11b\(^+\) cells was increased (Figure 8A). When we measured the expression of ARG1 and iNOS enzymes, which can strongly suppress CD8\(^+\) T cell response, we found increased levels of both proteins in Dab2\(^{-/-}\) CD11b\(^+\) tumor-infiltrating cells (Figure 8B). Considering how these enzymes are regulated in TAMs, we supposed that DAB2 could influence the macrophage M1/M2 polarization (Gordon and Taylor 2005). By analyzing the expression of several genes and surface markers associated with either M1 or M2 status, we found that DAB2 depletion enhanced the M2 polarization of TAMs (Figure 9). This effect could be explained considering that the Dab2 gene is transcriptionally repressed by the IFN-\(\gamma\)-responsive transcription factor IRF8 (Rosenbauer, Kallies et al. 2002). Although we do not know the meaning of this regulation, it is possible that DAB2 influences the macrophage response to IFN-\(\gamma\) and consequently, also their M1/M2 status. It should be noted that our data regarding macrophage polarization were obtained using the MCA203 tumor model, which paradoxically grew slower in Dab2\(^{-/-}\) mice with respect to C57Bl/6 littermates (Figure 2). However, injection of either MN/MCA\(_2\) or LLC/F4 tumor cells did not result in slower
tumor growth (Figure 10A and 10B), suggesting that other factors, specific of the MCA203 tumor model, concurred to alter the growth rate of tumor cells in Dab2<sup>−/−</sup> mice. Altogether these data suggest that DAB2 can potentially influence the immunologic equilibrium within the tumor. The nature of this influence could be addressed in an immunotherapy setting, for example by adoptively transferring tumor-specific cytotoxic CD8<sup>+</sup> T cells in Dab2<sup>−/−</sup> mice to monitor their survival with respect to wild type mice.

Abrogation of DAB2 functions in myeloid cells had the important consequence of hampering the metastatic potential of tumor cells, as assessed by studying two different metastatic tumor models (Figure 10). Considering the negative impact of DAB2 deficiency on macrophage differentiation, this is quite expected. However, we cannot exclude that DAB2 functionally participates to prometastatic functions of macrophages. From the invasion to the extravasation process into metastatic sites, macrophages have always proved to sustain complex interplays with metastatic cells (Joyce and Pollard 2009). These interplays require macrophages to respond to dynamically oriented stimuli in a spatial manner, thus conferring a fundamental role to the ability of the cells to orient its surface receptors and intracellular protein complexes where needed. The M-CSF/EGF paracrine loop described by Pollard and colleagues is an optimal example of this concept (Condeelis and Segall 2003; Wyckoff, Wang et al. 2004; Goswami, Sahai et al. 2005; Wyckoff, Wang et al. 2007). DAB2 could participate to this process in several ways, for example by concentrating CSF1R molecules toward extracellular M-CSF released by neighbouring tumor cells, or by docking integrins that interact with collagen fibers, thus allowing travelling through the stroma. Preliminary data obtained by our group support this hypothesis, as we found that DAB2 co-localizes with CSF1R<sup>+</sup> intracellular vesicles in bone marrow-derived macrophages and intratumoral CD11b<sup>+</sup> myeloid cells. Of interest, the DAB2 protein was found to be presented in the proteome of MDSCs isolated from metastatic mammary tumors (Boutte, McDonald et al. 2011).

In conclusion, the present study reveals DAB2 to be an appealing target to hamper the recruitment and functionality of intratumoral myeloid cells which are of support to tumor progression. Block of DAB2 functions in myeloid cells, by gene silencing or pharmacological inhibitors, should impair accumulation and function of TAMs in cancer patients, with potential benefits for disease outcome. We are currently developing
molecular strategies to efficiently deliver small interfering RNA molecules in intratumoral myeloid cells, in order to silence the *Dab2*. 
Appendix A: Gating strategy for tumor-infiltrating myeloid cells

Appendix B: Gating strategy for myeloid cells in the spleen and bone marrow
Abbreviations

AMBRA1 ................................................................. Autophagy/beclin 1 regulator 1
ANG ................................................................. Angiopoietin
APC ...................................................................... Antigen presenting cell
ARG1 ................................................................. Arginase 1
ATG ...................................................................... Autophagy-related
Atg1/ULK ............................................................ Atg1/Unc-51-like Kinase
AP2 ...................................................................... Adaptor protein 2
Bax ...................................................................... BCL2-associated X protein
Bcl2 ...................................................................... B-cell lymphoma 2
C/EBPβ ................................................................. CAAT-enhancer-binding protein-β
CCL ...................................................................... CC-chemokine ligand
CCR ...................................................................... CC-chemokine receptor
CDP ...................................................................... Common DC precursor
CMP ...................................................................... Common myeloid progenitor
COX2 ..................................................................... Cyclooxygenase 2
CSC ...................................................................... Cancer stem cell
CSF-1R .................................................................. CSF-1 receptor
CTL ...................................................................... Cytotoxic T lymphocyte
CXCL ..................................................................... CXC-chemokine ligand
CXCR ..................................................................... CXC-chemokine receptor
CX3CR1 ............................................................... CX3C chemokine receptor 1
DAB2 ................................................................ Disabled homolog 2, mitogen-responsive phosphoprotein
DAMP ................................................................ Damage-associated molecular pattern
DC ........................................................................ Dendritic cell
DFCP1 ................................................................. FYVE-containing protein 1
ECM ...................................................................... Extracellular matrix
EGF ...................................................................... Epidermal growth factor
EGR1 ................................................................ Early growth response 1
EMT ...................................................................... Epithelial-mesenchymal transition
Endoplasmic reticulum
Fibroblast growth factor
Focal adhesion kinase family interacting protein of 200 kDa
Granulocyte colony-stimulating factor
Granulocyte/macrophage colony-stimulating factor
Granulocyte-Macrophage progenitor
Hepatocyte growth factor
Hypoxia-inducible factor 1α
Hematopoietic stem cell
Inflammatory bowel disease
Intercellular adhesion molecule 1
Indoleamine 2,3-dioxygenase
Interferon γ
Interleukin
Inducible nitric oxide synthase
Interferon regulatory factor 8
c-Jun N-terminal kinase 1
Kruppel-like factor 4
Microtubule-associated protein 1 light chain 3-phospatidyl ethanolamine
Low-density lipoprotein receptor
Lysyl oxidase
Lipopolysaccharide
Macrophage colony-stimulating factor
Monocytic myeloid-derived suppressor cell
Macrophage activating factor
Metastasis-associated macrophage
mDab2-interacting protein
Macrophage/DC progenitor
Major histocompatibility complex
Matrix metalloproteinase
Target of rapamycin complex 1
Myeloblastosis oncogene
VEGFR1 ........................................................................................................ VEGF receptor 1
Vps15 ............................................................................................................. Vacuolar protein sorting
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