THE ROLE OF RGS2 IN HEMATOPOIESIS AND MYELOID DERIVED SUPPRESSOR CELLS IN CANCER

By

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CHAPTER I

INTRODUCTION

Cancer Progression and Tumor Microenvironment

Cancer is the second leading cause of death in the United States, with numbers of expected new cases and deaths rising every year. Research is directed at learning the mechanisms by which cancer progresses, and is leading us to novel treatments, but we still have a long way to go to eradicate cancer as a major cause of illness and death. Cancer progression entails several different processes, and is regulated by many different mechanisms (Figure 1) (Brooks et al., 2010; Gassmann and Haier, 2008). At the primary site, this involves tumor initiation and growth. Tissue remodeling occurs, mediated by processes such as new blood vessel growth by angiogenesis or vasculogenesis, and immune cell and bone marrow derived cell infiltration, underscoring the importance of the tumor microenvironment in cancer progression. As the tumor progresses, the tumor cells invade the surrounding tissues, and intravasate into the blood circulation. Once in the blood stream, these cells can arrest at distant sites, extravasate, and form secondary tumors, or metastases. While this process is very inefficient, with the majority of tumor cells dying before a metastatic growth can be established, the outcome of metastasis is severe, and the majority of cancer deaths are linked to metastases.

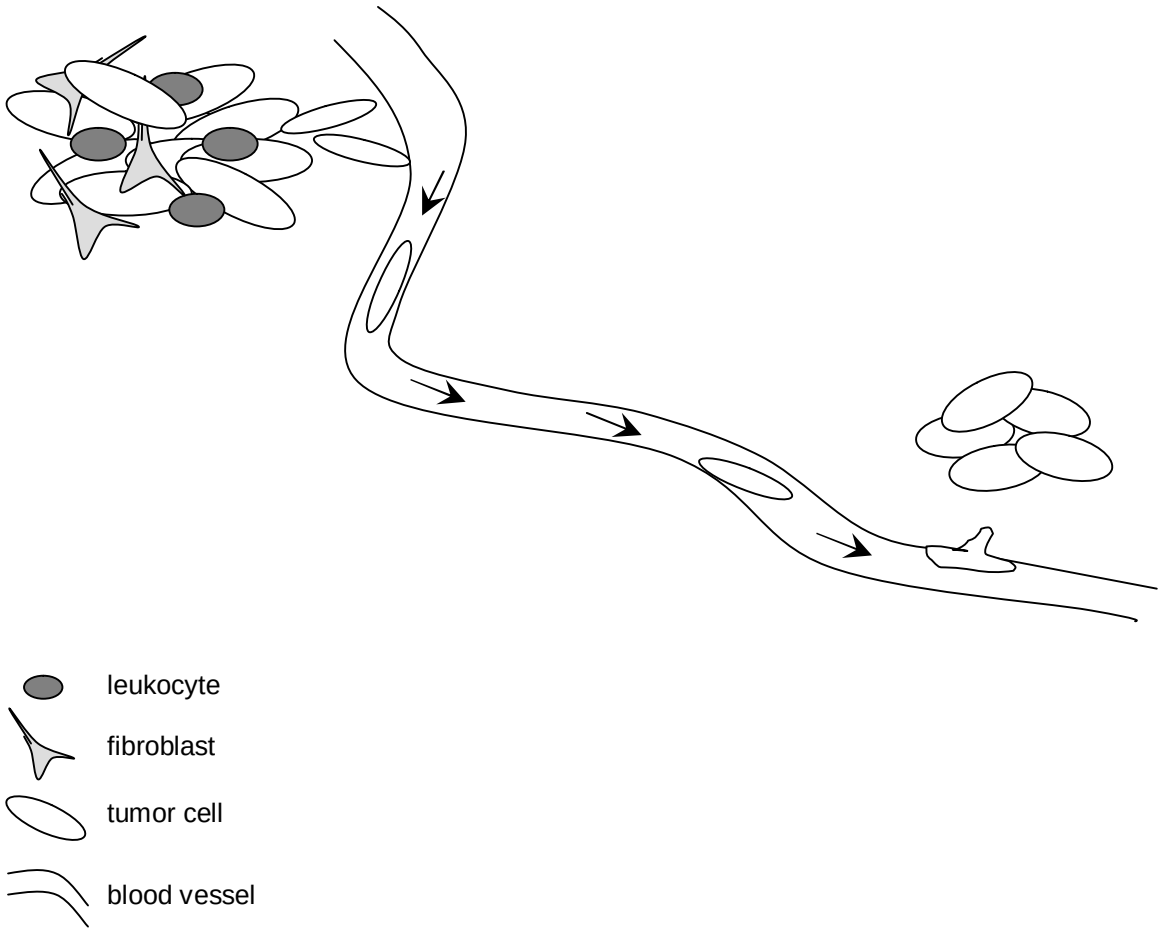

Figure 1. Model of cancer progression. At the primary site of tumor growth, the tumor cells are proliferating, immune cells are infiltrating, and other stromal cells, such as fibroblasts, are contributing to tumor growth. As the tumor grows, some tumor cells are able to invade the surrounding tissue and migrate to blood vessels. Once at a blood vessel, a tumor cell can intravasate, and move in the bloodstream to a distant site, where it can then extravasate, and move into the tissue outside of the blood vessel. There, the tumor cell can proliferate, and form a secondary tumor, or a metastatic growth.

Tumor progression is closely linked with host response, and is mediated by interactions between the many cell types in the stroma, such as leukocytes and fibroblasts, as well as the tumor cells. The tumor microenvironment also contains extracellular matrix and other factors secreted by the tumor and stromal cells that can greatly affect tumor progression. As progression occurs, the tumor cells secrete many cytokines and chemokines, leading to recruitment of leukocytes. Leukocytes that infiltrate into the tumor have been shown in many studies to be able to function in both pro-and anti-tumor fashions, indicating that they have an important role in tumor progression (DeNardo et al., 2008). While natural killer (NK) cells are in many cases tumoricidal, myeloid derived suppressor cells and T regulatory cells lead to immune tolerance. In addition, leukocytes in the tumor can secrete factors that lead to more leukocyte infiltration, as well as angiogenesis, leading to promotion of tumor growth. Infiltrating leukocytes have also been shown to be pro-metastatic through production of proteinases and cytokines, mediating intravasation of tumor cells (DeNardo et al., 2008).

Angiogenesis

As tumor cells proliferate at the primary site, the tumor quickly outgrows its nutrient and oxygen supply from nearby blood vessels. In order for the tumor to progress further, new blood vessel growth must occur. Blood vessels can form through a couple of processes. Vasculogenesis is the process by which blood vessels form *de novo*, usually involving bone marrow derived cells (BMDCs), or

endothelial progenitor cells (EPCs), that differentiate into endothelial cells and form vasculature at the site (Yancopoulos et al., 2000). Vasculogenesis occurs mostly in fetal development, but can occur in the tumor (Patenaude et al., 2010; Yancopoulos et al., 2000). More common in physiological and pathological settings in adults is angiogenesis, defined as the process of vessel formation by capillary sprouting from surrounding pre-existing blood vessels. In tumor angiogenesis, the cells comprising the nutrient starved and hypoxic tumor secrete factors which promote destabilization of existing blood vessels, endothelial cell proliferation and migration, and formation of new vessel structures, which can then provide blood supply to the growing tumor (Carmeliet and Jain, 2000; Yancopoulos et al., 2000) (Figure 2).

One major factor controlling blood vessel formation is vascular endothelial growth factor/vascular permeability factor (VEGF/VPF). VEGF, signaling through its receptor, VEGFR2, and to a smaller extent, VEGFR1, on endothelial cells, mediates endothelial cell survival and migration, as well as permeability of blood vessel structures (Ferrara, 2004). VEGF can also have an effect on hematopoiesis, promoting stem cell survival and blood cell migration (Ferrara, 2004) (Olsson et al., 2006). VEGF can be induced by hypoxia, through the transcription factor, hypoxia-inducible factor-1 (HIF-1), as well as by several growth factors and inflammatory cytokines, and is produced and secreted by both tumor and stromal cells (Ferrara, 2004; Olsson et al., 2006). While VEGFR1 signaling is not the major pathway indicated for VEGF driven angiogenesis, this signal has been shown to induce matrix metalloproteinase 9 (MMP9) production

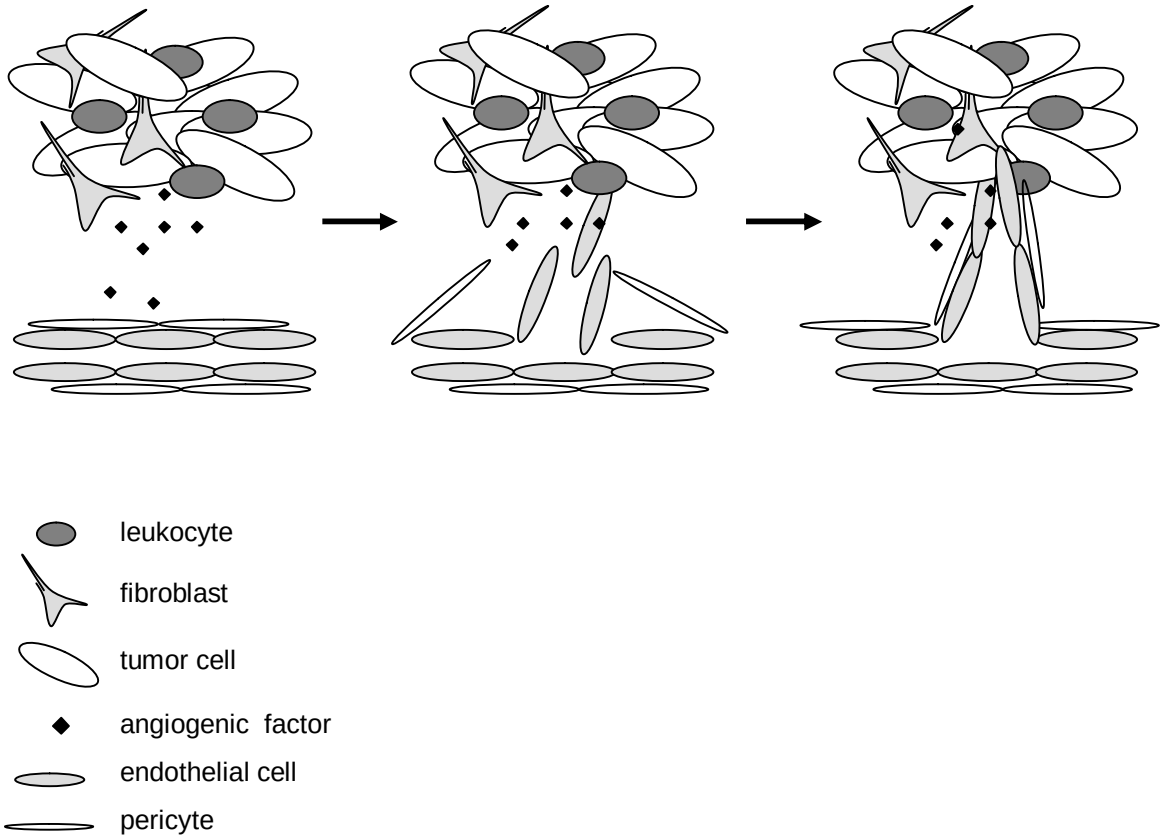

Figure 2. Model of tumor angiogenesis. As tumors grow and progress, their requirement for nutrients and oxygen, as well as waste removal, increases. The cells comprising the tumor, both tumor cells themselves and stromal cells, secrete factors such as vascular endothelial growth factor (VEGF) and angiopoietins. These factors mediate the destabilization of existing blood vessels by promoting the disassociation of pericytes from endothelial cells, endothelial cell proliferation and migration, and formation of new vessel structures. These new vessels, while abnormal in morphology, help provide blood supply to the growing tumor.

in endothelial cells (Hiratsuka et al., 2002), which could be involved in extracellular matrix remodeling, angiogenesis, and metastasis. Other factors that play a role in angiogenesis include fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), angiopoietins, and many inflammatory factors, among others (Ferrara, 2004). It is important to note that tumor blood vessels are structurally and functionally abnormal. They are generally leaky, enlarged, and tortuous when compared to normal blood vessels (Carmeliet and Jain, 2000). In addition, they often lack mature pericytes, indicating that the tumor vessels are unstable. The vascular network can also be used by tumor cells as a way to spread. As the tumor cells become invasive, they migrate towards the vessels, and intravasate into the bloodstream, where they can then move to distant locations and form metastases. Without the process of angiogenesis, tumor progression could not occur. Also important in tumor progression is lymphangiogenesis. Lymphangiogenesis describes the process of capillary sprouting from existing vessels made up of lymph endothelial cells (Werynska et al., 2009). These vessels support the lymph system, which is known to be important for metastasis, as tumor cells can also exit the tumor via lymphatic vessels (Sleeman et al., 2009; Werynska et al., 2009). Similar molecules regulate lymphangiogenesis, including VEGF receptor 3 (VEGFR3), VEGF-C, VEGF-D, FGF, and angiopoietins (Olsson et al., 2006; Sleeman et al., 2009; Werynska et al., 2009).

Many factors and types of cells can play a role in angiogenesis, particularly within a tumor setting. Under normal conditions, inhibitory factors

balance the angiogenic factors, keeping the endothelial cells quiescent and preventing angiogenesis from occurring (Carmeliet and Jain, 2000). Once a tumor has reached the point of needing new blood vessels, this balance shifts, throwing the “angiogenic switch” on. Infiltrating immune cells are major players in turning on the “angiogenic switch.” Monocytes, macrophages, neutrophils, etc., have been shown to promote vascularization of tumors by secreting factors such as VEGF, FGF, and TGF-β, all of which potently affect endothelial cells (Carmeliet and Jain, 2000; Coffelt et al., 2010; Murdoch et al., 2008; Okamoto et al., 2005; Sunderkotter et al., 1994). Further, there is a subset of monocytes that express Tie2, a receptor for angiopoietins, which have been shown to provide paracrine support for angiogenesis (Coffelt et al., 2010; Murdoch et al., 2008). These infiltrating cells also produce and secrete proteases, such as MMP-9, which enhance matrix remodeling, and therefore VEGF bioavailability and vascular remodeling associated with angiogenesis (Coffelt et al., 2010; Murdoch et al., 2008; Nozawa et al., 2006). In addition, infiltrating leukocytes produce inflammatory factors, such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), which also have been shown to promote angiogenesis (Coffelt et al., 2010; Kofler et al., 2005; Murdoch et al., 2008; Sunderkotter et al., 1994).

Immune response

The immune response to tumors mediated by leukocytes plays a major role in tumor progression. There are two major types of immune response

studied in response to cancer, although it is becoming clear that more types occur (DeNardo and Coussens, 2007; DeNardo et al., 2008; Tan and Coussens, 2007). The first is a type 1 response. A type 1 immune response is theorized to have developed to help combat intracellular pathogens such as viruses. As such, this type of immune response promotes cell killing. This response is mediated by CD4+ T helper cells (T_H1 cells) that produce factors such as IFNγ. Macrophages have an M1 phenotype, and produce cytokines such as IL-12 to help promote this response from T_H1 cells. CD8+ cytotoxic T lymphocytes (CTLs) and B cells respond by secreting lytic enzymes or antibodies, respectively, leading to killing of the tumor cell. The type 1 immune response is able to keep the tumor in check, if not lead to overall rejection altogether.

The immune response most seen under tumor conditions is the type 2 response (DeNardo and Coussens, 2007; DeNardo et al., 2008; Tan, 2007). This response is theorized to have developed in order to combat parasites and extracellular pathogens. It has been shown to be involved in wound healing, where a large amount of tissue remodeling must occur. In this response, innate cells, such as macrophages and myeloid derived suppressor cells (MDSCs), secrete cytokines, for example IL-10, which lead to polarization of T helper cells to a type 2 phenotype (T_H2). These T_H2 cells secrete cytokines such as IL-4, IL-6, and IL-13, which mediate the type 2 response. Macrophages acquire an M2 phenotype, where they secrete proteases and factors that support tumor growth and angiogenesis (Balkwill et al., 2005; Pollard, 2004). CTLs are inhibited, and a population of T cells that is immune suppressive, the T regulatory cells (Tregs),

accumulates. B cells produce large amounts of antibodies; however, these immunoglobulins form complexes that accumulate in the extracellular matrix, promoting recruitment of more leukocytes instead of leading to killing of tumor cells. Overall, these cells promote an environment of tolerance, considered to be anti-inflammatory, which leads to tumor promotion and progression.

Myeloid Derived Suppressor Cells

Immune response is a key factor in host response to tumor growth. Ideally, immune cells, such as NK cells and cytotoxic T cells should attack tumor cells. However, there is mounting evidence suggesting that tumors can “reprogram” immune response and produce an environment in which immune cells promote tumor progression. Myeloid derived suppressor cells (MDSCs) play an integral role in modulating the immune response to tumor cells, creating an environment to facilitate tumor progression (Figure 3). MDSCs increase in tumor bearing hosts, including human patients, at the expense of more mature cells, such as dendritic cells (Almand et al., 2001; Kusmartsev et al., 2000; Melani et al., 2003; Prins et al., 2002; Solheim et al., 2007; Yang et al., 2004). Accumulation has been shown to be mediated by inflammatory and angiogenic factors, for example, GM-CSF, PGE_2, IL-1β, and VEGF (Gabrilovich et al., 1998; Melani et al., 2003; Serafini et al., 2004a; Sinha et al., 2007b; Solheim et al., 2007; Varga et al., 2008; Bunt et al., 2006; Bunt et al., 2007; Huang et al., 2007b), and is likely due in part to Stat3 activity (Gabrilovich and Nagaraj, 2009). Stat1, Stat6, and NF-κB have all been implicated in the function of MDSCs (Gabrilovich and Nagaraj, 2009). These cells

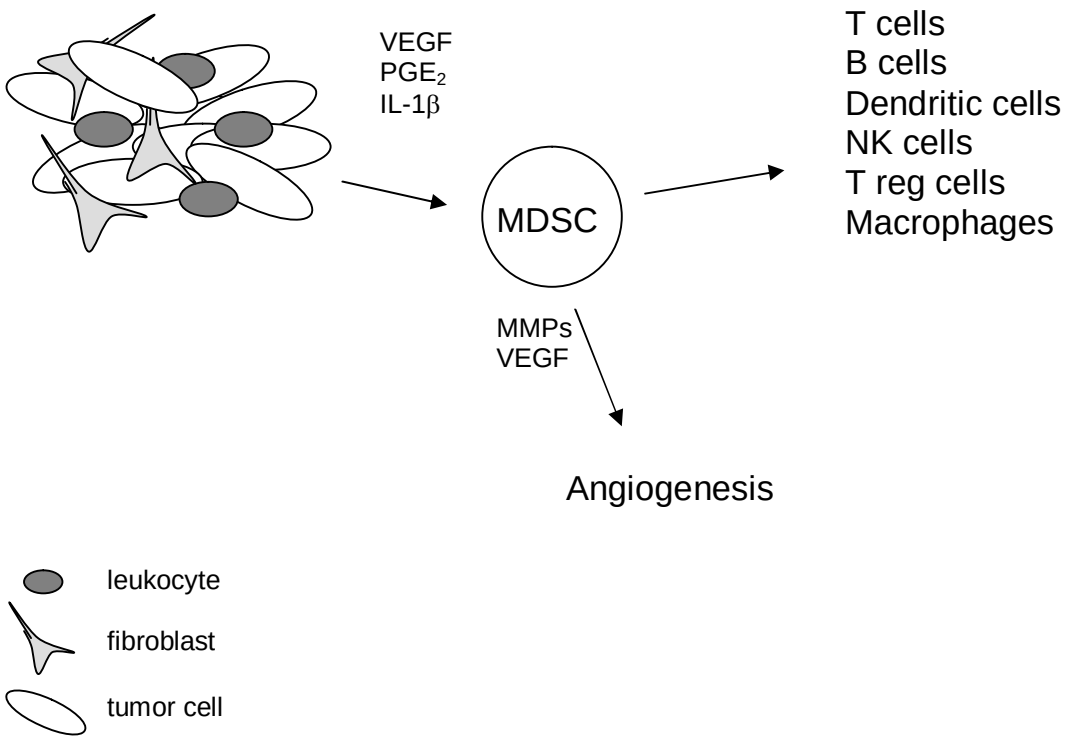

Figure 3. Role of MDSCs in tumor progression. Cells within the tumor secrete factors which lead to the accumulation of MDSCs. MDSCs can then affect immune response by inhibiting activation of T cells, B cells, NK cells, and dendritic cells. They can also mediate the accumulation of T regulatory cells, and promote a type 2 response from macrophages, further promoting immune tolerance of the tumor. MDSCs also secrete MMPs and factors such as VEGF, which can then enhance vascular remodeling and angiogenesis. In addition, remodeling of the matrix by MMPs secreted by MDSCs can enhance metastasis.

can promote tumor vascularization and enhance metastasis (Murdoch et al., 2008; Shojaei et al., 2007a; Yang et al., 2004; Yang et al., 2008). Furthermore, MDSCs accumulate in response to some tumor vaccine models in mice, promoting tumor progression and negating the effects of the vaccines, and can also play a role in refractoriness to anti-VEGF tumor therapy (Prins et al., 2002; Serafini et al., 2004a; Shojaei et al., 2007a).

MDSCs are composed of a heterogeneous population of Gr-1+CD11b+ immature cells in mice, and can be differentiated into various types of cells of hematopoietic origin, including dendritic cells and granulocytes (Bronte et al., 2000; Kusmartsev et al., 2003; Kusmartsev and Gabrilovich, 2002; Kusmartsev and Gabrilovich, 2005; Kusmartsev et al., 2005; Kusmartsev et al., 2000; Mazzoni et al., 2002; Van Ginderachter et al., 2006). Gr-1 expression is considered to be a marker of granulocytic differentiation, with expression increasing as granulocytes differentiate; however, its function is still under investigation. CD11b is the α_M integrin subunit of the complement receptor 3 (CR3), also known as Mac-1. In humans, the markers used to distinguish MDSCs are not as clear cut, especially since humans do not express Gr-1. Different studies have highlighted human cells with immunosuppressive function from tumor patients ranging from immature dendritic cells, to monocytes, to immature polymorphonuclear cells (Almand et al., 2001; Almand et al., 2000; Peranzoni et al., 2010). Several studies have attempted to assign particular functions to particular subsets. Most of the research has focused on Ly6C+CD11b+ versus Ly6G+CD11b+ subsets in mice. The antibody against the Gr-1 antigen

recognizes two different cell surface molecules, Ly6C, which is found on the mononuclear subset of MDSCs (MO-MDSCs), and Ly6G, which is found on the polymorphonuclear subset of MDSCs (PMN-MDSCs). Two separate studies determined that although both subsets have immune suppressive activity, they use different mechanisms, and the amount of MDSCs and proportions of MO-MDSCs and PMN-MDSCs is dependent on the specific tumor (Movahedi et al., 2008; Youn et al., 2008).

Moreover, another subset of these cells has been shown to express CD31, a marker for endothelial cells (Bronte et al., 2000), and when under angiogenic conditions, some MDSCs can be transdifferentiated into endothelial cells, indicated by cobblestone shape, VEGFR2 and VE-cadherin expression, and low-density lipoprotein uptake (Yang et al., 2004). This is not surprising as endothelial cells and hematopoietic cells are thought to have a common precursor (Choi et al., 1998; Park et al., 2005; Shaw et al., 2004). Also, bone marrow-derived cells, in particular myeloid cells, have been shown to become endothelial cells (Bailey et al., 2006; Conejo-Garcia et al., 2004; Coukos et al., 2005; Fernandez Pujol et al., 2000; Fernandez Pujol et al., 2001; Fujiyama et al., 2003; Graf, 2002; Harraz et al., 2001; Lyden et al., 2001; Schmeisser et al., 2001; Young, 2004). Alternatively, it has been shown that bone marrow cells contribute to the formation of vasculature through acting as pericytes and providing paracrine signals (De Palma et al., 2005; Rajantie et al., 2004). As mentioned above, MDSCs are an immature population of cells, and when these cells increase, mature myeloid cells decrease. When MDSCs were led to differentiate

into dendritic cells in mice *in vivo*, immune response improved (Li et al., 2004). In addition, in mice and human patients, treatment with all-trans retinoic acid (ATRA), a factor that induces differentiation, led to a decrease in immature myeloid cells while enhancing dendritic and T cell responses (Kusmartsev et al., 2003; Mirza et al., 2006). Thus, determining the mechanisms of differentiation and function of these cells may have an impact on patient survival.

MDSCs play an important role in tumor angiogenesis. MDSCs promote increased vascularization in tumors, leading to decreased apoptosis of tumor cells, and decreased hypoxic and necrotic regions in tumors (Yang et al., 2004). This correlates with increased tumor burden (Yang et al., 2004). The pro-angiogenic function of MDSCs has been linked in part to matrix metalloprotease-9 (MMP9) expression; MMP9 expression led to increased VEGF/VEGFR2 association, suggesting that the MDSC produced MMP9 enhanced the bioavailablilty of VEGF (Yang et al., 2004). Furthermore, culturing endothelial cells on a collagen matrix with MDSCs led to increased tube formation compared to endothelial cells alone (Kujawski et al., 2008). Kujawski et al. determined that this pro-angiogenic function of MDSCs was due to Stat3-dependent production of VEGF and basic FGF, both of which are potent angiogenic factors (Kujawski et al., 2008). These studies suggest that MDSCs may contribute to angiogenesis by production of factors which add to or alter the tumor microenvironment in a pro-angiogenic manner.

MDSCs are known to inhibit immune response by affecting other immune cells, including T cells, macrophages, NK cells, dendritic cells, and T regulatory

cells (Almand et al., 2001; Bronte et al., 2000; Bronte et al., 2003a; Bronte et al., 2003b; Bronte et al., 1998; Ezernitchi et al., 2006; Gabrilovich and Nagaraj, 2009; Kusmartsev and Gabrilovich, 2005; Kusmartsev et al., 2005; Kusmartsev et al., 2004; Liu et al., 2007; Mazzoni et al., 2002; Melani et al., 2003; Serafini et al., 2006a; Serafini et al., 2004a; Serafini et al., 2004b; Serafini et al., 2006b; Serafini et al., 2008). Gr-1+CD11b+ cells mediate apoptosis of CD8+ T cells, and IL-4 seems to promote this activity of MDSCs (Bronte et al., 2000; Bronte et al., 2003a; Bronte et al., 1998). One study has even shown that MDSCs can process and present antigen in the tumor to T cells, but instead of activating the T cells, they promote anergy, and therefore immune tolerance (Kusmartsev et al., 2005). MDSCs are known to express high levels of two enzymes, arginase 1 and inducible nitric oxide synthase (iNOS), which both use L-arginine as a substrate. Arginase 1 is induced by IL-4 or activated T_H2 cells, and iNOS is induced by IFNγ or T_H1 cells, indicating that a mixture of immune reactions occurs (Bronte et al., 2003a). Kusmartsev et al. also found that IFNγ plays a role in the inhibition of T cells by MDSCs (Kusmartsev et al., 2000). The resulting lack of L-arginine in the microenvironment leads to decreased T cell proliferation (Bronte et al., 2003a; Bronte et al., 2003b). In addition, a recent study has shown that MDSCs are able to deplete the microenvironment of other amino acids necessary for immune cell function (Srivastava et al., 2010). MDSCs make high levels of reactive oxygen species (ROS), nitric oxide (NO), and peroxynitrite, which can then affect the surrounding cells (Bronte et al., 2003b; Kusmartsev and Gabrilovich, 2003; Kusmartsev et al., 2004; Kusmartsev et al., 2000). Peroxynitrite can lead to

nitration and nitrosylation of several amino acids, which can affect protein function in target cells. For example, high levels of peroxynitrite led to nitration of the T cell receptor (TCR) and inhibited signaling (Nagaraj et al., 2007). MDSCs can also mediate the down-regulation of the ζ chain of the TCR, leading to immune suppression (Bronstein-Sitton et al., 2003; Ezernitchi et al., 2006). In addition, one study found that MDSCs upregulated heme-oxygenase-1 and IL-10 in response to lipopolysaccharide stimulation, mediating the immune suppressive activity of MDSCs (De Wilde et al., 2009).

MDSCs have been shown to present antigen to T regulatory cells, promoting their expansion and immune tolerance in an arginase-dependent manner (Serafini et al., 2008). Data indicate that MDSCs are able to inhibit NK cell cytotoxicity in a cell-cell contact dependent manner (Liu et al., 2007). Cell-cell contact has also been found to be necessary for T cell activity inhibition (De Wilde et al., 2009; Ezernitchi et al., 2006; Mazzoni et al., 2002). Sinha et al. showed that reducing the numbers of MDSCs and increasing levels of M1 macrophages could promote the rejection of metastases (Sinha et al., 2005). MDSCs produce IL-10, which along with a cell-cell contact dependent mechanism, leads to decreased production of IL-12 by macrophages, resulting in the skewing of immune response from an anti-tumor type 1 response, to a pro-tumor type 2 response (Sinha et al., 2007a). Furthermore, some data indicate that MDSCs can differentiate into tumor associated macrophages, which can also mediate death of T cells and immune suppression (Kusmartsev and Gabrilovich, 2005).

In addition to affecting prognosis by differentiating the MDSCs, several treatments appear promising. Gemcitabine, a nucleoside analog used for chemotherapy, selectively reduces the number of MDSCs in tumor bearing mice, leading to increased immune response to the tumor cells (Ko et al., 2007; Suzuki et al., 2005). Sunitinib, a tyrosine kinase inhibitor, reduced MDSC levels and enhanced immune response to tumors in human patients, suggesting that targeting the factors that lead to accumulation of MDSCs would be therapeutic (Ko et al., 2009). PGE_2 has also been implicated in MDSC function, leading to upregulation of arginase 1 (Rodriguez et al., 2005). Accordingly, cyclooxygenase-2 (COX-2) inhibitors can abrogate production of arginase 1, and enhance immune response (Talmadge et al., 2007; Zea et al., 2005). In addition, phosphodiesterase 5 inhibitors, such as sildenafil, are able to decrease the production of both arginase 1 and iNOS, again, blocking the tumor-promoting functions of MDSCs and enhancing immune response in the mice (Serafini et al., 2006b). Another drug, nitroaspirin, shown to inhibit production of reactive oxygen species, also inhibits arginase 1 and iNOS, and therefore MDSC suppressive function (De Santo et al., 2005). Several of the drugs that have been found to target MDSCs are already in use for cancer treatment. When used in combination with other tumor therapies, these drugs should not only affect the tumor cells themselves, but also prevent the tumor enhancing capabilities of MDSCs, leading to a better prognosis for the patient.

Monocyte Chemoattractant Protein – 1

Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, is a chemokine important for cell migration (Loetscher et al., 1996; Melgarejo et al., 2009; Rollins, 1997; Rollins et al., 1991). It signals through CCR2, a G protein coupled receptor found on monocytes, endothelial cells, T cells, etc. (Loetscher et al., 1996; Melgarejo et al., 2009; Salcedo et al., 2000) (Figure 4). Early studies correlated higher levels of MCP-1 with inflammatory conditions, such as arthritis, fibrosis in asthma patients, infections, etc., and found that inflammatory factors such as IL-1β and TNFα, which as mentioned above are found produced under tumor conditions, induce MCP-1 expression (Proost et al., 1996). Increased MCP-1 production in tumors has been found to be associated with increased tumor progression, and has been suggested to play a role in metastasis, by acting on stromal cells such as macrophages, as well as the tumor cells themselves (Craig and Loberg, 2006; Loberg et al., 2006; Raman et al., 2007). The MCP-1/CCR2 chemokine axis is implicated in the recruitment of myeloid suppressor cells, likely similar to MDSCs, to tumors (Huang et al., 2007a). In addition, MCP-1 is a potent angiogenic factor, promoting angiogenesis through indirect effects, such as through monocyte migration or induction of molecules like VEGF and MMP9 (Goede et al., 1999; Raman et al., 2007; Varney et al., 2005), or by directly functioning on endothelial cells (Salcedo et al., 2000). Furthermore, blocking MCP-1 with a neutralizing antibody inhibited angiogenesis, and led to decreased tumor metastases and increased survival in a mouse tumor model (Salcedo et al., 2000).

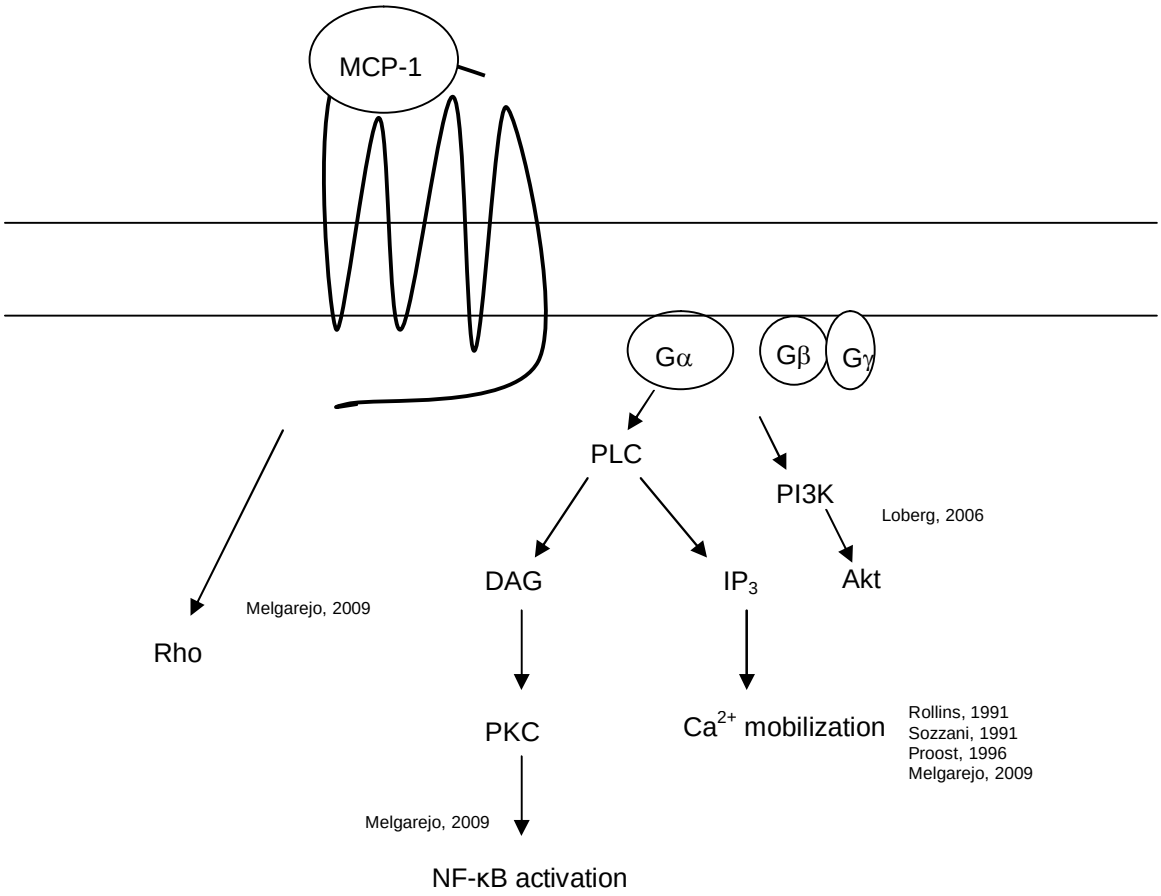

Figure 4. MCP-1 signaling through CCR2. MCP-1 binds to CCR2 as a dimer, and activates downstream signaling. CCR2 has been shown to couple to $G\alpha_q$ and $G\alpha_i$ proteins. Among the pathways known to be activated by MCP-1/CCR2 signaling are Rho, PKC, NF-κB, Ca^{2+} mobilization, and Akt pathways. References are indicated for the signaling pathways.

While MCP-1 is known to mediate cell migration, studies also report an important role of MCP-1 in modulating immune response. Mice deficient in MCP-1 are not able to mount a T_H2 response (Gu et al., 2000). In contrast, mice deficient in the receptor for MCP-1, CCR2, exhibit both reduced T_H1 and T_H2 responses, and are considered to be defective in T_H1 immunity (Boring et al., 1997; Conti and Rollins, 2004; Warmington et al., 1999). However, MCP-1 is not the only chemokine that can signal through the CCR2 receptor, suggesting that these other chemokines may be playing a role with CCR2 in determining immune response (Luther and Cyster, 2001). Blocking or knocking out MCP-1 in several models led to the decrease of T_H2 mediators, particularly IL-4 (Gonzalo et al., 1998; Gu et al., 2000; Lu et al., 1998), and induction or addition of MCP-1 led to decreased T_H1 and increased T_H2 responses (Karpus et al., 1998; Karpus et al., 1997). Due to the effects of MCP-1 on tumor cells, immune cell infiltration, type of immune response induced, and angiogenesis within a tumor, this molecule is likely an important player in the tumor microenvironment.

Regulator of G protein Signaling Family of Proteins

Signaling through G protein coupled receptors (GPCRs) mediates many responses within the cell. GPCRs are seven transmembrane-domain receptors that are coupled to intracellular heterotrimeric guanine nucleotide binding proteins (G proteins) (McCudden et al., 2005). The heterotrimer consists of 3 subunits: Gα, Gβ, and Gγ. Gα in its inactive state is bound by GDP. When a signal is received by the GPCR, it acts as a guanine nucleotide exchange factor

(GEF), leading to the exchange of GDP for GTP. The conformational change instigated by bound GTP then leads to the dissociation of Gβγ from Gα, and both are now free to interact with effector proteins. The Gα subunit has intrinsic GTPase activity, resulting in the hydrolysis of GTP to GDP, and the subsequent reassociation of the Gα and Gβγ subunits and cessation of signaling. GPCR signaling is tightly regulated by several mechanisms.

Regulator of G protein signaling (RGS) proteins act as GTPase-activating proteins (GAPs), enhancing the intrinsic GTPase activity of the Gα subunit, and thereby decreasing the time that the G protein subunits are dissociated (De Vries and Gist Farquhar, 1999; De Vries et al., 2000; Hollinger and Hepler, 2002; Ross and Wilkie, 2000). These proteins function through their RGS domain, a conserved 128 amino acid domain (Burchett, 2003; Ross and Wilkie, 2000). The RGS domain functions to stabilize the transition state of Gα-GTP by stabilizing the switch regions of Gα, promoting hydrolysis (Srinivasa et al., 1998; Tesmer et al., 1997). The proteins in this family, approximately 30 proteins, all contain the RGS domain, but they are further classified into subfamilies based on structures outside of the RGS domain, with the larger RGS proteins containing domains for interactions with other proteins. Rgs2 is one of the smaller RGS proteins, consisting of the RGS domain with short N- and C-terminal extensions (Kehrl and Sinnarajah, 2002).

Regulator of G protein signaling-2

Rgs2 was first discovered through a screen of blood mononuclear cells treated with lectin and cyclohexamide in an attempt to discover genes which may act as regulators of the transition from G0 to G1 (G0 switch genes). Siderovski et al. found that the mRNA of this gene accumulated in stimulated cells, and named the gene G0S8 (Siderovski et al., 1990; Siderovski et al., 1994). The gene was found to encode a protein product of 211 amino acids derived from 5 exons, generating a basic, hydrophilic protein with a molecular weight of about 24 kDa (Figure 5A). Through sequence analyses and protein database comparisons, this gene was predicted to code for a putative basic helix-loop-helix (bHLH) protein with several potential phosphorylation sites (Siderovski et al., 1994). In 1996, several groups independently discovered that proteins with a conserved domain, the RGS domain, could function as GAPs for Gα subunits; G0S8 was found to belong to this group of proteins and was renamed Rgs2 (Druey et al., 1996; Koelle and Horvitz, 1996; Siderovski et al., 1996).

Expression of Rgs2

Rgs2 is known to be widely expressed, with mRNA detected in multiple murine organs, including the heart, brain, spleen, lung, kidney, white blood cells, bone marrow, adrenal gland, ovary, prostate, bladder, uterus, brown fat, and murine bone marrow-derived dendritic cells (Chen et al., 1997; Park et al., 2002; Reif and Cyster, 2000) (Shi et al., 2004; Su et al., 2002). In human tissues, Rgs2 mRNA was found in human monocyte-derived dendritic cells, blood cells, B cells,

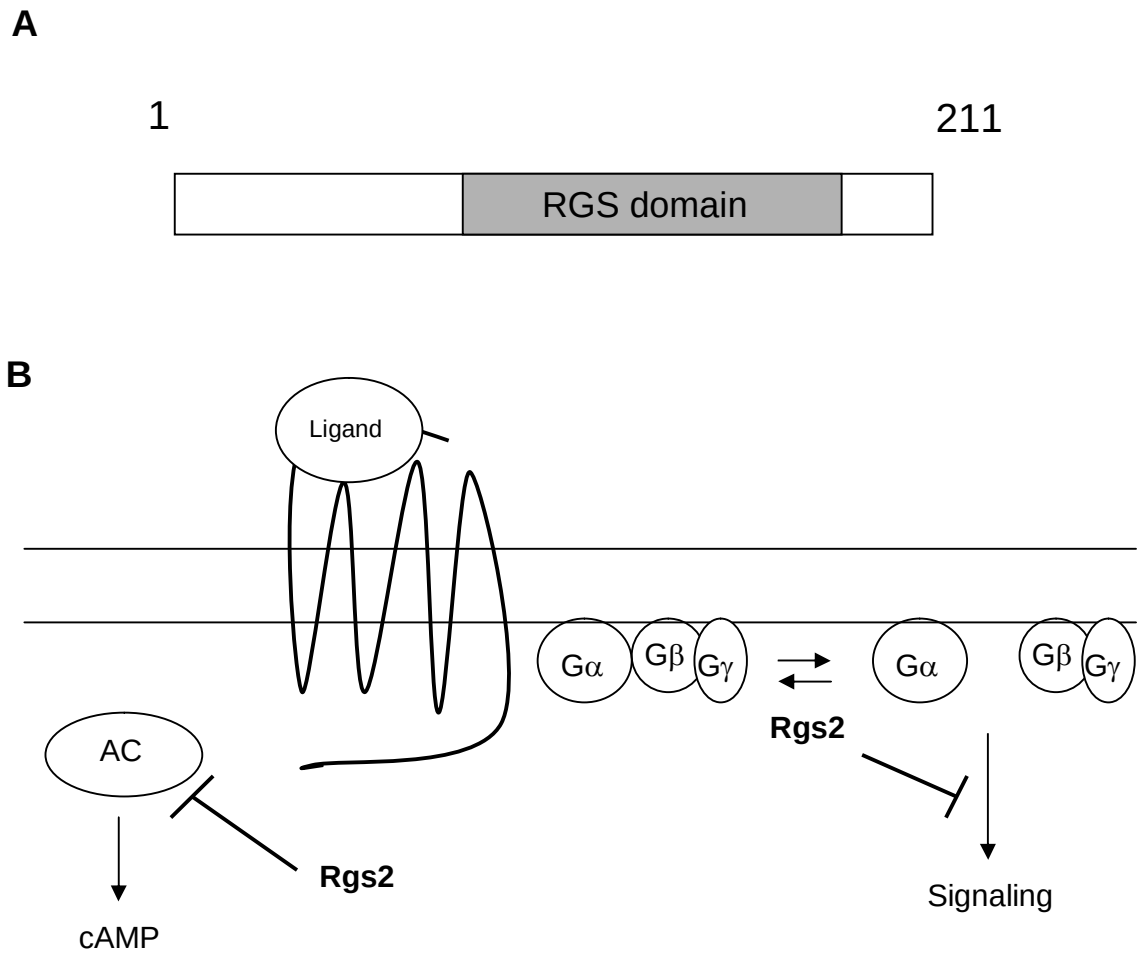

Figure 5. Role of Rgs2 in GPCR signaling. (A) Scheme of Rgs2 protein. Rgs2 is one of the smaller members of the Regulator of G Protein Signaling (RGS) family, comprised of 211 amino acids with a molecular weight of approximately 24 kDa. Outside of its RGS domain, shown in gray, it has a small N-terminal region, and even smaller C-terminal region. (B) Diagram of Rgs2 in signaling. Rgs2 functions on Gα subunits, enhancing the intrinsic GTPase activity of the subunit, leading to decreased time that the trimer is dissociated, and therefore leading to cessation of signaling. Rgs2 can also directly inhibit some forms of adenylyl cyclase (AC), preventing cAMP production.

NK cells, dendritic cells, myeloid cells, uterus, ovary, prostate, pancreatic islets, pituitary gland, and adrenal gland (Shi et al., 2004; Su et al., 2002). In addition, Rgs2 mRNA levels are lower in bone marrow cells of AML patients compared to bone marrow of normal individuals and in common and blastoid variant mantle cell lymphoma compared to B cells from normal individuals (Larramendy et al., 2002; Schwable et al., 2005; Zhu et al., 2002).

Rgs2 functions in a negative feedback loop with regard to GPCRs; ligand stimulation of the GPCR leads to increased expression of Rgs2, which then attenuates signaling through the GPCR (De Vries and Gist Farquhar, 1999). A summary of stimuli that induce Rgs2 in various cell types and cell lines can be found in Table 1. Rgs2 has been shown to be transcriptionally regulated, and expression is rapidly induced, but quite transient (Grant et al., 2000; Song et al., 1999; Tsingotjidou et al., 2002). Several reports indicate mRNA stabilization is not involved (Heximer et al., 1997a; Homme et al., 2003). There is no evidence that alternative splicing of Rgs2 occurs; however, Gu et al. have reported alternative translation sites producing different sized proteins (Gu et al., 2008). While Rgs2 is expressed in a wide variety of cell and tissue types, the signals involved in regulation appear to vary between cell types, and perhaps even between primary cells and cell lines.

Heximer et al. found no response of Rgs2 mRNA with TPA stimulation in isolated human blood mononuclear cells, which is in contrast to the results of Schwable et al., which demonstrated that Rgs2 mRNA levels were affected by

Table 1. Upregulation of Rgs2 mRNA. The signals that stimulate upregulation are listed with cell type. Con A- concanavalin A, PMA- Phorbol 12-Myristate 13-Acetate, PGE- prostaglandin E, ATRA- all-trans retinoic acid, DMSO- dimethyl sulfoxide, PTH- parathyroid hormone, PTHrP- parathyroid hormone-related peptide, PB- peripheral blood, S1P- sphingosine-1-phosphate, TLR3- toll like receptor 3

Cell Type	Signal	References
Human blood mononuclear cells	Lectin, Con A, ionomycin (not PMA)	Heximer et al., 1997a Siderovski et al., 1990 Siderovski et al., 1994
Human PB T cells	cAMP (decreased by IL-2)	Beadling et al., 1999
U937, HL-60, NB4 cell lines	PGE_1, PGE_2, ATRA, PMA, DMSO	Beadling et al., 1999 Schwable et al., 2005
Murine B cells	activation by antigen	Reif and Cyster, 2000
Bone marrow derived macrophages	poly(I:C) TLR3 activation	Riekenberg et al., 2009
Rat primary cardiomyocytes	Forskolin, isoproterenol, PMA, phenylephrine/timolol	Hao et al., 2006 Zou et al., 2006
Rat vascular smooth muscle cells	angiotensin II, PMA, S1P	Grant et al., 2000 Hendriks-Balk et al., 2009
human primary thyroid cells	thyrotropin	Eszlinger et al., 2004
PC12, 1321N1, SH-SY5Y cell lines	Forskolin (not SH-SY5Y cells), carbachol, isoproterenol, PMA	Pepperl et al., 1998 Zmijewski et al., 2001b Song et al., 1999
Rat brain tissue	Neuronal stimuli, amphetamine	Burchett et al., 1998 Ingi et al., 1998
Rat C6 astrocytoma cell line, primary astrocytes	Isoproterenol	Kim et al., 2006
Primary murine osteoblasts	PTH, Forsklin, PMA, PTHrP, ATP	Tsingotjidou et al., 2002 Roy et al., 2006b
Rat bone and primary osteoblasts	PTH, PTHrP, PGE_2	Miles et al., 2000
ROS 17/2.8, MC3T3-E1, Py1a, UMR 106-01 cell lines	PTH, forskolin, dibutyryl cAMP	Miles et al., 2000 Tsingotjidou et al., 2002 Homme et al., 2003

TPA stimulation in myeloid cell lines (Heximer et al., 1997a; Schwable et al., 2005). This indicates that freshly isolated cells may have different expression of Rgs2 than cell lines. Heximer et al. also found that Rgs2 levels were responsive to ionomycin stimulation, showing a transient increase with stimulation by the calcium ionophore, suggesting that Rgs2 mRNA expression may be regulated by calcium dependent mechanisms.

Similar to other studies, Beadling et al. found that Rgs2 mRNA levels are increased in human peripheral blood T cells that have undergone the G0/G1 transition (Beadling et al., 1999; Siderovski et al., 1990; Siderovski et al., 1994). Also, IL-2 and cAMP levels within the cell have opposite effects on Rgs2 levels; IL-2 decreases the response of Rgs2 mRNA, and subsequent increase in intracellular cAMP can increase Rgs2 mRNA (Beadling et al., 1999). Stimulation of U937 cells with PGE_1 and PGE_2 increases Rgs2 expression while blocking proliferation of these cells. Treatment of PC12 cells with forskolin to increase cAMP levels led to an increase in Rgs2, but treatment with ionomycin had no effect on Rgs2 levels, in contrast to the study above (Pepperl et al., 1998).

Hao et al. demonstrated that $G\alpha_q$ signaling and increased intracellular cAMP (by forskolin stimulation) can lead to increased expression of Rgs2 mRNA in cardiomyocytes (Hao et al., 2006). Zmijewski et al. found that Rgs2 levels are increased in a rapid and transient manner in 1321N1 cells by stimulation of the inositol signaling pathway and by increased cAMP (Zmijewski et al., 2001b). Reif et al. found that *in vivo* activation of B cells led to increased Rgs2 mRNA expression (Reif and Cyster, 2000). Ingi et al. found that Rgs2 mRNA is

upregulated in brain neurons in response to synaptic activity (Ingi et al., 1998). Parathyroid hormone induced Rgs2 mRNA in UMR 106-01 cells by a cAMP/PKA pathway, and pretreatment with vitamin D enhanced this effect (Homme et al., 2003). Thyrotropin was found to increase Rgs2 mRNA in primary human thyroid cells, which could inhibit thyrotropin receptor mediated IP_3 accumulation, but had no affect on cAMP accumulation (Eszlinger et al., 2004). Sphingosine-1-phosphate (S1P) treatment of primary rat vascular smooth muscle cells led to increased Rgs2 mRNA levels, likely through S1P receptor 2, but not through $G\alpha_{i/o}$ (Hendriks-Balk et al., 2009).

Song et al. found that Rgs2 mRNA levels increase in SH-SY5Y cells upon stimulation of muscarinic receptors through a protein kinase C (PKC) dependent mechanism (Song et al., 1999); when PKC activity was inhibited, the increase in Rgs2 mRNA was blocked. Stimulation with PMA, which leads to the activation of PKC, led to induction of increased Rgs2 mRNA expression, which was also blocked by inhibition of PKC activity. Rgs2 levels increased rapidly due to increased transcription, much like in other studies. However, unlike other studies, levels remained high for a long period of time. In direct contrast with other studies, forskolin-induced increase in intracellular cAMP did not increase Rgs2 mRNA levels in these cells (Song et al., 1999). Heat shock leads to a rapid and transient increase in Rgs2 mRNA levels in SH-SY5Y cells, indicating that cell stress can regulate Rgs2 levels (Song et al., 2001). After differentiation of SH-SY5Y cells, Rgs2 levels decreased. Treatment of the differentiated SH-SY5Y cells with

carbachol, PMA, or heat shock led to subsequent increases in Rgs2 levels. Again, forskolin treatment had no effect.

Toll-like receptor (TLR) signaling can also modulate Rgs2 levels. In bone marrow derived macrophages and a macrophage cell line, stimulation and activation of TLR2 and TLR4 with lipopeptides or lipopolysaccharides, or stimulation of TLR9 with CpG oligo-nucleotide, led to downregulation of Rgs2 mRNA levels (Riekenberg et al., 2009). However, stimulation of TLR3 with poly(I:C) increased Rgs2 mRNA levels (Riekenberg et al., 2009). Furthermore, LPS stimulation of RAW264.7 macrophages with LPC led to decreased Rgs2 mRNA levels through TLR4 signaling involving phospholipase D2 (PLD) and protein kinase C-η (PKC) (Lee et al., 2010). Activation of natural killer cell receptors Ly49A or Ly49D both led to increased Rgs2 mRNA and protein (Kveberg et al., 2005). These data suggest that Rgs2 may have a role outside of GPCR signaling, or that it is upregulated in response to other signals in order to provide crosstalk with GPCR signaling.

Cellular localization of Rgs2

RGS proteins localize to various compartments within the cell, which has been postulated to be a mechanism by which RGS protein function is regulated (De Vries et al., 2000). Rgs2 has been shown to localize predominantly in the nucleus, with some localization at the plasma membrane. Zmijewski et al. determined that Rgs2 is mainly localized to the nucleus in 1321N1 cells, examining endogenous expression by immunocytochemistry and cell

fractionation, and exogenous expression of a tagged Rgs2 by fluorescent and confocal microscopy (Zmijewski et al., 2001b). Rgs2 could also be seen lining the plasma membrane in these cells, as would be expected for interactions with Gα subunits. Song et al. also saw a mostly nuclear localization of GFP-Rgs2 in SH-SY5Y cells by confocal microscopy (Song et al., 2001). In addition, transfection of L1-2, 3T3, or COS-7 cells with GFP-tagged or c-myc-tagged Rgs2 indicated a nuclear localization for this protein (Bowman et al., 1998; Chatterjee and Fisher, 2000). Gu et al. found that some Rgs2 protein can be found to colocalize with nucleolar proteins (Gu et al., 2008).

For Rgs2 to function as a GAP for the Gα subunit, it would need to be present at the plasma membrane in order to interact with its target; yet, data suggest that this is a nuclear protein. Rgs2-GFP expressed in HEK 293 cells localized mainly to the nucleus, but to some degree in the cytoplasm and at the plasma membrane (Heximer et al., 2001). When Rgs2-GFP was co-expressed with a constitutively active $G\alpha_q$ mutant, Rgs2-GFP was found to be localized more to the plasma membrane, an effect not seen when the N-terminal domain of Rgs2 is deleted. These results were confirmed in a later study as well. Roy et al. found that GFP-Rgs2 localized to the nucleus in HEK 293 cells, but when co-expressed with $G\alpha_q$ or $G\alpha_s$ (but not $G\alpha_i$), it was recruited to the plasma membrane (Roy et al., 2003). They further demonstrated that when GFP-Rgs2 was co-transfected with receptors that activate $G\alpha_q$ or $G\alpha_s$, recruitment to the plasma membrane was observed. These data suggest that Rgs2 is recruited to the plasma membrane when the pertinent signaling pathway is activated, lending

further evidence to the idea that cellular localization may be a way in which RGS proteins are regulated.

How does this recruitment occur? The RGS domain of Rgs2 contains a consensus nuclear localization sequence, and GFP-tagged RGS domains localized to the nucleus, implying that nuclear localization is controlled by this domain (Chatterjee and Fisher, 2000). In addition, Rgs2, having a molecular weight of only 24 kDa, can diffuse freely into and out of the nucleus, without the assistance of nuclear transport, suggesting that a nuclear retention mechanism exists for this protein (Siderovski et al., 1994). Heximer et al. demonstrated that Rgs2 is not actively transported, but rather passively diffuses into the nucleus where it is then retained (Heximer et al., 2001). They attached a GST tag to the Rgs2-GFP fusion protein, which then made the protein too large to passively diffuse; as a result, no Rgs2-GFP was seen in the nucleus. In addition, exclusion from the nucleus did not affect the GAP function of Rgs2. Perhaps signaling from the GPCR leads to inhibition of nuclear retention, allowing Rgs2 to leave the nucleus and move to the plasma membrane.

Heximer et al. also found that a domain within the N-terminus of Rgs2 was necessary and sufficient for localization of this protein to the plasma membrane, and found that this region was conserved in some small RGS family members (Heximer et al., 2001). Further analysis indicated that this region forms an amphipathic α-helix, which has the ability to associate with vesicles *in vitro*. A subsequent study found that the hydrophobic residues adjacent to the amphipathic helix in the N-terminus of Rgs2 also contributed to plasma

membrane association, explaining why Rgs2 is better at membrane association than other members of its subfamily (Gu et al., 2007). Heximer et al. also suggested that the N-terminal domain may play a role in directing nuclear accumulation, as several of the mutations created there resulted in loss of or changes in nuclear localization (Heximer et al., 2001). It is conceivable that Rgs2 is retained in the nucleus by a protein binding at this domain and concealing this region, and that upon stimulation, binding is disrupted, allowing for recruitment of Rgs2 to the plasma membrane.

Functions and Interactions of Rgs2

There are different isoforms of Gα, generally classified as $G\alpha_s$, $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$ (McCudden et al., 2005). RGS proteins were first found to act upon $G\alpha_i$, then $G\alpha_q$ (Berman et al., 1996; Hepler et al., 1997). Rgs2 was at first only thought to act upon $G\alpha_q$. Heximer et al. demonstrated that Rgs2 binds to $G\alpha_q$, and not $G\alpha_i$, as well as performed GTPase assays to confirm this result (Heximer et al., 1997b). Furthermore, they showed that Rgs2 could inhibit the activation of phospholipase C (PLC), indicating that $G\alpha_q$ signaling was inhibited. It has since been found that Rgs2 can indeed act upon $G\alpha_i$. Ingi et al. found that Rgs2 not only acted upon $G\alpha_q$ *in vitro*, but also upon $G\alpha_i$ when the M2 muscarinic acetylcholine receptor, which couples to $G\alpha_i$, was also present in reconstituted phospholipid vesicles *in vitro* (Ingi et al., 1998). In COS cells expressing this receptor, Rgs2 was also able to inhibit G protein signaling. However, Rgs2 functions more efficiently as a GAP for $G\alpha_q$ than for $G\alpha_i$ (Heximer et al., 1999).

Although some evidence points toward a direct interaction of Rgs2 with $G\alpha_s$, it is still uncertain whether this interaction occurs (Abramow-Newerly et al., 2006). However, Rgs2 is known to interact with a downstream signaling molecule of the $G\alpha_s$ signaling pathway. Rgs2 interacts with certain isoforms of adenylyl cyclase (AC) and inhibits production of the second messenger cAMP (Roy et al., 2006a; Roy et al., 2003; Salim et al., 2003; Sinnarajah et al., 2001). Rgs2 affects AC III, V, and VI, but not AC I and II *in vitro* (Sinnarajah et al., 2001). Rgs2 functions to decrease cAMP in cells through direct interaction with AC. The interaction of Rgs2 with AC V has been characterized, and it was determined that the N terminus of Rgs2 is important for this interaction (Salim et al., 2003). Smaller Rgs2 proteins generated by alternative translation sites are missing portions of the N-terminus, and therefore cannot inhibit AC (Gu et al., 2008). Furthermore, signaling through $G\alpha_s$ and AC V specifically upregulates the full length Rgs2, which can feedback to inhibit signaling (Gu et al., 2008). Roy et al. found that co-expression of GFP-Rgs2 and AC isoforms (I, II, V, VI) in HEK 293 cells led to the localization of GFP-Rgs2 at the plasma membrane. They further demonstrated through the utilization of bioluminescence resonance energy transfer (BRET) in these cells that Rgs2 can exist in association with $G\alpha_s$ and AC II and IV, and with AC III when $G\alpha_s$ is also present (Roy et al., 2006b). However, these results do not indicate a direct interaction, just a close association.

Hao et al. found that Rgs2 selectively regulates $G\alpha_q$ signaling in cardiomyocytes (Hao et al., 2006). They also found that this molecule does not appear to affect AC directly in these cells. Rgs2 attenuated $G\alpha_q$ signaling, but not

$G\alpha_i$ signaling in HEK 293 cells as determined by examining the IL-8/CXCR1/MAPK pathway (Beadling et al., 1999). These data suggest that Rgs2 function may be cell type-specific. Also, the receptor itself plays a role in selectivity; Rgs2 was found to bind directly to the third intracellular loop of the M1 muscarinic acetylcholine receptor, an interaction that was mediated by the N-terminal region of Rgs2 (Bernstein et al., 2004). Rgs16 did not bind this receptor, suggesting that the interaction of the receptor and Rgs2 is selective (Bernstein et al., 2004). Rgs2 was also found to bind to the third intracellular loop of the α_{1A}-adrenergic receptor, whereas Rgs16 did not (Hague et al., 2005). This interaction required the N-terminus of Rgs2, as well. Furthermore, when Rgs2 was truncated to only express the RGS domain, it was no longer able to inhibit signaling through GPCRs, indicated by increased inositol phosphate accumulation, suggesting the interaction of the N-terminus is important for function (Tikhonova et al., 2006). While Rgs2 has been shown to bind to the third intracellular loop of receptors by several studies, one study found that it binds to spinophilin, an adaptor molecule that binds to GPCRs in the same region (Wang et al., 2005b). Wang et al. found that the N-terminus of Rgs2 was involved in this interaction, which led to increased inhibitory activity of Rgs2 (Wang et al., 2005b).

Interactions with the plasma membrane and receptors are not the only mechanisms involved in modulation of Rgs2 activity. Rgs2 inhibited activation of ERK and Akt downstream of M_3 muscarinic receptors, but Rgs3 could only inhibit Akt activation (Anger et al., 2007). While Rgs3 could inhibit Akt and ERK activation downstream of M_2 muscarinic receptors, Rgs2 could only inhibit Akt

activation (Anger et al., 2007). These data indicate that other mechanisms are in place to regulate Rgs2 protein function. Indeed, one study has shown that palmitoylation of Rgs2, mediating a conformational change, inhibits its function as a GAP for G proteins (Ni et al., 2006). Phosphorylation of Rgs2 by protein kinase C (PKC) also leads to decreased function as a GAP (Cunningham et al., 2001). Phosphorylation by type Iα cGMP-dependent protein kinase (cGKIα) leads to increased Rgs2 GAP activity (Tang et al., 2003); however, cGKIα activity also mediates proteasomal degradation of Rgs2 in a manner not dependent on phosphorylation of Rgs2, providing another level of feedback in this signaling pathway (Osei-Owusu et al., 2007).

As Rgs2 is highly expressed in hematopoietic cells, which are known to undergo chemotaxis due to GPCR chemokine receptor activation, its role in cell migration has been assessed. Bowman et al. found that Rgs2 has no effect on migration of lymphocytic cells (Bowman et al., 1998). They co-transfected pre-B lymphocytic L1-2 cells with vectors encoding the chemokine receptors CXCR1 and CCR-2B, along with different RGS proteins. When migration towards IL-8 or MCP-1 was assayed, Rgs2 did not inhibit migration, whereas Rgs1 and Rgs3 did. Reif et al. found that in the B cell line 2PK3, exogenous Rgs2 had little to no effect on migration of these cells to the B cell chemokines stromal-derived factor-1 (SDF-1), B lymphocyte chemoattractant (BLC), or EBV-induced molecule 1 ligand chemokine (ELC) (Reif and Cyster, 2000). These results may be explained by the findings that Rgs2 is not a strong GAP for $G\alpha_i$, which is the main α subunit utilized by chemokine receptors.

There has been accumulating evidence that RGS proteins have functions other than their GAP activity. One other way by which RGS proteins might act to inhibit G protein mediating signaling is to inhibit G protein-effector interactions (Hepler et al., 1997). Rgs2, and its subfamily member Rgs4, were found to compete for binding to $G\alpha_q$ with GRK2, a $G\alpha_q$ effector, suggesting they can indeed function as allosteric inhibitors (Shankaranarayanan et al., 2008). Cunningham et al. designed experiments aimed at separating the effects of Rgs2 GAP activity from its potential effector antagonist activity, and found that Rgs2 was better at inhibiting signaling when it was able to function as a GAP (Cunningham et al., 2001). Other studies have focused on roles for Rgs2 outside of GPCR signaling. Sullivan et al. found Rgs2 and Rgs4 bind to β'-COP, a subunit of the COPI complex involved in Golgi trafficking (Sullivan et al., 2000). While they did not report a role for Rgs2, Rgs4 binding to COPI inhibited transport and secretion of proteins, suggesting Rgs2 may have a similar role (Sullivan et al., 2000). Rgs2 interacts with TRPV6, a Ca^{2+} channel, through its N-terminus, and attenuates the ion current, providing evidence that Rgs2 can affect Ca^{2+} levels outside of functioning as a GAP alone (Schoeber et al., 2006). Lastly, Rgs2 has been shown to bind to eukaryotic initiation factor 2B ε subunit (eIF2Bε) and inhibit protein translation (Nguyen et al., 2009). The binding site is within the RGS domain, but binding does not occur with Rgs4, suggesting this is specific for Rgs2 (Nguyen et al., 2009). More studies are likely to follow examining roles of Rgs2 outside of its capability as a GAP for Gα proteins. One common theme that

emerges, though, is that the various roles of Rgs2 all indicate that it is an inhibitory molecule.

Rgs2 in Cell Stress and Cell Cycle

There is mounting evidence in the literature that Rgs2 may play a role in the cell other than as a GAP for G proteins. Heat shock can lead to a rapid and transient increase in Rgs2 mRNA levels in SH-SY5Y cells, indicating that cell stress can regulate Rgs2 levels (Song et al., 2001). After differentiation of SH-SY5Y cells, Rgs2 levels decreased (Song et al., 2001). In addition, hydrogen peroxide, peroxynitrite, and heat shock all quickly increase Rgs2 mRNA in 1321N1 cells (Zmijewski et al., 2001a). Protein levels of Rgs2 also increased, and it was found mainly in the nucleus in the hydrogen peroxide treated cells. Furthermore, treatment of SH-SY5Y cells with camptothecin to induce DNA damage increases Rgs2 mRNA levels (Song and Jope, 2006). Addition of cdk2 inhibitors abrogated the Rgs2 increase, and treatment with an inhibitor of Hsp90 increased Rgs2 mRNA alone and in an additive manner when applied in conjunction with camptothecin treatment (Song and Jope, 2006). Stimulation of U937 cells with PGE_1 and PGE_2 increases Rgs2 expression while blocking proliferation of these cells (Beadling et al., 1999). Further, Rgs2 inhibited growth of androgen-independent LNCaP cells (Cao et al., 2006). Chemically induced ischemia in astrocytes mediated upregulation of Rgs2 mRNA, which was associated with increased cell death (Endale et al., 2010). These data indicate a

role for Rgs2 in the cell cycle that may not be related to its role as a GAP for G proteins.

Role of Rgs2 in Differentiation and Cancer

Rgs2 is suspected to act in differentiation of myeloid cells. In one study, Schwable et al. examined the receptor activating Flt3-ITD mutation in acute myelogenous leukemia (AML), and found that Rgs2 expression was downregulated in cells with this mutated receptor compared to cells with the wild type receptor. Also, when comparing Rgs2 levels in the bone marrow of healthy patients with that of patients with AML, it was found that the bone marrow of healthy patients had much higher levels of Rgs2 (Schwable et al., 2005). They also found that the expression levels of Rgs2 in AML cells matched that of CD34+ progenitor cells, suggesting that Rgs2 levels are low in more immature cells. This then led them to examine the role that Rgs2 may be playing in signaling though the Flt3 receptor. They demonstrated that Rgs2 was able to negatively regulate proliferation of Flt3-ITD/32D cells. Additionally, Rgs2 was able to reverse the block in differentiation that is caused by the Flt3-ITD mutation. They further confirmed in differentiation studies of cells lines that Rgs2 is upregulated during myeloid differentiation (Schwable et al., 2005). Rgs2 has also been implicated in macrophage foam cell production; lipopolysaccharide (LPS) induced foam cell formation by downregulating Rgs2 levels (Lee et al., 2010). When Rgs2 was overexpressed, foam cell production was inhibited (Lee et al., 2010).

As transformed cells are speculated to exist at less differentiated states, repression of Rgs2, which may positively regulate differentiation, may play an important role in tumorigenesis. Indeed, Rgs2 inhibited growth of androgen-independent LNCaP cells (Cao et al., 2006). However, the role of Rgs2 in tumor progression is not clear. Whereas Schwable et al. were able to detect Rgs2 in normal bone marrow by real time reverse transcriptase-PCR (RT-PCR), Wu et al. were unable to detect this mRNA in bone marrow by RT-PCR, but they detected Rgs2 in leukemic and lymphoblastic cells lines as well as in the majority of AML and acute lymphoblastic leukemia (ALL) cases (Wu et al., 1995). Rgs2 was detected in cases of chronic myelogenous leukemia (CML), but only those in the blast crisis phase. In addition, Rgs2 was detected in several other cancerous cell lines, including HeLa (cervical), OVCAR-3 (ovarian), and A-172 (glioblastoma) (Wu et al., 1995). Furthermore, Rgs2 was found to be expressed at higher levels in human breast cancer cells compared to normal breast cells (Smalley et al., 2007). However, in human colorectal cancer, Rgs2 was found to be downregulated, with low Rgs2 expression being an indicator of poor prognosis (Jiang et al., 2010). The presence of Rgs2 in these tumor cells is in direct contradiction with the idea that Rgs2 downregulation may have a role in promoting tumor growth, and demonstrates that there is much more to be learned about the functions of this molecule.

Rgs2 appears to play a role in differentiation of cell types other than myeloid cells. James et al. demonstrated by microarray analysis and confirmed by RT-PCR and in situ hybridization that Rgs2 is upregulated for a period of time

during chondrocyte differentiation (James et al., 2005). Rgs2 has even been implicated in adipocyte differentiation. Imagawa et al. found that Rgs2 was expressed early in adipocyte differentiation, using mouse 3T3-L1 cells, and that this protein was only expressed in stimulated cells that were not proliferating (Imagawa et al., 1999). The same group continued to examine this role for Rgs2, showing that in NIH-3T3 fibroblast cells, Rgs2 only functions to promote adipocyte differentiation in the presence of ligand for the peroxisome proliferator-activated receptor γ (PPAR γ) (Nishizuka et al., 2001). In addition, Rgs2 was found to bind to tubulin and increase microtubule formation, playing a role in neuron differentiation (Heo et al., 2006). While the possibility for a role for Rgs2 in differentiation of several cell types is becoming evident, the exact nature of this role is still unknown. Evidence points toward an anti-proliferative role for Rgs2. As cells must stop proliferation as they differentiate, perhaps with further study, the role of Rgs2 in differentiation will be found to be related to this anti-proliferative effect.

Rgs2 in mammals

In an effort to further understand the roles of this molecule, mice deficient in Rgs2 were generated by replacing exons 4-5 (which contain the RGS domain) and part of the 3” UTR with a Neo resistance cassette (Oliveira-Dos-Santos et al., 2000). These mice are fertile and appear to grow normally. Curiously, these mice seem to have no defects in hematopoietic differentiation. As this gene product was first identified in T cells, the effect of Rgs2 deficiency was first examined in

these cells. Development of T cells appeared to be comparable to wild type mice, but when activation was examined, differences were noticed. Proliferation of T cells and IL-2 production were mildly impaired in Rgs2 deficient mice upon T cell activation. The decreased ability to produce activated T cells led to a lower level of response *in vivo* to viral challenge. T cell response to chemokines appeared unaffected, and B cell production and activation also were not affected. Outside of the immune system, polymorphisms in Rgs2 and CREB regulated transcriptional coactivator 3 (CRTC3), a molecule that upregulates Rgs2 expression, have been linked to adiposity and metabolic disorder in humans (Freson et al., 2007; Song et al., 2010).

Studies have shown upregulation of Rgs2 in vascular smooth muscle cells and cardiomyocytes in response to different stimuli, suggesting that this molecule may play a role in the cardiovascular system (Grant et al., 2000; Hao et al., 2006). Indeed, studies in the knockout mice indicate that these mice, even with a heterozygous phenotype, develop hypertension (Heximer et al., 2003). The hypertensive mice displayed signs of vascular smooth muscle cell hypertrophy or hyperproliferation in the aorta and renal arteries, prolonged constriction in response to vasoconstrictors, and increased Ca^{2+} signaling in vascular smooth muscle cells (Heximer et al., 2003; Sun et al., 2005). In vascular smooth muscle cells, cGMP-dependent protein kinase I-α (PKGI-α) phosphorylates and activates Rgs2, which can then turn off constrictor signaling through protease-activated receptor-1 (PAR-1), enhancing the vasodilatory effects of nitric oxide and cGMP (Obst et al., 2006; Osei-Owusu et al., 2007; Sun et al., 2005; Tang et al., 2003).

Angiotensin II treatment of Rgs2 deficient mice led to increased blood pressure, likely though decreased attenuation of signaling through the angiotensin II type 1 receptor (Hercule et al., 2007). One recent study used kidney transplantation to establish that lack of Rgs2 in the kidneys alone is sufficient to mediate development of hypertension (Gurley et al., 2010). Rgs2 has been shown to reduce hypertrophy caused by α-adrenergic signaling in ventricular rat cardiomyocytes, and decreased Rgs2 levels are detected before hypertrophy occurs in models of increased $G\alpha_q$ signaling (Zhang et al., 2006; Zou et al., 2006). Transverse aortic constriction led to increased cardiac hypertrophy, heart failure, and mortality in Rgs2-/- mice (Takimoto et al., 2009). Moreover, mutations and single nucleotide polymorphisms in Rgs2 have been linked to hypertension in humans (Li et al., 2010; Riddle et al., 2006; Yang et al., 2005b). One study has linked a mutation found in hypertensive patients to increased proteasomal degradation of Rgs2, providing a mechanism for the hypertensive phenotype (Bodenstein et al., 2007).

Rgs2-/- mice exhibit differences in behavior, indicating a role for Rgs2 in the central nervous system, specifically in the hippocampus (Oliveira-Dos-Santos et al., 2000). The male null mice are less aggressive and more anxious than their wild type counterparts. Decreased spine density in the CA1 neurons in the hippocampus of Rgs2 deficient mice has been observed, indicating a smaller number of synapses (Oliveira-Dos-Santos et al., 2000). Overexpression of Rgs2 increased neurite growth, and Rgs2 localizes in the neurites of PC12 cells (Heo et al., 2006). Furthermore, Rgs2 is present in the synapses of neurons, and has

been found to regulate $G\alpha_{i/o}$ and Ca^{2+} influx, leading to modulation of vesicle release in synapses (Han et al., 2006). Several single nucleotide polymorphisms in Rgs2 were found to correlate with anxiety disorders in humans (Koenen et al., 2009; Leygraf et al., 2006). However, a different study found no correlation between anxiety disorders and polymorphisms in Rgs2 (Mouri et al., 2010). Recently, a microRNA, miR-22, was found to correlate with anxiety disorders, and one of the targets of miR-22 is Rgs2 (Muinos-Gimeno et al., 2010). Further study is warranted to understand the role of Rgs2 in human anxiety disorders.

Rgs2 Summary

In summary, Rgs2 functions as a GAP for $G\alpha_q$, and to some degree for $G\alpha_i$. It is localized in the nucleus until stimulation of GPCRs and activation of Gα mediates recruitment to the plasma membrane (Figure 5B). Once at the membrane, Rgs2 interacts with Gα or AC, leading to the attenuation of signaling and cAMP production, respectively. In addition, interactions of Rgs2 and other molecules have been shown. While the exact mechanisms are unknown, Rgs2 appears to play a role in differentiation of many cell types. Rgs2 regulation appears to occur primarily at the transcriptional level, but regulation by post-translational modifications, such as phosphorylation of the protein, have also been shown to occur. Rgs2 is proving to be an important molecule in many cells, and future studies are likely to elucidate more functions for this molecule.

Summary and Dissertation Goals

MDSCs play an important role in cancer progression, and elucidating the mechanisms involved in the accumulation and function of these cells is important in the fight against cancer. In a microarray comparing splenic Gr-1+CD11b+ cells from nontumor bearing mice and tumor bearing mice, Rgs2 was found to be upregulated in the tumor derived cells. Based on published data, we hypothesized that Rgs2 plays a role in myelopoiesis, and as such, would play a role in MDSC production or differentiation. In Chapter II, the role of Rgs2 in hematopoiesis and myeloid cell differentiation was examined. Overall hematopoiesis was not affected by loss of Rgs2, and mature myeloid cells were not altered. While lack of Rgs2 did promote a delay in granulocytic differentiation, the delay was small and quickly overcome by the Rgs2-/- cells. In addition, while Rgs2-/- mice are more sensitive to serial 5-fluorouracil treatment, further studies found that lack of Rgs2 did not greatly affect stem cell or multipotent progenitor cell production. These data suggest that Rgs2 does not play a major role in hematopoiesis or myelopoiesis. In Chapter III, the role of Rgs2 in MDSCs was investigated. Rgs2 deficiency in MDSCs abrogated the tumor promoting roles of these cells, leading to retarded tumor growth along with decreased vascular density. While lack of Rgs2 did not affect the production or heterogeneity of MDSCs within the spleens of tumor mice, it did lead to decreased MCP-1 production. Further, MCP-1 deficiency of Rgs2-/- MDSCs led to decreased tube formation and migration of endothelial cells, and a shift from IL-4 production to IFNγ production in splenocytes cultured with the tumor MDSCs. Together, these

data suggest that Rgs2 affects the tumor promoting functions of MDSCs through mediating MCP-1 production. Understanding the mechanisms by which MDSCs promote tumor growth and progression is important in determining how to target these cells. In targeting the signaling pathway involving Rgs2 in MDSCs, we can not only prevent the promotion of angiogenesis and immune tolerance caused by these cells, but also perhaps shift these cells to mediators that promote killing of tumor cells by the immune system.

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CHAPTER II

RGS2 DOES NOT AFFECT HEMATOPOIESIS UNDER NORMAL OR STRESS CONDITIONS

Summary

Hematopoiesis is a process involving many different molecules and many different signaling pathways, all coming together to produce mature, functional cells such as red blood cells, myeloid cells, and lymphocytes. Rgs2 for years has been suspected to play a role in hematopoiesis, either through affecting proliferation or differentiation of myeloid cells. Here, we have examined the role of Rgs2 in several aspects of hematopoiesis. We found that although Rgs2-/- mice are more sensitive to serial 5-fluorouracil treatment, they do not have any major defects in stem cell development or function. We also found that although there is a slight delay in granulocytic differentiation, there is no difference in levels of mature granulocytes, or other hematopoietic cells. There is also no difference in numbers of myeloid progenitors in the bone marrow of wild type and Rgs2-/- mice measured in methocellulose assays. All together, our data point to a small to no role for Rgs2 in hematopoiesis.

Introduction

Hematopoiesis is the process by which blood cells develop and mature. It is a tightly regulated process, starting with stem cells. Hematopoietic stem cells (HSCs) are characterized by the ability to be quiescent most of the time, but proliferate and undergo self-renewal with the right signals, as well as being able to repopulate each of the cells of the hematopoietic system. Studies have shown that hematopoietic stem cells reside in the bone marrow (BM) of adult mammals in a hematopoietic niche (Eliasson and Jonsson, 2010; Levesque et al., 2010). Early studies also indicated that some HSCs and early progenitor cells can be found moving from the BM to the blood, and back again (Goodman and Hodgson, 1962; Levesque et al., 2010; Wright et al., 2001). Homing into the niche involves cell-cell interactions between the HSCs and the endothelial cells of the vessels in the BM, as well as cytokine and chemokine gradients in the BM (Levesque et al., 2010). Once in the BM, the HSCs are kept there by interactions with the surrounding cells, such as mesenchymal stem cells, osteoblasts, and osteoblastic progenitors (Levesque et al., 2010). One interaction known to be involved is the SDF-1/CXCR4 chemokine axis (Aiuti et al., 1997; Hattori et al., 2001; Jo et al., 2000; Mohle et al., 1998; Peled et al., 1999; Zou et al., 1998). Treatment with G-CSF, known to mediate mobilization of stem cells (Morrison et al., 1997), leads to upregulation of proteases within the BM that can cleave SDF-1 and CXCR4, among other important proteins, disrupting the retention signal

(Kopp et al., 2005; Lapidot and Petit, 2002; Levesque et al., 2010; Levesque et al., 2003; Petit et al., 2002).

Recent studies have uncovered the importance of osteoblasts and osteoblastic progenitors in the hematopoietic niche (Levesque et al., 2010). Indeed, long term repopulating HSCs have been found to reside in the endosteal region of the BM, close to osteoblasts, and are deeply quiescent (Kubota et al., 2008; Levesque et al., 2010; Nilsson et al., 2001; Wilson et al., 2008). The HSC niche in the endosteal region is hypoxic, creating an environment for the HSCs conducive to decreased damage from reactive oxygen species, as well as decreased proliferation (Chen et al., 2008; Eliasson and Jonsson, 2010; Levesque et al., 2010; Parmar et al., 2007; Winkler et al., 2010). Those HSCs found in the less hypoxic vascular areas of the BM, or further from the endosteum, have been found to be more proliferative (Levesque et al., 2010; Lo Celso et al., 2009; Winkler et al., 2010). Several intracellular molecules have been shown to regulate stem cell proliferation, self-renewal, and quiescence, including c-myc (Eliasson and Jonsson, 2010; Levesque et al., 2010; Wilson et al., 2004). HSCs express lower levels of c-myc compared to progenitor cells, and deletion of c-myc leads to increased self renewal (Wilson et al., 2004). However, the lack of c-myc in HSCs also prevented differentiation of more mature cells, and overexpression of c-myc in HSCs was detrimental in BM reconstitution assays (Baena et al., 2007; Wilson et al., 2004). Interestingly, HIF-1α has been shown in fibroblast cell lines to displace c-myc from the promoter of the gene encoding the $p21^{waf1/cip1}$ protein, derepressing it and leading to cell cycle

arrest (Koshiji et al., 2004). Perhaps a similar mechanism of hypoxia induced HIF-1α is leading to p21 production and function in HSCs. Without p21, stem cells exhibit increased proliferation and subsequent exhaustion (Cheng et al., 2000). It is important for stem cells to remain quiescent in order to prevent exhaustion, or loss of the ability to self-renew, due to multiple rounds of cell cycles (Eliasson and Jonsson, 2010; Harrison and Astle, 1982; Mauch and Hellman, 1989). As such, a recent study suggests that in addition to more replicative HSCs, there is a “highly dormant” pool of stem cells than only replicate about 5 times during the life of the mouse under normal conditions, but can rapidly respond to stimulation, and then return to dormancy (Wilson et al., 2008).

HSCs, often defined as Lineage- Sca-1+ cKit+ (LSK) cells by flow cytometry, can be broken into groups by the presence of Flt3 receptor (Flt3R) on the cell surface. LSK cells negative for Flt3R are considered to be long-term stem cells (LT-HSCs) capable of fully repopulating the hematopoietic system, and LSK cells positive for Flt3R comprise the short term HSCs (ST-HSCs) and multipotent progenitor cells (MPPs) incapable of reconstituting the hematopoietic system for the long term (Adolfsson et al., 2001; Christensen and Weissman, 2001; Guenechea et al., 2001; Wilson et al., 2008). MPPs include cells that can differentiate into the erythroid, myeloid, and lymphoid lineages (Lai and Kondo, 2006; Lai et al., 2005). Committed progenitor cells, the common lymphoid progenitors (CLPs) and the common myeloid progenitors (CMPs), are considered to have made a “decision” to progress along a particular lineage, and produce the cells of the lymphoid and myeloid lineages, respectively (Akashi et al., 2000;

Kondo et al., 1997). The resulting mature cells depend on intrinsic and extrinsic factors, and the interplay of transcription factors, such as C/EBP family members, PU.1, c-Myb, GATA family members, among others, is extremely important in modulating lineage paths (Anderson, 2006; Friedman, 2002; Laiosa et al., 2006; Lenny et al., 1997; Tenen et al., 1997).

Rgs2 has been suggested to play a role in hematopoiesis, specifically in the myeloid lineage, or myelopoiesis. Studies have indicated that Rgs2 expression increases in differentiating myeloid cell lines, such as NB4, U937, and HL-60 cell lines (Schwable et al., 2005). Also, when comparing Rgs2 levels in the bone marrow of healthy patients with that of patients with AML, it was found that the bone marrow of healthy patients had much higher levels of Rgs2, whereas the levels of Rgs2 in the AML samples matched that of CD34+ progenitor cells, suggesting that Rgs2 levels are low in more immature cells (Schwable et al., 2005).

AML is exemplified by increased proliferation and decreased differentiation of myeloid progenitor cells, and almost one third of AML cases involve activating mutations in the Flt3 receptor (Gilliland and Griffin, 2002). Schwable et al. examined the receptor activating Flt3-ITD mutation in AML, and found that Rgs2 expression was downregulated in cells with this mutated receptor compared to cells with the wild type receptor (Schwable et al., 2005). They also demonstrated that Rgs2 was able to negatively regulate proliferation of Flt3-ITD/32D cells. Additionally, Rgs2 overexpression was able to reverse the block in differentiation that is caused by the Flt3-ITD mutation. These data led us

to hypothesize that Rgs2 plays a role in hematopoiesis by regulating proliferation and/or differentiation of myeloid cells.

Methods

Mice: Rgs2-/- mice were obtained from Dr. Josef Penninger at the Institute of Molecular Biotechnology GmbH, have previously been characterized (Oliveira-Dos-Santos et al., 2000), and were bred at Vanderbilt. Age and gender matched wild type control mice were purchased from Jackson Laboratories. Mice were kept in clean housing according to IACUC regulations.

Cell Lines: The HL-60 cell line was cultured in RPMI 1640 + 10% FBS + 1% penicillin/streptomycin at 37 °C, 5% CO_2. 32D cells were cultured in RPMI 1640 + 10% FBS + 1% penicillin/streptomycin, supplemented with medium from CHO-IL-3 cells to provide IL-3 at 37 °C, 5% CO_2. OP9-DL1 cells were cultured in αMEM with 20% heat inactivated FBS and 1% penicillin/streptomycin.

Differentiation of cell lines: HL-60 cells were treated with 1.3% DMSO in growth medium to induce granulocytic differentiation. Medium was replaced with fresh medium every other day. 32D cells were switched to IMDM medium, supplemented with 15% FBS, 1% glutamine, 1% penicillin/streptomycin, and CHO-IL3 cell conditioned medium. To differentiate these cells to granulocytes, 50

ng/ml G-CSF (R&D Systems) was added. Cells were given fresh medium every other day.

Flow cytometry and MACS: Tissues were prepared into single cell suspensions and labeled with antibodies for markers of mature and immature blood cells (BD Biosciences). Cells were analyzed using a BD LSRII or BD FACScan. For magnetic activated cell sorting (MACS) of lineage negative or lineage negative Sca-1+ cells, bone marrow was first isolated from femurs and tibias of mice with phosphate buffered saline (PBS). The cells were labeled using a lineage depletion kit (Miltenyi), and lineage negative cells were collected as they exited a depletion column (Miltenyi). For further Sca-1+ isolation, the cells were labeled using a Sca-1 isolation kit (Miltenyi), and collected from a column (Miltenyi).

Methocellulose assay: Tibias and femurs were isolated from mice, and bone marrow was flushed with cold IMDM supplemented with 2% FBS. Marrow was pooled from two mice per group. A single cell suspension was prepared, and added to Methocult 3234 (StemCell Technologies) + 50 ng/ml SCF (R&D Systems), 10 ng/ml IL-3 (R&D Systems), 10 ng/ml IL-6 (R&D Systems), and 3 U/ml EPO (StemCell Technologies) at $5x10^4$ cells/ml. Colonies were scored after nine days of culture in a CO_2 incubator at 37°C. Each assay was performed in duplicate.

Granulocytic differentiation assay: Tibias and femurs were isolated from mice, and bone marrow was flushed with cold DMEM supplemented with 15% FBS and 1% antibiotics. Bone marrow cells were cultured in DMEM, 15% FBS, 1% antibiotics + 10 ng/ml IL-3 (R&D Systems), 50 ng/ml Flt3L (R&D Systems), 100 ng/ml SCF (R&D Sytems), and 10 ng/ml TPO (R&D Systems) at < $1x10^7$ cells/ml in a CO_2 incubator at 37°C. After three days in culture, the light density cells were purified using Histopaque (Sigma), and Lin- Sca-1+ cells were isolated with kits from Miltenyi Biotec. The cells were cultured in DMEM, 15% FBS, 1% antibiotics + 100 ng/ml SCF overnight. The next day, Day 0 of the assay, the cells were washed, and cultured in 100 ng/ml G-CSF (R&D Systems) and 100 ng/ml SCF. Medium was changed every other day, using fresh G-CSF and SCF. Samples were taken at different time points for cytospin, stained with the Three Step Stain (Richard Allan Scientific), and examined by a hematopathologist for morphology.

5-fluorouracil (5-FU) serial treatment: Mice were injected intraperitoneally (I.P.) with 150 mg/kg of 5-FU or 10% DMSO in PBS vehicle once per week until the mice began to show signs of distress. Upon signs of ruffled fur, hunched back, etc., the mice were sacrificed.

BrdU incorporation: Mice were injected with 1 mg of BrdU I.P. two hours before being sacrificed. Femurs and tibias were removed, and bone marrow cells were isolated using PBS. The cells were depleted of lineage marker positive cells, and BrdU antibody labeling was performed using a kit (BD Biosciences). Briefly, c-Kit

and Sca-1 surface proteins were labeled with antibody, and the cells were fixed and permeabilized. The cells were then subjected to DNase treatment at 37 °C, followed by labeling with anti-BrdU.

Annexin V labeling: Femurs and tibias from mice were removed, and bone marrow cells were isolated using PBS. The bone marrow cells were depleted of cells expressing lineage markers by MACS, and labeled with antibodies against Annexin V, c-Kit, and Sca-1, then analyzed.

OP9-DL1 co-culture: The assay was performed as previously described (de Pooter and Zuniga-Pflucker, 2007; Holmes and Zuniga-Pflucker, 2009; Wang et al., 2006). Briefly, bone marrow cells were isolated, and Lineage- Sca-1+ cells were isolated using MACS. The isolated cells were seeded on top of irradiated OP9-DL1 cells in αMEM + 20% heat inactivated FBS + 1% penicillin/streptomycin, supplemented with 5 ng/ml Flt3 ligand (R&D Systems) and 5 ng/ml IL-7 (Miltenyi Biotec). The cells were filtered and passaged onto fresh irradiated OP9-DL1 cells with fresh growth factors on Day 7, and on 4 day intervals afterwards. The cells were counted on the days indicated, and the numbers of cells at each time point were plotted.

Statistical analysis: Data were averaged and compared using Student's t test. Error bars on graphs represent standard error across experiments.

Results

Rgs2 mRNA levels increase in myeloid cells as they differentiate

Previous data from other groups has suggested a role for Rgs2 in differentiation of myeloid cells, and Schwable et al. have shown that in some myeloid cell lines, Rgs2 mRNA levels increase with differentiation (Schwable et al., 2005). We sought to determine if this is true in human and mouse myeloid cell lines. To test this, we first differentiated HL-60 human acute promyelocytic leukemia cells with DMSO treatment for 7 days. We isolated RNA from samples taken at the indicated time points, and with subsequent real time PCR, we found that Rgs2 mRNA levels increased with differentiation of these cells (Figure 6A, left). We confirmed differentiation on Day 7 by comparing CD11b expression levels on undifferentiated and DMSO differentiated cells by flow cytometry (Figure 6A, right). The differentiated HL-60 cells express more CD11b, a molecule acquired during differentiation. We also examined Rgs2 levels in 32D murine progenitor cells as they were differentiated into granulocytes with G-CSF treatment. Again, Rgs2 mRNA levels increased with differentiation (Figure 6B, left). We confirmed differentiation by examining the morphology of the 32D cells with and without G-CSF treatment (Figure 6B, right). The undifferentiated cells have round, uniform nuclei when stained and visualized. The G-CSF differentiated 32D cells have lobed nuclei, indicating that the cells have become granulocytes.

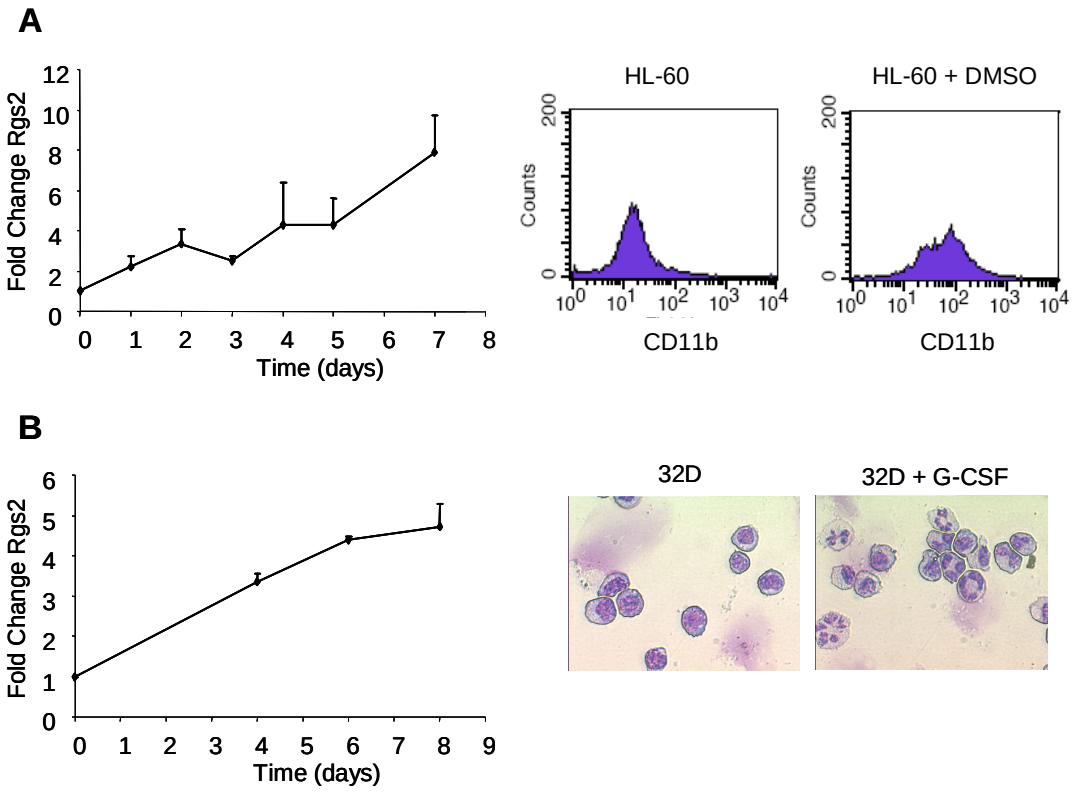

Figure 6. Rgs2 mRNA levels increase as myeloid cells differentiate. (A) HL-60 human leukemia cells were differentiated with 1.3% DMSO over a course of 7 days. Samples were taken at the time points indicated, and Rgs2 levels were measured by real time PCR. Samples were normalized to β-actin, and compared to Rgs2 levels in undifferentiated controls (left). Flow cytometry was used to confirm differentiation on Day 7 by measuring upregulation of CD11b, a maturation marker (right). (B) 32D mouse hematopoietic progenitor cells were differentiated into granulocytes with 50 ng/ml G-CSF over a course of 8 days. Samples were taken at the time points indicated, and Rgs2 levels were measured by real time PCR. Expression was normalized to β-actin, and compared to Rgs2 levels in undifferentiated controls (left). Differentiation was confirmed by examining morphology of undifferentiated and differentiated cells stained with Three Step Stain (Richard Allan Scientific) (right).

Rgs2 deficiency does not affect production of mature or progenitor hematopoietic cells

In order to study the role of Rgs2 in hematopoiesis further, we utilized Rgs2 -/- mice. These mice have no major defects; they are fertile and viable (Oliveira-Dos-Santos et al., 2000). We examined the mature immune cell populations using flow cytometry and antibodies against cell surface molecules characteristic to B lymphocytes, T lymphocytes, T_H lymphocytes, cytotoxic T lymphocytes, dendritic cells, monocytes, and granulocytes. We did not observe differences in the populations between wild type and Rgs2 null mice within the spleen (Figure 7). As Rgs2 has been proposed to be a stress-response gene, we decided to determine if there would be a difference in hematopoiesis if we stress the mice. We gave the mice a one time injection of 5-fluorouracil (5-FU) at 200 mg/kg intraperitoneally. 5-FU is a pyrimidine analog of thymidine, and inhibits DNA replication, leading to death of rapidly proliferating cells, such as hematopoietic progenitor cells. After 24 hours, we examined the populations of mature cells in the spleens by flow cytometry. As before, we did not observe any difference in the mature cells studied (Figure 7).

In addition, when we tested the progenitors in the bone marrow using a methocellulose assay, we did not observe any significant differences in the colony forming units, indicating no difference in the levels of progenitors present (Figure 8). We also tested the role of Rgs2 in myeloid differentiation using an *ex vivo* granulocytic differentiation assay. We isolated Lineage - Sca-1+ stem cells from bone marrow of wild type and Rgs2 -/- mice, and used G-CSF to

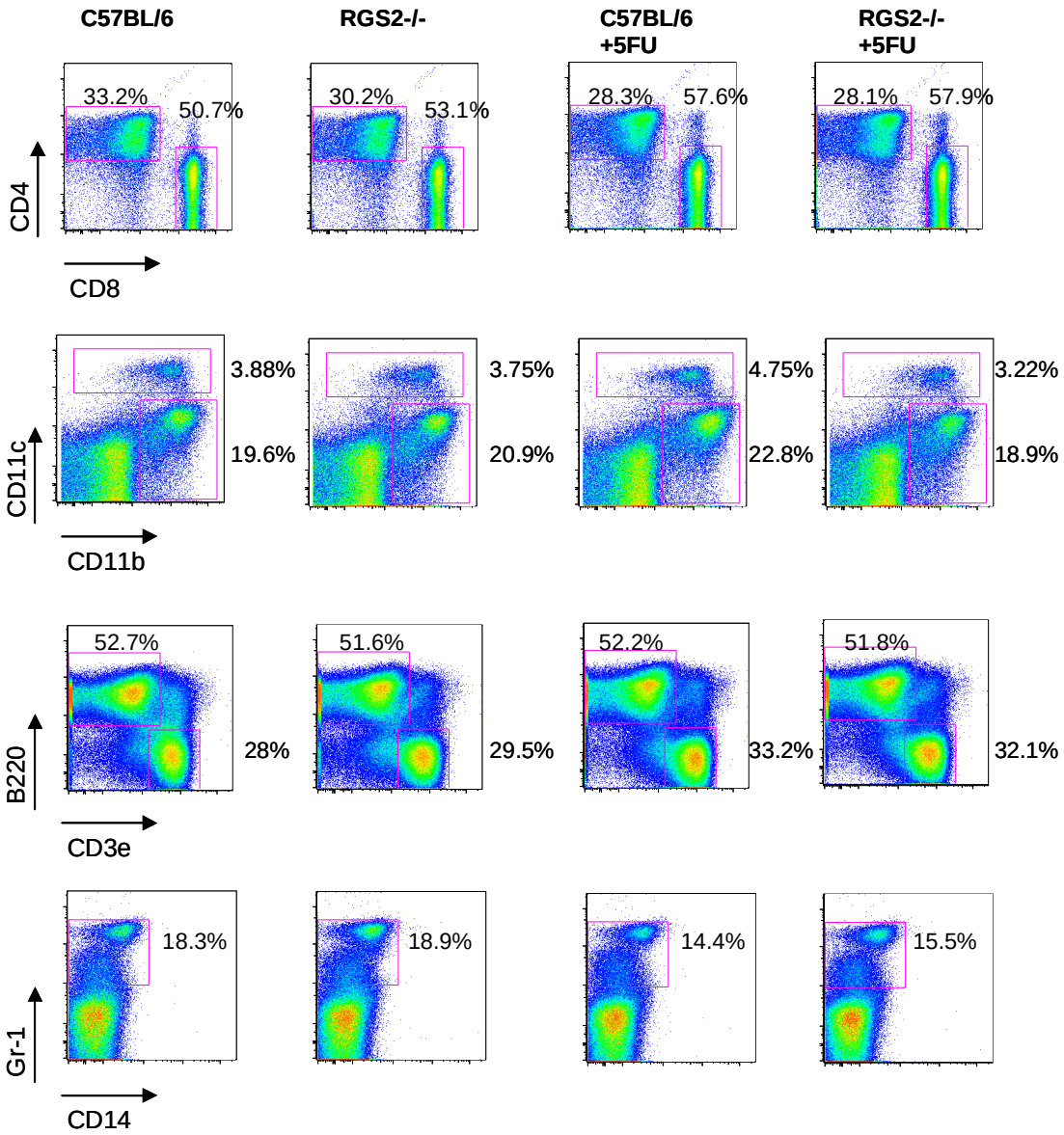

Figure 7. Lack of Rgs2 does not affect populations of mature leukocytes. Mice were treated with 200 mg/kg of 5-FU intraperitoneally for 24 hours. Spleens were isolated from control and 5-FU treated WT and Rgs2-/- mice, processed into single cell suspensions, labeled with the indicated antibodies, then analyzed by flow cytometry. Experiment was performed 3 times with 3-4 mice per group. Representative graphs are shown.

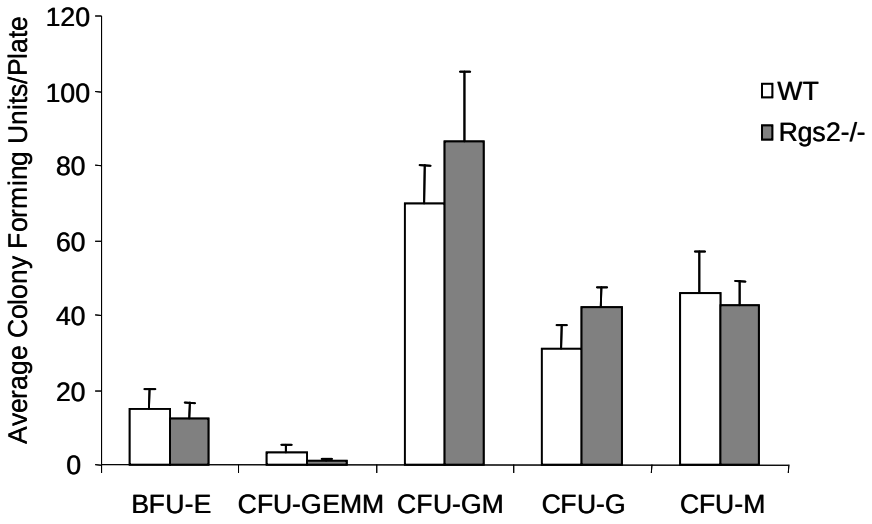

Figure 8. Rgs2 deficiency does not affect numbers of hematopoietic progenitors. Bone marrow from femurs and tibias of wild type and Rgs2-/- mice was isolated and cultured in methocellulose with growth factors to produce colony forming units. The graph shows the average of 3 independent experiments performed in duplicate with bone marrow pooled from 2 mice each. BFU-E, burst forming unit- erythroid; GEMM-CFU, granulocyte erythroid macrophage megakaryocyte- colony forming unit; GM-CFU, granulocyte macrophage- colony forming unit; G-CFU, granulocyte- colony forming unit; M-CFU, macrophage- colony forming unit.

differentiate them *in vitro*. We found that monocyte production was not affected by Rgs2 deficiency (Figure 9A). When looking at the neutrophil series, we found that Rgs2 deficiency led to a short delay in differentiation, beginning at Day 3 and more evident at Day 4 (Figure 9B). However, the Rgs2 deficient cells recover and catch up by Day 6. Together, the data point to no major role for Rgs2 in hematopoietic progenitor or mature cell production or differentiation.

Rgs2 deficiency leads to increased sensitivity to serial 5-fluorouracil treatment

In an effort to determine if lack of Rgs2 could have an effect on hematopoiesis under stressful conditions, we treated the mice serially with 150 mg/kg of 5-FU once per week. As mentioned, 5-FU leads to the death of rapidly proliferating cells, such as hematopoietic progenitor cells. By treating the mice multiple times with 5-FU, we are creating a situation in which the stem cells must remain active to replenish the dying progenitor cells. This eventually leads to exhaustion of the stem cells, and the mice cannot recover. Rgs2 deficient mice are more sensitive to serial 5-FU treatment, succumbing to 5-FU more quickly than wild type counterparts (Figure 10). Control mice injected with 10% DMSO in PBS were not affected (data not shown). These data suggest that there may be a defect in Rgs2 -/- hematopoietic stem cells.

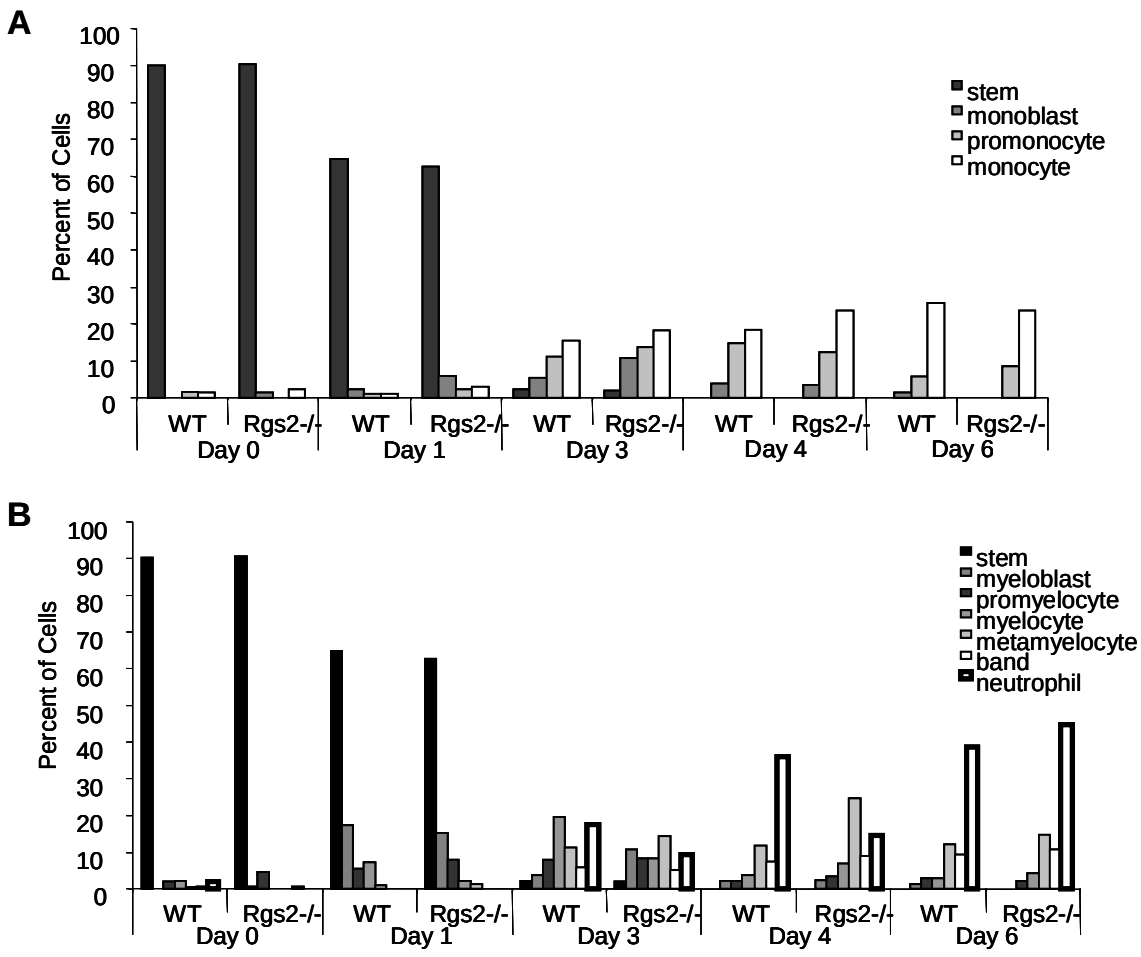

Figure 9. Rgs2 deficiency leads to a small delay in *in vitro* granulocytic differentiation. (A) Monocytic lineage. (B) Granulocytic lineage. Bone marrow was isolated from femurs and tibias of wild type and Rgs2-/- mice, and depleted of cells with lineage markers, followed by purification of Sca-1+ cells. The Lin-Sca-1+ cells were cultured in medium containing SCF and G-CSF to promote differentiation, and samples were taken on the indicated days, placed on slides using a cytospin centrifuge, and stained for morphology. The slides were scored by a hematopathologist in a blind fashion. The experiment was performed two times with bone marrow pooled from 8-10 mice per group.

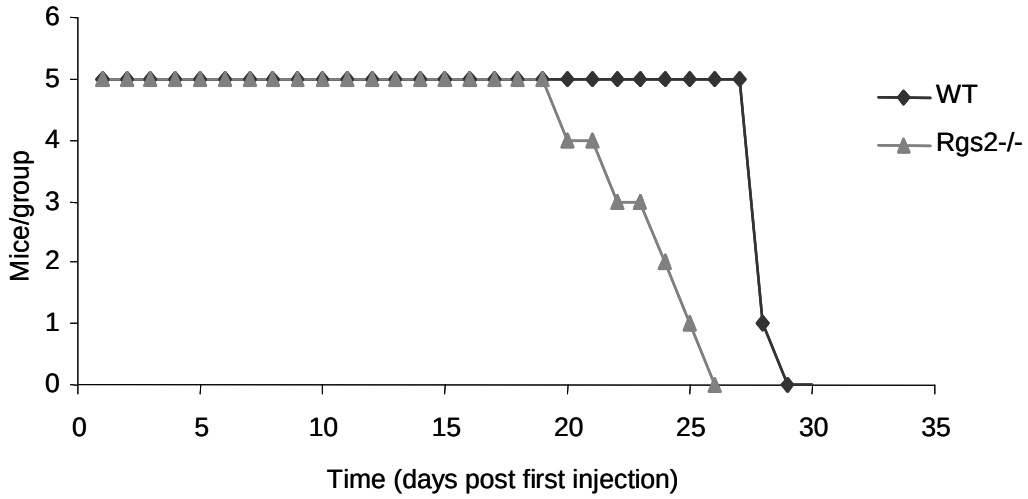

Figure 10. Rgs2-/- mice are more sensitive to serial 5-FU treatment. Mice were injected intraperitoneally with 150 mg/kg of 5-FU or 10% DMSO in PBS (vehicle) once per week until the mice began to show signs of distress. Upon signs of ruffled fur, hunched back, etc., the mice were sacrificed. A survival curve was plotted. Experiment was performed twice. n=5

Lack of Rgs2 does not have a major impact on stem cell function

In order to examine function of Rgs2 deficient stem cells, we first examined the levels of Sca-1+cKit+Flt3- long term stem cells and Sca-1+cKit+Flt3+ short term stem cells and multipotent progenitor cells. We isolated bone marrow from wild type and Rgs2 -/- mice, depleted the bone marrow cells of Lineage+ cells by magnetic activated cell sorting (MACS), and then used flow cytometry to observe the populations. We found that there was no difference in the percentages of stem cells and multipotent progenitors (Figure 11A). Next, we analyzed proliferation of Rgs2 deficient stem cells. Mice were injected with BrdU intraperitoneally, and bone marrow was isolated from the mice two hours later. The cells were then depleted of Lineage+ cells using MACS, followed by processing for BrdU, Sca-1, and cKit labeling. We used flow cytometry to observe BrdU labeling of the Lineage-Sca-1+cKit+ (LSK) stem cells. We did not observe a significant difference in proliferation as measured by BrdU incorporation between wild type and Rgs2 null stem cells (Figure 11B). We also investigated turnover of LSK cells between wild type and Rgs2-/- mice. We removed the bone marrow and isolated LSK cells as before, then used an Annexin V antibody to label dying cells. We did find that Rgs2-/- stem cells are significantly more positive for Annexin V (Figure 11C). However, the amount of Annexin V positive cells in either group is very low, and the increase in apoptosis in the Rgs2-/- stem cells is not reflected in the overall numbers of stem cells. These data, together, indicate that stem cell function is not substantially different between wild type and Rgs2-/- mice.

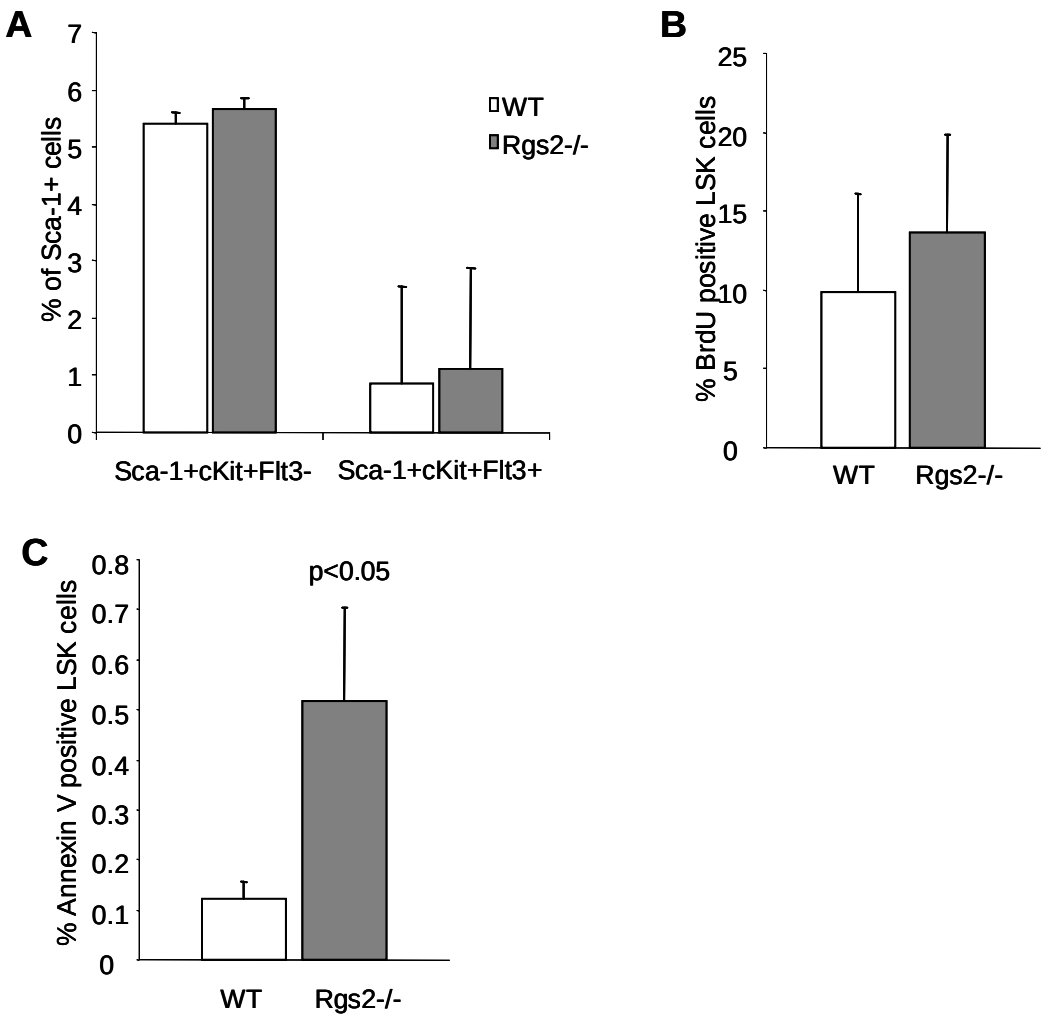

Figure 11. Rgs2-/- mice do not exhibit defects in stem cell function. (A) Bone marrow from wild type and Rgs2-/- mice was isolated from the femur and stained with antibodies against B220, c-Kit, Sca-1, and Flt-3, then analyzed by flow cytometry. Gates were placed on the B220-, Sca-1+ cells, and the percentages of c-Kit+Sca-1+Flt-3+ and c-Kit+Sca-1+Flt-3- cells were calculated and graphed. (B) Wild type and Rgs2-/- mice were injected with 1 mg of BrdU intraperitoneally. After 2 hours, bone marrow was isolated from the femurs and tibias, and depleted of cells with lineage markers. The Lin- cells were stained with antibodies against Sca-1 and c-Kit, fixed and permeabilized, treated with DNase, and labeled with anti-BrdU. Samples were read by flow cytometry. (C) Bone marrow was isolated from femurs and tibias of wild type and Rgs2-/- mice, and depleted of cells with lineage markers. The Lin- cells were then labeled with antibodies against Sca-1, c-Kit, and Annexin V, and analyzed by flow cytometry.

Rgs2 deficiency does not affect multipotent progenitor cells

As we observed a difference in 5-FU sensitivity, but did not see a difference in stem cell number or proliferation, and only a small difference in stem cell death, we then focused on the multipotent progenitor cells (MPPs). These cells are directly affected by 5-FU, and typically express the Flt3 receptor (Adolfsson et al., 2001; Christensen and Weissman, 2001; Guenechea et al., 2001; Wilson et al., 2008). Rgs2 has previously been implicated to play a role in Flt3 signaling (Schwable et al., 2005), suggesting that MPPs may be affected by loss of Rgs2. To examine this hypothesis, we first analyzed the levels of these cells in the bone marrow after a one time 5-FU injection at 48 and 72 hours (Figure 12A). We did not observe a difference in the levels of MPPs at either time point.

We next examined Flt3R responsiveness in an assay dependent on Flt3 signaling. Bone marrow cells were removed from tibias and femurs, and Lineage-Sca-1+ cells were isolated using MACS. The isolated cells were seeded on top of irradiated OP9-DL1 cells, and supplemented with 5 ng/ml Flt3 ligand and 5 ng/ml IL-7, to induce development and proliferation of T lymphocytes. The cells were filtered and passaged onto fresh OP9-DL1 cells with fresh growth factors on Day 7, and then again in 4 day intervals. Flt3 ligand was previously shown to promote proliferation without influencing differentiation in this co-culture assay, so we counted cells to assess response to Flt3 signaling (Wang et al., 2006). The cells were counted on the days indicated, and the numbers of cells at each time point were plotted (Figure 12B). We did not observe a difference in proliferation

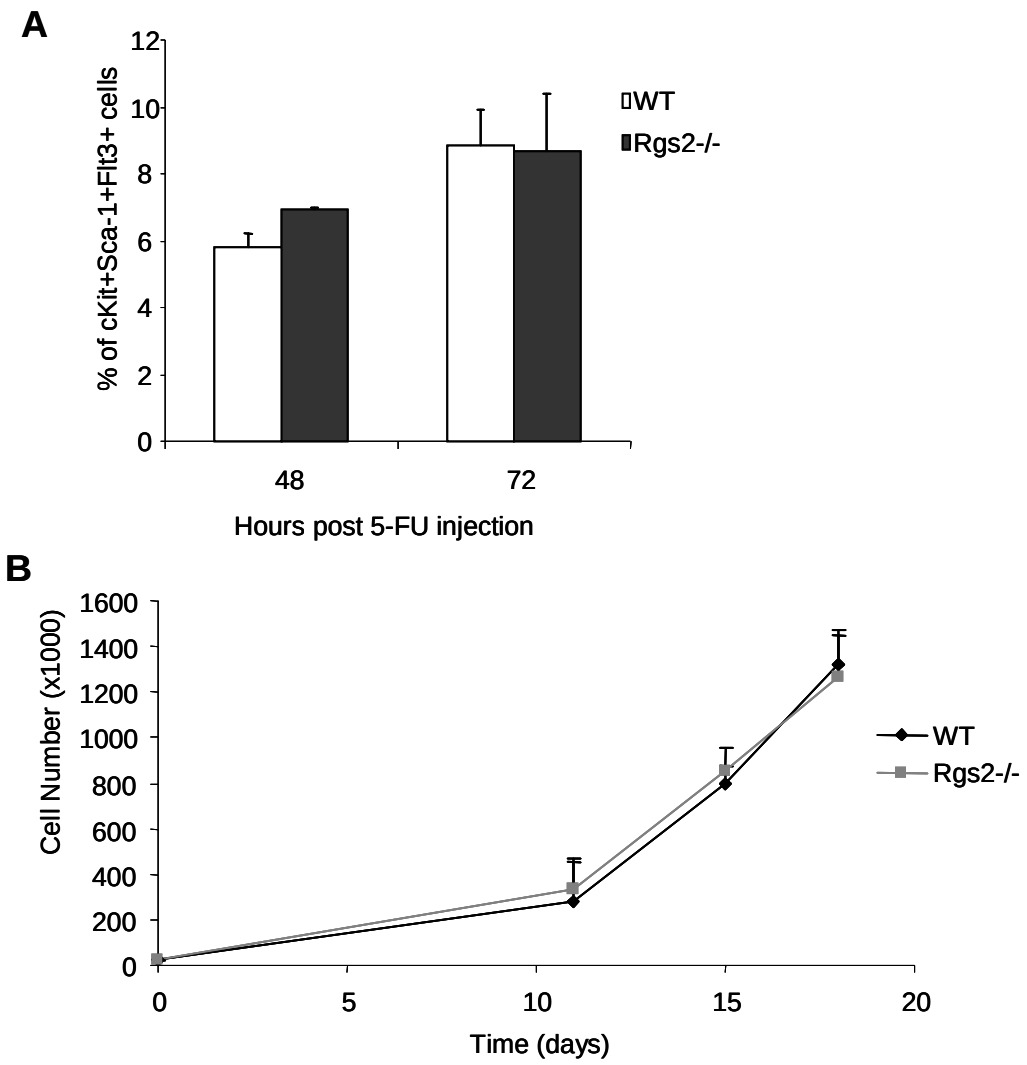

Figure 12. Rgs2 deficiency does not affect numbers of multipotent progenitors. (A) Wild type and Rgs2-/- mice were injected intraperitoneally with 150 mg/kg of 5-FU. At 48 and 72 hours post-injection, bone marrow was isolated and labeled with antibodies against c-Kit receptor, Sca-1, and Flt3 receptor, then analyzed by flow cytometry. The average percentages of triple positive cells at each time point is shown. n=3 (B) Bone marrow cells were isolated from wild type and Rgs2-/- mice, and Lineage- Sca-1+ cells were further isolated using MACS. The isolated cells were seeded on top of irradiated OP9-DL1 cells, supplemented with 5 ng/ml Flt3 ligand and 5 ng/ml IL-7. The cells were filtered and passaged onto fresh OP9-DL1 cells with fresh growth factors on Day 7, and then again in 4 day intervals. The cells were counted on the days indicated, and the average numbers of cells at each time point were plotted. n=3

between wild type and Rgs2 deficient cells, indicating that there is no difference in responsiveness to Flt3, as the role for this growth factor is one of proliferation in these cells.

Discussion

Based upon data presented in the literature, we hypothesized that Rgs2 played a role in hematopoiesis. We found that in accordance with published data, Rgs2 mRNA levels do increase as myeloid cell lines differentiate. We examined the populations of mature hematopoietic cells in wild type and Rgs2-/- mice by flow cytometry, and did not observe any differences. We also determined that while Rgs2 deficiency led to a delay in granulocytic differentiation *in vitro*, this delay was small and quickly overcome. Furthermore, the numbers of progenitor cells, analyzed by methocellulose assays, are not different between wild type and Rgs2 deficient mice. These data together indicate that Rgs2 does not play a role in hematopoiesis under normal conditions.

Since Rgs2 has been implicated as a stress response gene in other cell types, we wondered if Rgs2 might be involved in the hematopoietic response to stress. While we did not see a difference in mature cell populations after 5-FU treatment for 24 hours, we found that serial treatment with 5-FU led to increased lethality in Rgs2-/- mice, suggesting that stem cells in mice deficient for Rgs2 are exhausted more quickly. However, when we assayed stem cells directly, we did not find any major differences between Rgs2-/- and wild type mice. Since serial 5-FU had an increased effect on Rgs2 deficient mice, and 5-FU directly affects

actively proliferating cells, we wondered if the MPPs may have defects. If we were to see a difference in MPP production or differentiation, this might explain the delay in granulocytic differentiation we observed *in vitro*. We began with lineage negative, Sca-1 positive cells in that assay, which would include stem cells that had to move past the MPP stage, as well as MPPs. In AML cases with activating Flt3-ITD mutations, Rgs2 has been found to negatively regulate signaling through the Flt3-ITD receptor (Schwable et al., 2005). MPPs express the Flt3 receptor, and Flt3 signaling is important in proliferation of these cells. This led us to examine the role of Rgs2 in MPPs and their Flt3 ligand responsiveness. We did not observe a difference in numbers of MPPs after 5-FU treatment, suggesting that lack of Rgs2 did not affect MPP production or differentiation. Furthermore, we did not observe a difference in proliferation of MPPs in response to Flt3 ligand stimulation, indicating that Rgs2 may not play the same role in Flt3 wild type receptor signaling as in activated Flt3-ITD receptor signaling.

Our data collectively suggest that Rgs2 does not play a role in hematopoiesis under normal conditions or under stressful conditions. We have not ruled out the possibility that another RGS protein is compensating for the lack of Rgs2 in hematopoiesis. However, studies have shown that other closely related family members, such as Rgs3 and Rgs16, cannot inhibit the same signaling pathways or bind to the same receptors, respectively, in response to the same stimuli, indicating that compensation likely is not complete or that it may take several family members (Anger et al., 2007; Bernstein et al., 2004;

Hague et al., 2005). While Rgs2 mRNA levels increase as myeloid cells are differentiated, this could be a side effect of differentiation, possibly due to increased expression of a receptor upstream of Rgs2. In addition, the increased sensitivity to serial 5-FU treatment could be a result of a cell type other than hematopoietic cells. 5-FU is also known to have cardiac toxicity in humans, and several studies have highlighted important roles for Rgs2 in cardiac function (Ang et al., 2010; Hercule et al., 2007; Jensen et al., 2010; Stewart et al., 2010; Takimoto et al., 2009; Zhang et al., 2006; Zou et al., 2006). Our challenge of the mice with 5-FU could have led to an increase in cardiac toxicity in the Rgs2-/- mice compared to the wild type mice, enhancing illness in the mice. Further study is necessary to determine the underlying cause of increased serial 5-FU treatment sensitivity in the Rgs2 deficient mice.

CHAPTER III

RGS2 REGULATES PRO-ANGIOGENIC AND IMMUNE SUPPRESSIVE FUNCTIONS OF MYELOID DERIVED SUPPRESSOR CELLS IN THE TUMOR MICROENVIRONMENT

Summary

Tumor growth is intimately linked with stromal interactions, such as angiogenesis and immune response. Myeloid derived suppressor cells (MDSCs) are dramatically elevated in cancer patients and tumor bearing mice. MDSCs modulate the tumor microenvironment through attenuating host immune response and increasing vascularization. In searching for molecular mediators responsible for these pro-tumor functions, we found that Rgs2 is highly increased in tumor-derived MDSCs compared to control MDSCs. Genetic deletion of Rgs2 in mice resulted in a significant retardation of tumor growth, and the tumors exhibit increased cell death, decreased vascular density, and increased leukocyte infiltration. Consistently, while wild type MDSCs promoted tumor growth in reconstitution assays, Rgs2-/- MDSCs did not, showing similar growth as with 3LL cells alone. Molecular profiling identified that Rgs2-/- tumor MDSCs produce less MCP-1, leading to decreased angiogenesis, which could be restored with addition of recombinant MCP-1. Additionally, stimulated T cells in co-culture with Rgs2-/- MDSCs produced more IFNγ and less IL-4 than those

cultured with wild type MDSCs, indicating a switch from a type 2 to a type 1 immune response. Together, our data identify Rgs2 as a critical regulator of pro-angiogenic and immune suppressive functions of MDSCs in tumor conditions, likely through MCP-1 production.

Introduction

Over the past several years, it has become clear that the tumor microenvironment plays an important role in tumor progression. Tumors are comprised of several cell types, including fibroblasts, smooth muscle cells, endothelial cells, immune cells, and epithelial cells, each contributing to the microenvironment in ways we are only beginning to understand (Tlsty and Coussens, 2006). In addition to the cells present, the tumor microenvironment contains extracellular matrix (ECM) and other factors secreted by the tumor and stromal cells that can greatly affect tumor progression.

Immune suppression and promotion of angiogenesis are essential for tumor growth and progression. Interestingly, myeloid derived suppressor cells (MDSCs) possess both properties, and create an environment to facilitate tumor progression. MDSCs increase in tumor bearing hosts, including cancer patients, and this accumulation is mediated by inflammatory and angiogenic factors (Gabrilovich and Nagaraj, 2009). MDSCs are known to inhibit immune response by affecting other immune cells, including T cells, macrophages, etc. (Gabrilovich and Nagaraj, 2009). MDSCs are also known to promote a shift to a type 2, tumor-promoting response in macrophages (Sinha et al., 2007a). In addition, they also

infiltrate into tumors, and promote tumor vascularization, tumor growth, and metastasis through modulating VEGF bioavailability and protease activity in the tumor microenvironment (Murdoch et al., 2008; Shojaei et al., 2007b; Yang et al., 2004; Yang et al., 2008). The pro-angiogenic function of these myeloid cells is sufficient to confer tumor refractoriness to anti-VEGF treatment (Shojaei et al., 2007a), a common target for anti-angiogenic therapy. This further illustrates the importance of MDSCs in tumor progression, as well as in molecular therapies for cancer.

MDSCs are composed of a heterogeneous population of Gr-1+ CD11b+ immature myeloid cells. When MDSCs were led to differentiate into dendritic cells in mice *in vivo*, immune response improved (Li et al., 2004). In addition, in mice and human patients, treatment with all-trans retinoic acid (ATRA), a factor that induces differentiation, led to a decrease in immature myeloid cells while enhancing dendritic and T cell responses, underscoring the importance of these cells in the immune response to tumors (Kusmartsev et al., 2003; Mirza et al., 2006).

Rgs2 contains a conserved Regulator of G protein Signaling domain, and functions as a GTPase-activating protein (GAP) for several Gα subunits of G proteins (Druey et al., 1996; Koelle and Horvitz, 1996; Siderovski et al., 1996). Rgs2 enhances the intrinsic GTPase activity of the Gα subunit, and thereby decreases the time that the G protein subunits are dissociated, leading to decreased signaling (De Vries et al., 2000; Ross and Wilkie, 2000). Rgs2 is widely expressed, and is detected in most cell types and tissues (Chen et al.,

1997; Park et al., 2002; Reif and Cyster, 2000; Shi et al., 2004; Su et al., 2002). A variety of stimuli could induce Rgs2 expression, most of which signal through G proteins. Therefore, Rgs2 functions in a negative feedback loop with regard to G protein coupled receptors (GPCRs). In addition, cell stress, such as heat shock or DNA damage, can also increase Rgs2 levels (Song and Jope, 2006; Song et al., 2001; Zmijewski et al., 2001a). Rgs2 inhibits cell proliferation, and is a known mediator of cell differentiation in several cell types, including myeloid cells (Schwable et al., 2005).

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine important for cell migration (Loetscher et al., 1996; Melgarejo et al., 2009; Rollins, 1997; Rollins et al., 1991). It signals through CCR2, a GPCR found on monocytes, endothelial cells, T cells, etc. (Loetscher et al., 1996; Melgarejo et al., 2009; Salcedo et al., 2000). In part due to a migratory effect on endothelial cells, MCP-1 is a potent angiogenic factor, promoting vascularization *ex vivo* and *in vivo* (Salcedo et al., 2000). Blocking MCP-1 with a neutralizing antibody inhibited angiogenesis, and led to decreased tumor metastases and increased survival in a mouse tumor model (Salcedo et al., 2000). In addition, MCP-1 can affect T helper cell response. Mice deficient in MCP-1 cannot mount a T_H2 response, and several studies have linked MCP-1 expression with upregulation of type 2 cytokines, such as IL-4, and downregulation of type 1 cytokines, such as IFNγ (Gonzalo et al., 1998; Gu et al., 2000; Karpus et al., 1998; Karpus et al., 1997; Lu et al., 1998).

Here, we report a novel role of Rgs2 in tumor growth and progression. Tumor conditions upregulate Rgs2 expression in MDSCs, and inactivation of Rgs2 leads to a significant reduction of MCP-1 in MDSCs, which retards tumor angiogenesis and tumor progression, as well as mediates a shift in T cell response. Thus, this study identifies Rgs2 as a critical mediator of pro-angiogenic and immune suppressive functions associated with MDSCs in the tumor microenvironment. Therapeutically targeting Rgs2 activity could potentially not only inhibit tumor angiogenesis, but also reverse immune suppression, decreasing tumor progression.

Methods

Mice: Rgs2-/- mice were obtained from Dr. Josef Penninger at the Institute of Molecular Biotechnology GmbH, have previously been characterized (Oliveira-Dos-Santos et al., 2000), and were bred at Vanderbilt. Age and gender matched wild type control mice were purchased from Jackson Laboratories. Mice were kept in clean housing according to IACUC regulations.

Cell Lines: Human HL-60 and murine 3LL cell lines were cultured in RPMI 1640 + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% CO_2. The 3LL cell line is a subclone of the LLC (Lewis lung carcinoma) cell line from C57Bl/6 mice. The melanoma B16 cell line, also from C57Bl/6 mice, was grown in DMEM + 10% FBS + 1% penicillin/streptomycin at 37°C, 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza, and cultured in EGM-2

Bulletkit medium, also from Lonza, at 37°C, 5% CO_2. HUVECs were used between passages 3 to 7. HUVECS were transfected using the HUVEC AMAXA nucleofector kit according to manufacturer's instructions.

RT-PCR and Real Time PCR: RNA was isolated from cells using the RNeasy kit from Qiagen according to the manufacturer's protocol. When starting with primary cells, on column DNase digestion (Qiagen) was also performed according to the manufacturer's protocol. iScript cDNA synthesis kit (BioRad) was used according to the manufacturer's protocol to produce cDNA. cDNA was then used for PCR using the Hi-Fidelity PCR mix (Promega), or for real time PCR using the SYBR green kit (BioRad) and an iCycler or MyiQ machine (BioRad). For real time PCR, B-actin was used as an internal control.

Tumor cell injection: For tumor growth studies, 3LL or B16 cells were injected subcutaneously into the left hindlimbs of mice. Tumor size was measured by caliper, and tumor volume was calculated as volume= length x $(\text{width})^2$ x 0.5. For the reconstitution assay, 3LL cells were mixed with wild type or Rgs2-/- MDSCs isolated by fluorescence activated cell sorting (FACS) at a ratio of 10:1 and injected as before. Again, tumor size was measured by caliper and volume was calculated.

Tumor sections: Tumor samples were flash frozen in OCT (Sakura) or fixed in formalin and embedded in paraffin, then sectioned. Sections were incubated with

primary antibodies overnight (CD45, Gr-1, CD31 from BD Biosciences, PCNA from Santa Cruz, von Willebrand Factor from Dakocytomation, and active caspase-3 from Promega), and then either fluorescent or biotin-labeled secondary antibodies for immunofluorescent or immunohistochemical analysis, respectively.

Isolation of lung microvascular cells: Procedure was performed as previously described (DeBusk et al., 2010). Briefly, mice were sacrificed, and the chest cavity was opened. The right ventricle of the heart was punctured with a 25 gauge butterfly needle and syringe. The heart and lungs were flushed with PBS + 2 mM EDTA until the lungs were white, followed by 0.25% trypsin + 2 mM EDTA until the lungs were pink. Lungs were removed, pumped with more trypsin, and allowed to incubate at 37°C for 20 minutes. The lungs were then diced, and the tissue was pipetted several times in DMEM + 10% FBS. After centrifugation, the cell pellet was resuspended in EGM, and seeded into plates. Medium was changed the next day to remove cell debris and red blood cells.

Flow cytometry, FACS, and MACS: Tissues were prepared into single cell suspensions and labeled with antibodies for markers of mature and immature blood cells (BD Biosciences). Cells were analyzed using a BD LSRII or BD FACScan.

For fluorescence activated cell sorting (FACS), spleen cells were labeled with Gr-1 (Miltenyi Biotec) and CD11b (BD Biosciences) and sorted in the VA Flow Cytometry Resource.

For magnetic activated cell sorting (MACS) of MDSCs, tumors were digested with 3 mg/ml collagenase A (Sigma) and 100 units/ml hyaluronidase (Sigma) and processed into single cell suspensions. Spleens were processed into single cell suspensions. The cells were labeled with anti-Gr-1-PE (Miltenyi), then anti-PE multisort beads, and run through a column. Then, the beads were cleaved enzymatically, and the Gr-1+ cells were labeled with CD11b beads, and run through another column. The resulting cells were > 98% pure for spleen MDSCs, and > 80% pure for tumor MDSCs.

MDSC morphology: MDSCs were isolated from spleens of wild type and Rgs2-/- tumor bearing mice by MACS. The samples were placed on a slide using a cytospin, stained with the Three Step Stain (Richard Allan Scientific), and analyzed for morphology by a histopathologist.

Migration assays: Tumor MDSCs were isolated by MACS and cultured in RPMI, 1% FBS, 2% penicillin/streptomycin overnight. Transwells with 8 micron pores (Corning) were coated with fibronectin in 0.1% gelatin at 37°C one hour prior to addition of HUVECs. Transwells were placed over the cultured MDSCs, and HUVECs were added in basal RPMI at 1 x 10^5/ well and allowed to migrate for 3.5 hours. The Transwells were then fixed in formalin and stained with crystal

violet. Migrated HUVECs were counted under microscopy. For primary endothelial cells and HUVECs overexpressing Rgs2, $1x10^5$ cells were placed in the top chamber of a Transwell in EBM, and migrated to EGM for 4 hours. Cells on the bottom of the Transwell were fixed, stained with crystal violet, and counted.

Tube formation assay: MDSCs were isolated by MACS from tumor tissue, and cultured in EBM (Lonza) plus 1% FBS and 2% penicillin/streptomycin overnight. Wells of 48-well plates were coated with Matrigel (BD Bioscience) and incubated at 37°C until Matrigel was firm. HUVECs were suspended in MDSC conditioned medium and plated over Matrigel at $8x10^4$ cells/ well. Tube formation was scored by counting branch points at 48 and 72 hours.

Cytokine array: Tumor MDSCs were isolated using MACS, and the cytokine array (RayBiotech) was performed according to the manufacturer's protocol. Briefly, the cells were lysed in the presence of protease inhibitors, and the lysates at equal protein amounts were incubated on membranes overnight. The membranes were incubated with biotin-conjugated cytokine antibodies, then HRP-conjugated strepavidin, then detection buffer, before exposure to X-ray film. Film was developed, and analyzed by densitometry, with each band being normalized to internal controls.

MDSC and splenocyte co-culture: MDSCs were isolated from tumors using MACS, and incubated overnight in 96-well tissue culture treated plates at

125,000 cells/ well in 100 ul RPMI 1640 plus 10% heat inactivated FBS, 2mM L-glutamine, and 1% penicillin/streptomycin. 96-well ELISA plates were coated with 2 ug/ml CD3 antibody (BD Biosciences) for 3 hours at room temperature, and then washed. Splenocytes from C57Bl/6 mice were isolated, and plated at 125,000 cells/well in 100 ul in the coated plates. The MDSCs were then transferred to the wells containing splenocytes, and they were co-cultured for 24 hours. The cells were then removed from stimulation, and cultured for 48 hours. The cells were transferred to a new CD3 antibody-coated plate for restimulation, and incubated for 48 hours. Media was removed and assayed by ELISA for IL-4 and IFNγ.

Statistical analysis: Data were averaged and compared using Student's t test. Error bars on graphs represent standard error across experiments.

Results

Rgs2 is dramatically increased in tumor derived MDSCs

It is well documented that MDSCs from tumor bearing mice function differently from those from non-tumor bearing mice. In an effort to investigate the mechanism the tumor utilizes to condition the MDSCs, we isolated MDSCs from spleens of BALB/c mice, either tumor bearing or non-tumor bearing, using magnetic activated cell sorting. We were able to purify the Gr-1+CD11b+ cells to 98% purity (Figure 13A). We then compared Rgs2 mRNA levels. We found that Rgs2 was dramatically upregulated in MDSCs from tumor bearing mice

compared to control mice (Figure 13B-C). Further analysis showed that hypoxia, a stress commonly associated with tumors, significantly increased Rgs2 mRNA levels in the human myeloid cell line, HL-60, *in vitro* (Figure 13D), consistent with published studies indicating that Rgs2 may be a stress response gene, increasing rapidly under conditions which are stressful for the cell. In addition, we found Rgs2 mRNA levels were affected in HL-60 cells by treatment with various factors (Figure 14). One hour treatment with GM-CSF, M-CSF, and IL-3 all significantly decreased Rgs2 levels compared to untreated control. In addition, treatment with 3LL tumor conditioned medium (containing 10% FBS), mediated a small, but significant increase in Rgs2 levels. However, treatment with inflammatory factors, such as IL-1β or TNFα, did not affect Rgs2 mRNA levels.

Lack of Rgs2 in MDSCs retards tumor growth

Because MDSCs of tumor bearing mice upregulate Rgs2 expression, we sought to understand the role Rgs2 plays in MDSCs in tumor progression using Rgs2 knockout mice. Mice without Rgs2 are viable, healthy, and fertile, but have defects in hippocampal development (Oliveira-Dos-Santos et al., 2000). We injected Rgs2-/- mice and syngeneic C57BL/6 wild type controls with 3LL cells subcutaneously in the hindlimb, and measured tumor growth over time. The null mice exhibited significantly lower tumor volumes than the wild type mice (Figure 15A). Similar results were achieved when the mice were injected with a melanoma tumor line, B16 (Figure 15B). These results reveal a positive role of Rgs2 in tumor growth and progression.

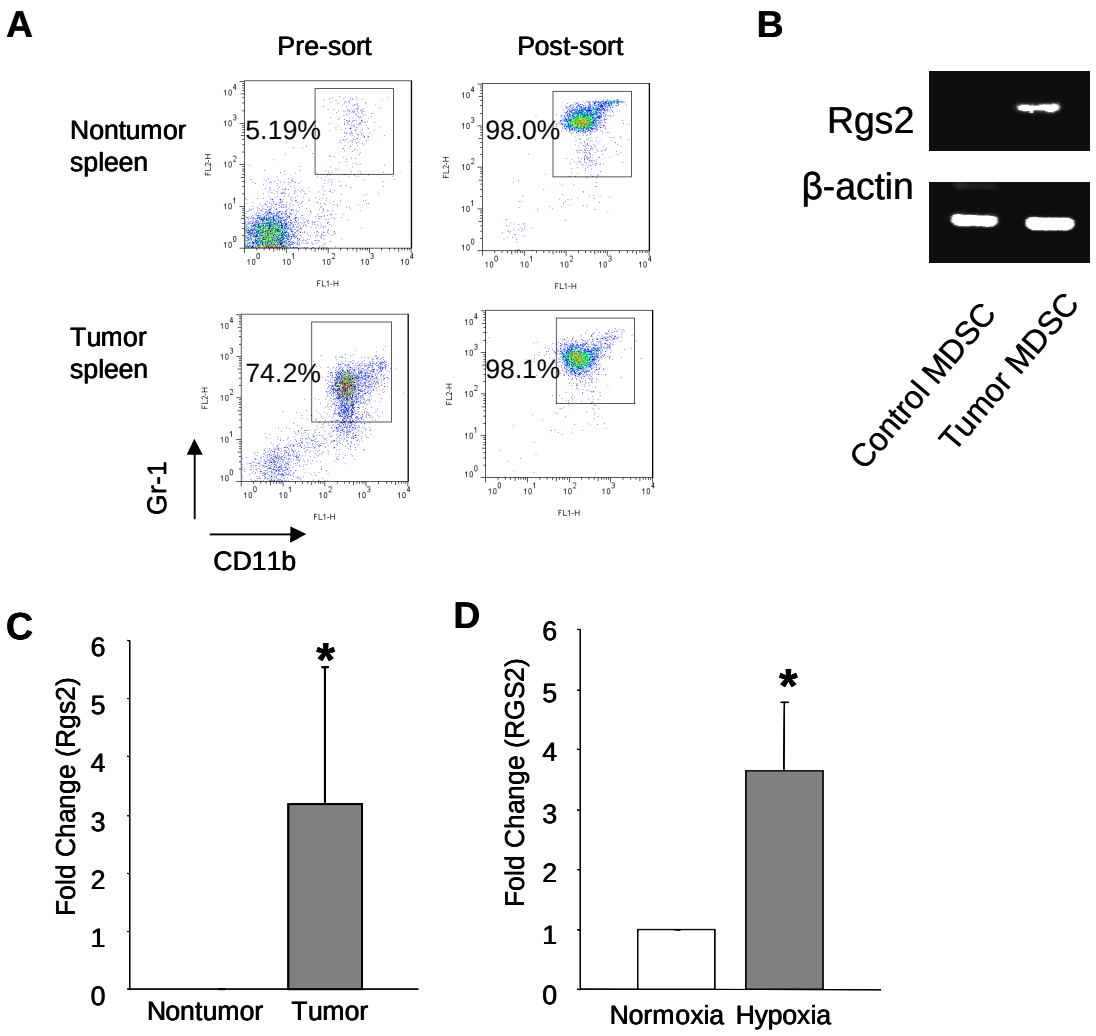

Figure 13. Induction of Rgs2 in tumor derived MDSCs. (A) Purity of cells from isolation of Gr-1+CD11b+ cells using MACS. Spleens from non-tumor bearing and MC26 tumor bearing BALB/c mice were isolated and processed into single cell suspensions, followed by sorting using MACS, as described in the Methods section. (B) and (C) Gr-1+CD11b+ cells were isolated from non tumor bearing and tumor bearing mice, MC26 tumors in BALB/c (B) and 3LL tumors in C57BL/6 (C), by magnetic sorting of pooled splenocytes from 5-10 mice, generating MDSCs of greater than 98% purity. Cells were then subjected to RNA isolation and RT-PCR (B) and real time PCR (C) for Rgs2 expression. These experiments were repeated 3 times. (D) HL-60 cells were incubated under normoxic (20% O2) or hypoxic (1.0-2% O2) conditions for an hour, and RNA was isolated, followed by real time PCR analysis. This experiment was performed 5 times. * $p < 0.05$.

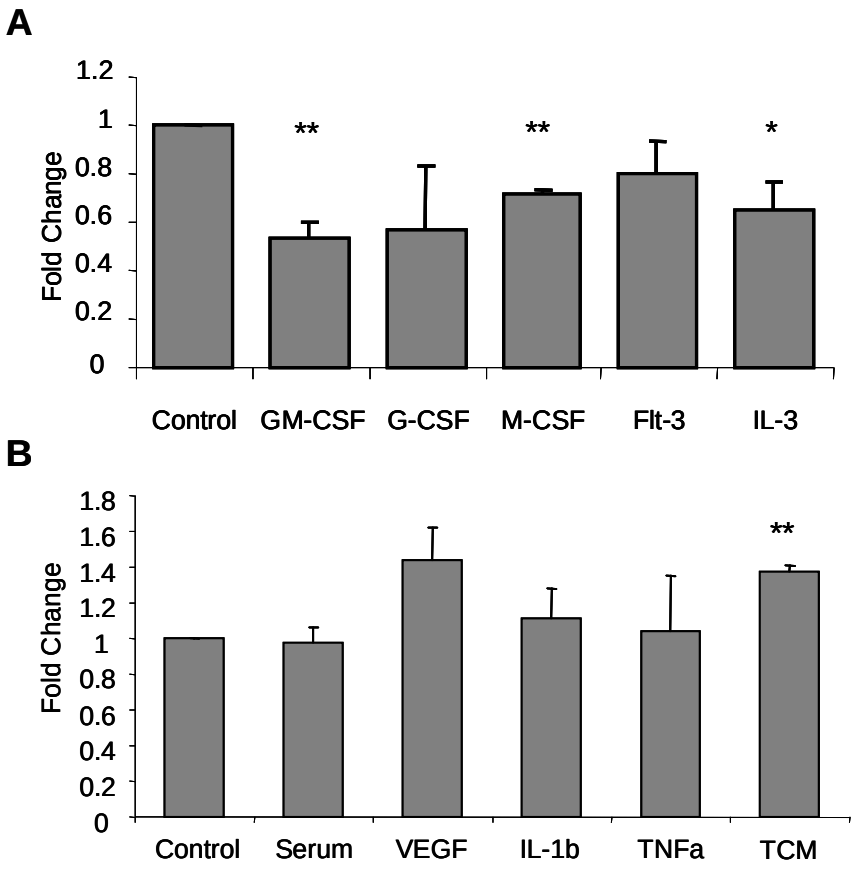

Figure 14. Rgs2 mRNA levels in HL-60 cells are affected by angiogenic and inflammatory factors. (A) HL-60 cells were treated for one hour with GM-CSF (10ng/ml), G-CSF (10ng/ml), M-CSF (10ng/ml), Flt-3 ligand (50ng/ml), or IL-3 (20ng/ml) in growth medium. Control is HL-60 cells in growth medium. Cells were collected, and RNA was isolated. RT-PCR was performed, followed by real time PCR in duplicate. n=2 (B) HL-60 cells were treated with the following factors: Serum (20%), VEGF (20 ng/ml), IL-1beta (10 ng/ml), TNFalpha (10 ng/ml), or 3LL tumor conditioned medium. Cells were collected after 30 min, and RNA was isolated. RT-PCR was performed, followed by real time PCR in duplicate. β-actin was used as an internal control, and fold changes were calculated and plotted. * $p<0.05$, ** $p<0.01$

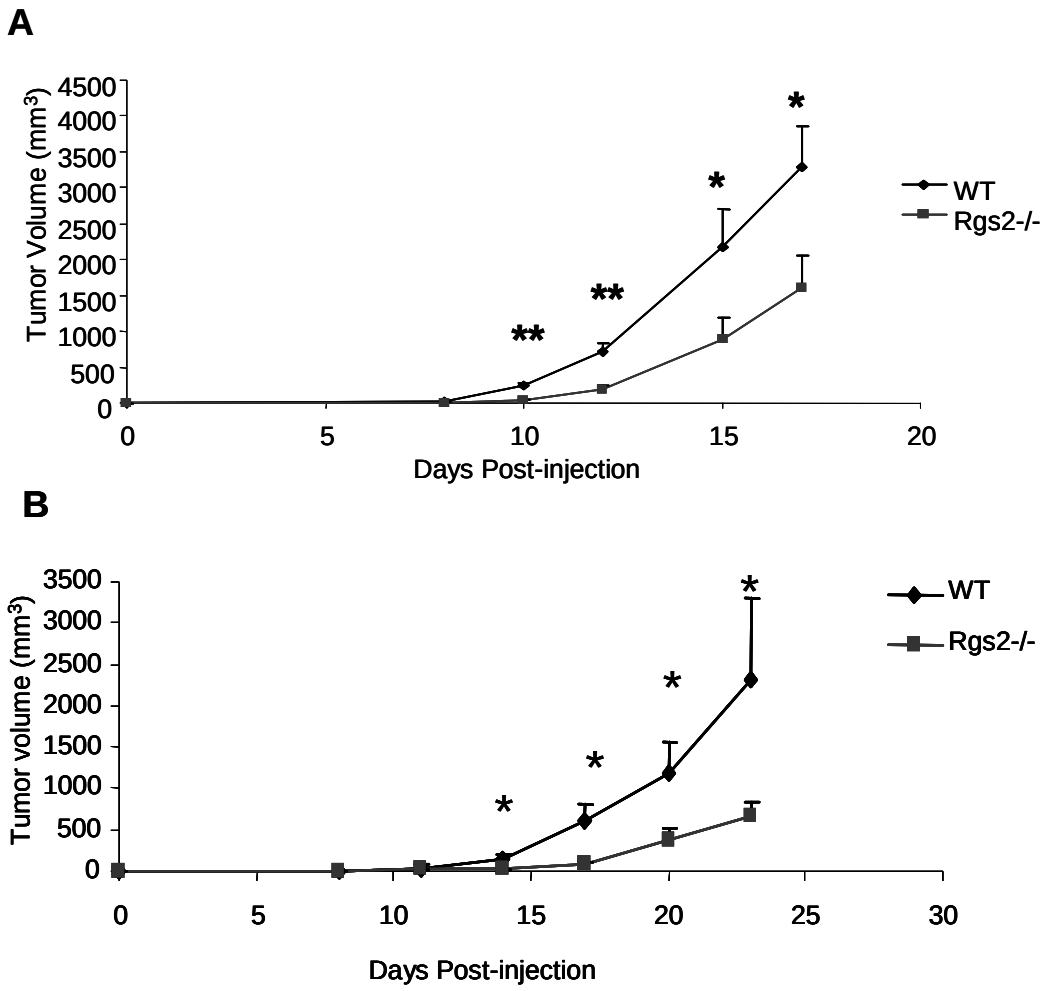

Figure 15. Tumor growth is retarded in Rgs2 deficient mice. (A) Rgs2-/- and C57BL/6 wild type mice were injected with $5x10^5$ 3LL tumor cells subcutaneously in the hindlimb, and tumor size was measured by caliper over time (n=12 mice per group). This experiment was repeated 3 times. (B) Rgs2-/- and C57Bl/6 wild type mice were injected with $1x10^5$ B16 tumor cells subcutaneously in the hindlimb, and tumor size was measured by caliper over time. n=5 mice per group. * $p< 0.05$.

To examine the role Rgs2 plays specifically in MDSCs, we performed a reconstitution experiment. MDSCs were isolated from spleens of tumor bearing wild type or null mice by magnetic sorting using antibodies against Gr-1 and CD11b. We achieved greater than 95% purity (data not shown). These MDSCs were co-injected with 3LL cells subcutaneously in the hindlimb of wild type mice, and tumor growth was measured over time (Figure 16). We found that while wild type MDSCs were able to promote tumor growth, Rgs2-/- MDSCs lost this tumor promoting function, compared to growth of the tumor cells alone or in the presence of wild type MDSCs (Figure 16). These data demonstrate an essential role of Rgs2 in the tumor promoting function associated with MDSCs *in vivo*.

Tumors from Rgs2-/- mice exhibit decreased vascularization and increased cell death

MDSCs are known to infiltrate into tumors and promote tumor angiogenesis. Histological analysis of size-matched tumors from wild type and Rgs2-/- mice revealed a significantly lower vascular density, as measured by CD31-positive vessel structures, in tumors harvested from null mice than from wild type controls (Figure 17A-B). Similar results were observed when we used another vascular marker, von Willebrand factor (data not shown). Consistently, there is a significant increase in cell death, as indicated by cleaved caspase-3 staining in tumors from the null mice (Figure 17C-D). We did not see any significant difference in cell proliferation, as measured by PCNA staining,

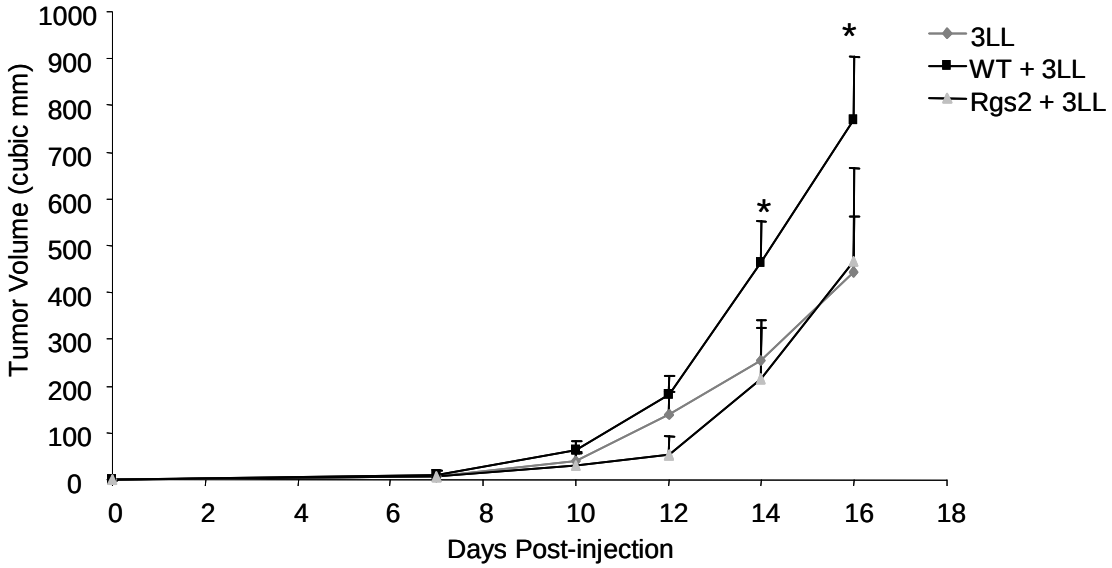

Figure 16. Decreased tumor growth in Rgs2-/- mice is due to lack of Rgs2 in MDSCs. Wild type mice were injected subcutaneously in the hindlimb with $1x10^5$ 3LL cells alone, or 3LL cells combined with $1x10^4$ wild type or Rgs2-/- MDSCs sorted by flow cytometry (>95% purity; data not shown) from spleens of tumor-bearing mice. Tumor growth was measured by caliper over time. n=8 mice per group. This experiment was performed twice. * $p<0.05$.

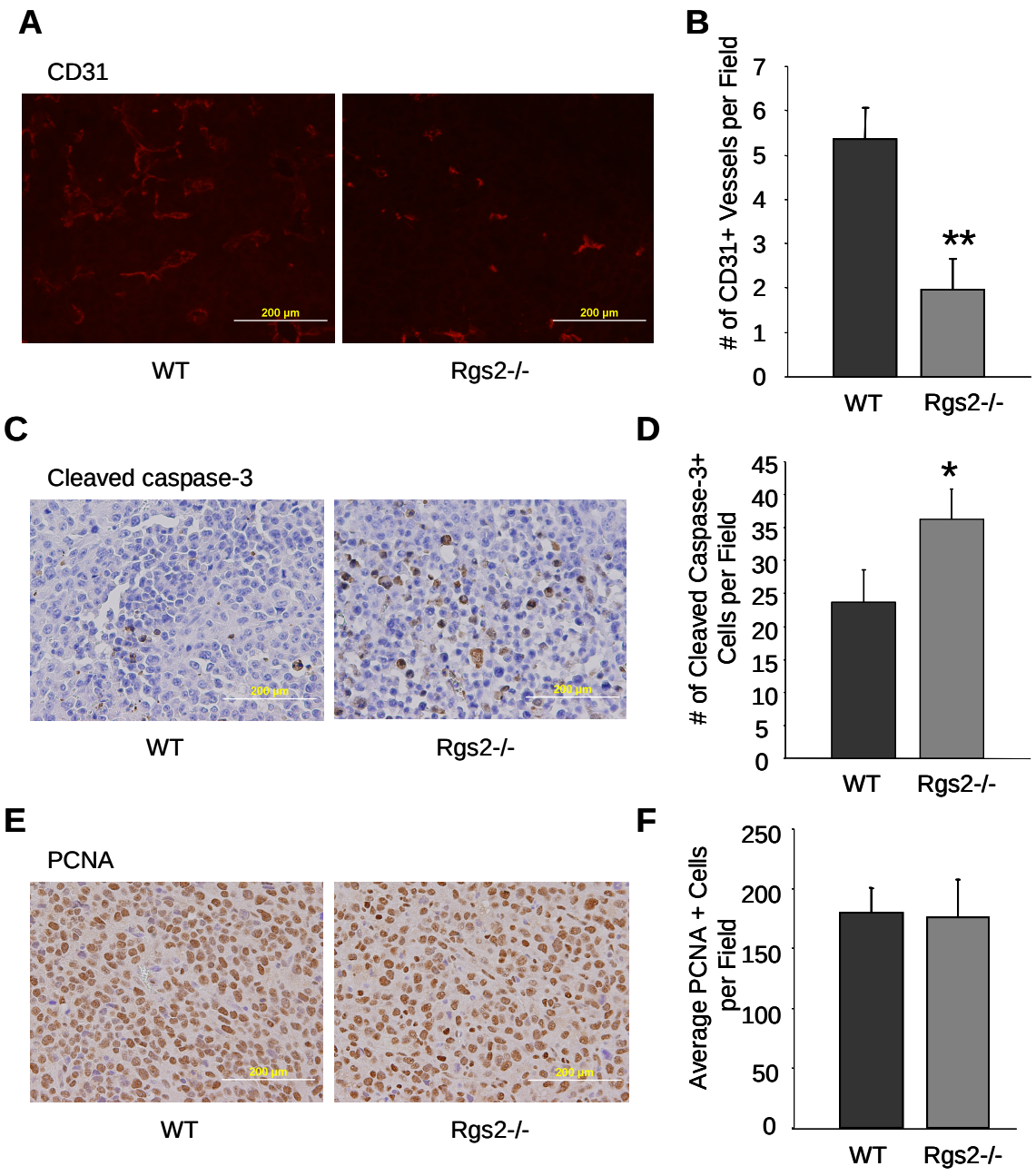

Figure 17. Tumors in Rgs2-/- mice exhibit decreased vascular density and increased cell death. (A), (C), (E) Sections from size matched 3LL tumors grown in wild type mice and syngeneic Rgs2 null mice were stained for CD31, active caspase-3 and PCNA, respectively. Representative images are shown. (B), (D), (F) The numbers of CD31 positive vascular structures, active caspase-3 positive cells, and PCNA positive cells, respectively, were quantified in 10 randomly selected fields under microscopy. These experiments were repeated 3-4 times. ** $p<0.005$, * $p<0.05$.

between the two groups (Figure 17E-F). These findings point to an angiogenic role of Rgs2 in MDSCs in tumor growth and progression.

Since we were interested in the immune cells present in the tumor, tumor infiltrating leukocytes were examined using a pan leukocyte marker, CD45. Surprisingly, we found that the null tumors exhibited significantly higher levels of leukocytes (Figure 18A-B). Furthermore, we found that the tumors in null mice had significantly higher numbers of Gr-1 positive cells, a marker for granulocytic myeloid cells, including MDSCs (Figure 18C-D). Together, these data are indicative of an important role of Rgs2 in modulating MDSC function and/or differentiation.

Lack of Rgs2 in endothelial cells does not seem contribute to the tumor phenotype

We first examined how the lack of Rgs2 affects endothelial cells, since there is a decrease in vessel density, and the mice are not a conditional knockout model. We performed assays with endothelial cells (ECs) isolated from the lungs of wild type and Rgs2-/- mice. We were not able to detect a difference in migration of wild type and Rgs2-/- ECs using a Transwell migration assay (Figure 19A). Furthermore, we did not observe a difference in BrdU incorporation or proliferation, measured *in vitro* (Figure 19B-C). We also did not observe a difference in survival (data not shown). Lastly, we did not observe a difference in tube formation on Matrigel between the wild type and Rgs2-/- ECs (Figure 19D).

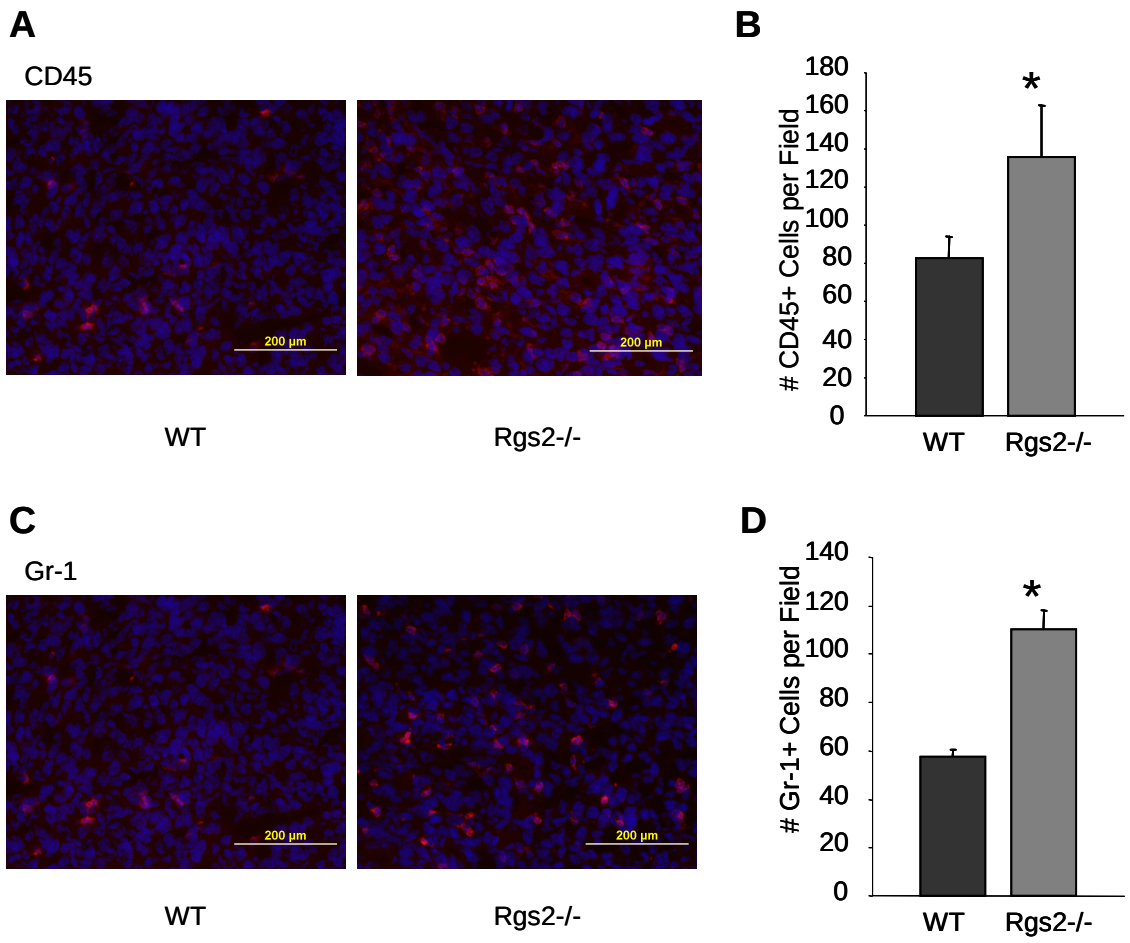

Figure 18. Tumors from Rgs2-/- mice exhibit increased leukocyte infiltration. (A),(C) Size matched 3LL tumors grown in wild type and syngeneic Rgs2 null mice were sectioned and stained for CD45 and Gr-1, respectively, and examined by immunofluorescent microscopy. (B),(D) Representative images were shown. The number of CD45 and Gr-1 positive cells were quantified in 10 randomly selected fields under microscopy. Experiments were performed 4 times each. * $p<0.05$.

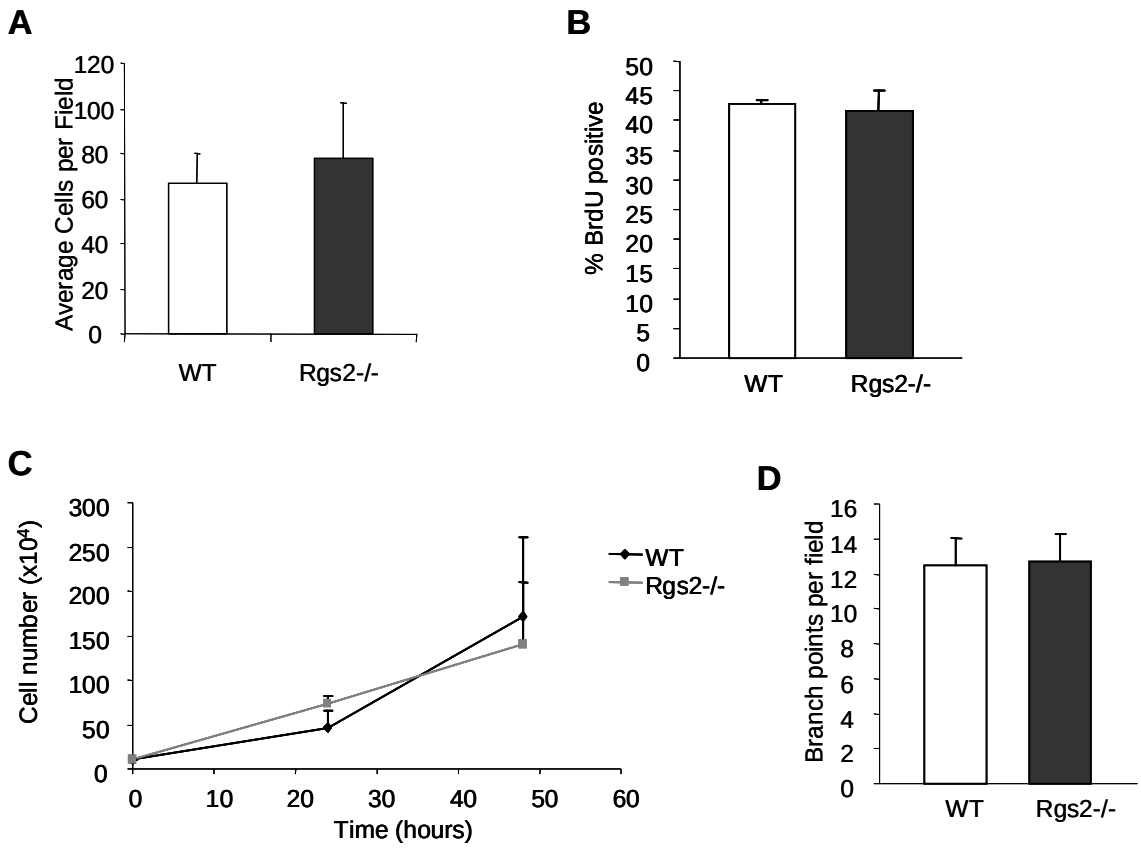

Figure 19. Lack of Rgs2 in endothelial cells does not affect migration, BrdU incorporation, proliferation, or tube formation. Lung microvascular endothelial cells were isolated from lungs of wild type and Rgs2-/- mice and cultured. (A) $1x10^5$ cells were placed in the top chamber of a Transwell, and migrated to growth medium for 4 hours. Cells on the bottom of the Transwell (migrated cells) were fixed, stained with crystal violet, and counted. Experiment was performed 3 times in duplicate. (B) Cells were pulsed with 10 uM BrdU for 2 hours, then collected, processed, and analyzed by flow cytometry. The average percentages of BrdU positive cells were plotted. Experiment was performed 2 times in duplicate. (C) $1x10^5$ cells were seeded in growth medium and proliferation was scored by counting cells at 24 and 48 hours. The average number of cells at each time point was plotted. Experiment was performed twice in duplicate. (D) Cells were seeded on top of Matrigel in growth medium and allowed to form tube structures for 48 hours. Branch points were counted, and the average number per field was graphed.

These data suggest that the phenotype we observe in the tumor is not due to lack of Rgs2 in ECs. As such, we focused on the MDSCs.

Rgs2 does not play a major role in MDSC expansion and differentiation

MDSCs are elevated in tumor bearing hosts, and display different differentiation profiles compared to cells from non-tumor bearing hosts (Yang, 2004). Since Rgs2 is elevated in tumor derived MDSCs, we first determined whether Rgs2 has a role in MDSC expansion by analyzing spleens from tumor bearing wild type and null mice by flow cytometry. We found that spleens of both mice contained similar numbers of MDSCs (Figure 20A). Then, to determine if the lack of Rgs2 affects the proportions of the cells that comprise the heterogeneic Gr-1+CD11b+ fraction, we isolated Gr-1+CD11b+ cells from spleens of tumor bearing wild type and Rgs2-/- mice and phenotyped them by morphology (Figure 20B). We observed a small, but significant decrease in the more monocytic MDSCs. However, no other cell types were different. We also analyzed MDSCs from spleen (Figure 21) and bone marrow (data not shown) by flow cytometry using various myeloid markers, and consistent with our other findings, there were no significant differences between wild type and null MDSCs. In addition, we examined the role of Rgs2 deficiency in production of T cells, B cells, monocytes, dendritic cells, NK cells, and granulocytes within the spleen (Figure 22) and bone marrow (data not shown) of wild type and Rgs2 -/- mice with or without tumors, and we saw no difference in any of these populations. These data together indicate that Rgs2 plays a role other than expansion and

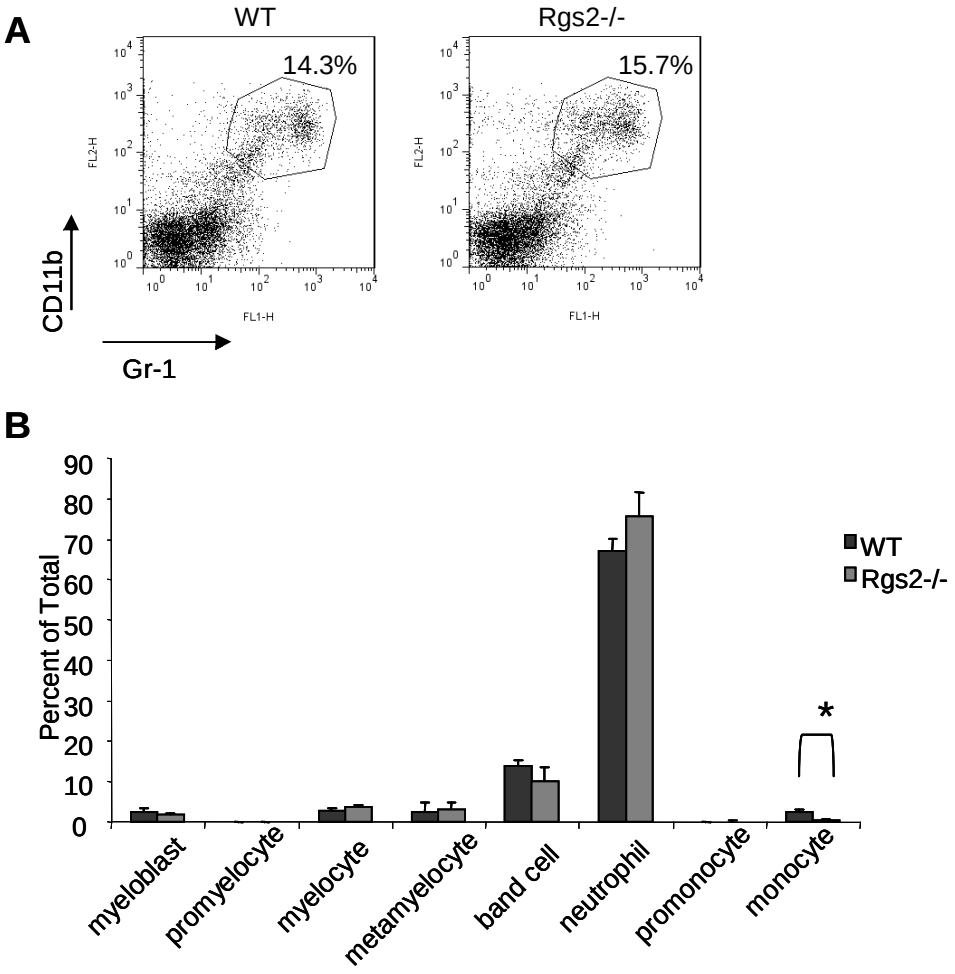

Figure 20. Rgs2 deficiency has minimal effects on MDSC expansion and differentiation. (A) Wild type and Rgs2-/- mice were injected with $1x10^5$ 3LL cells in the hindlimb, and 15-20 days later, spleens were isolated and analyzed by flow cytometry for Gr-1+CD11b+ MDSCs. This experiment was performed at least 3 times, and the graphs shown are results from pooling of 3 mice per group. (B) MDSCs were isolated from spleens of tumor bearing Rgs2-/- and wild type mice using the MACS system, spun onto slides using a cytospin centrifuge, and stained. The slides were read by a hematopathologist in a blind fashion, and cells were categorized by morphology. This experiment was performed 4 times. * p< 0.01.

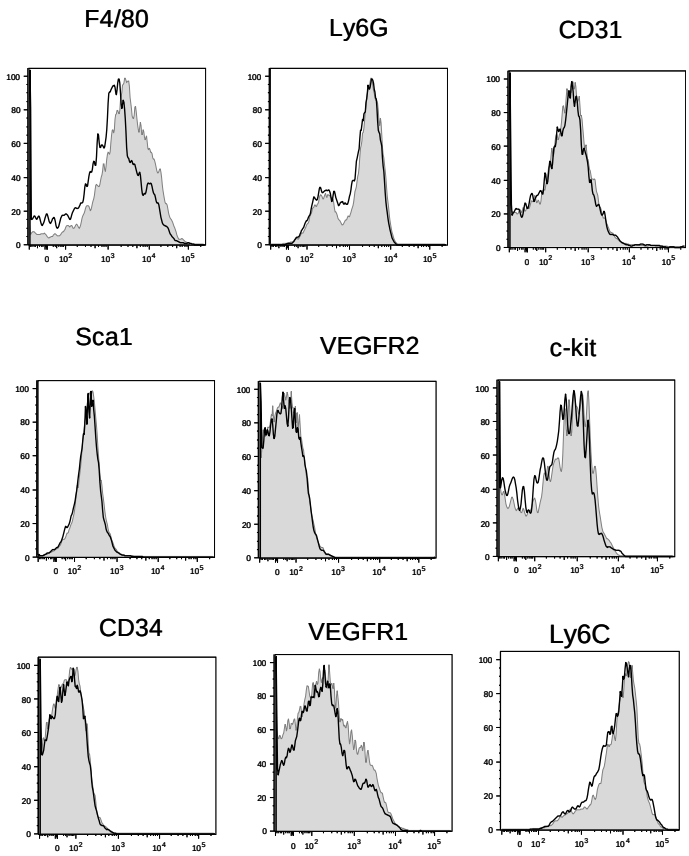

Figure 21. Analysis of cell surface molecules on Gr-1+CD11b+ MDSCs. Spleens were harvested from 3LL tumor bearing WT or Rgs2-/- mice between days 17-21 post-injection, processed into single cell suspensions, and labeled with antibodies against the indicated cell surface molecules. Representative plots are shown. Experiment was performed 3 times with 3-4 mice per group. Shaded = WT, open = Rgs2-/-.

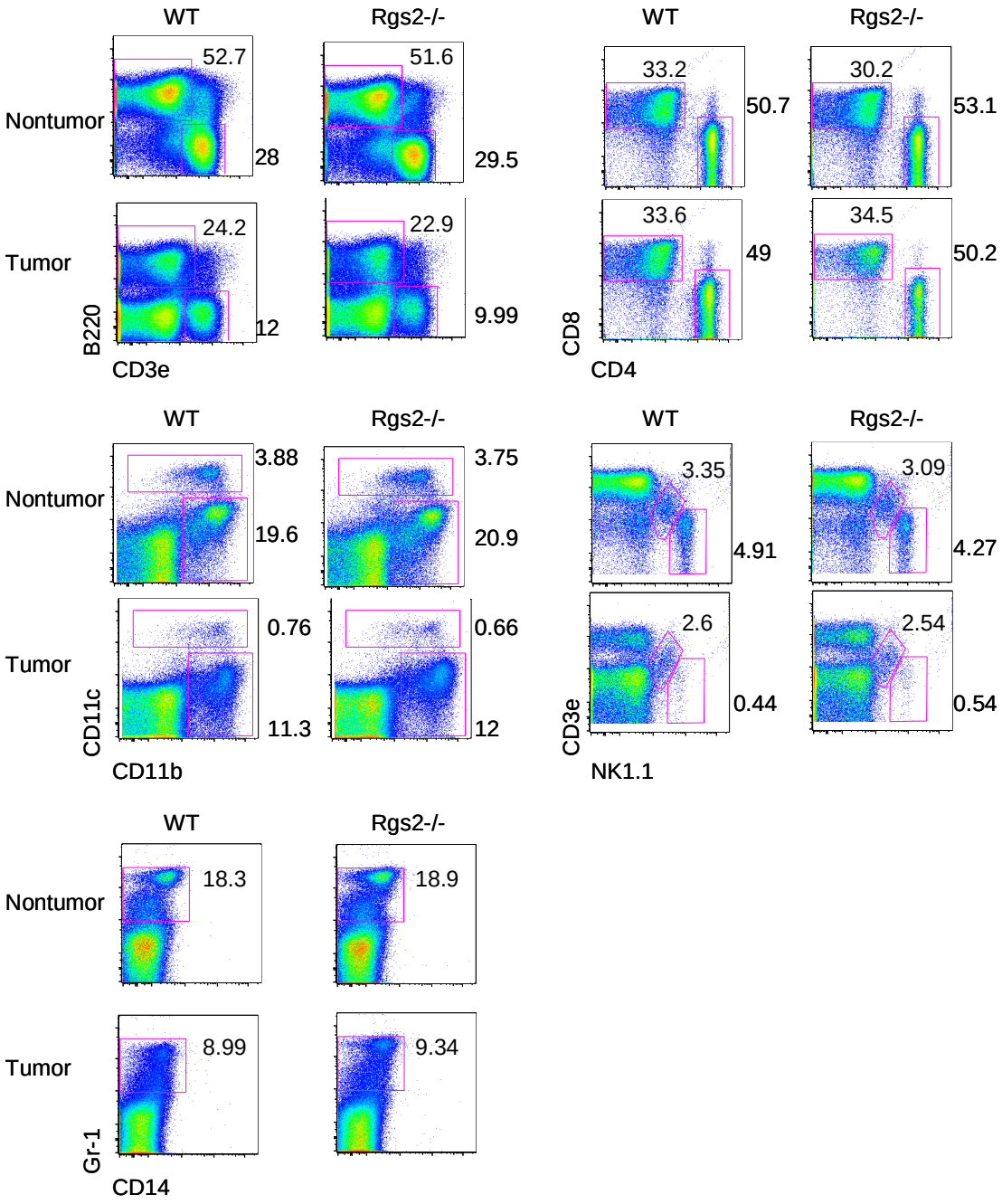

Figure 22. Lack of Rgs2 does not affect populations of mature leukocytes. Spleens were isolated from non-tumor bearing and 3LL tumor bearing WT and Rgs2-/- mice, processed into single cell suspensions, and labeled with the indicated antibodies, then analyzed by flow cytometry. Experiment was performed 3 times with 3-4 mice per group.

differentiation in MDSCs. This led us to consider a role of the gene in regulating MDSC function.

Rgs2 positively regulates MCP-1 in MDSCs

A major role for MDSCs is secretion of factors, contributing to the cytokine milieu that promotes tumor growth. To determine if lack of Rgs2 affects cytokine production, we performed a protein cytokine array on the lysates of wild type and null MDSCs isolated directly from tumor tissue. We found a dramatic reduction (~9-10-fold) in MCP-1 in null MDSCs compared to wild type MDSCs (Figure 23A). Furthermore, real time RT-PCR using RNA isolated from tumor-derived MDSCs confirmed that, similar to the cytokine array, MCP-1 mRNA levels were significantly reduced in Rgs2 null MDSCs compared to wild type MDSCs (Figure 23B). Moreover, cultivation of MDSCs isolated from tumors of wild type and Rgs2-/- mice for 48 hours revealed a dramatic reduction in the MCP-1 levels secreted by Rgs2-/- MDSCs (Figure 23C). We also correlated MCP-1 mRNA levels to Rgs2 mRNA levels (Figure 23D). We analyzed RNA from spleens of nontumor bearing wild type mice and tumors of tumor bearing wild type mice, and found that as Rgs2 levels increase in MDSCs from tumors, so do MCP-1 levels.

Rgs2 mediates pro-angiogenic function in MDSCs through induction of MCP-1

MCP-1 is a potent angiogenic factor, and we found that tumors in Rgs2-/- mice have decreased vascular density compared to tumors in wild type mice

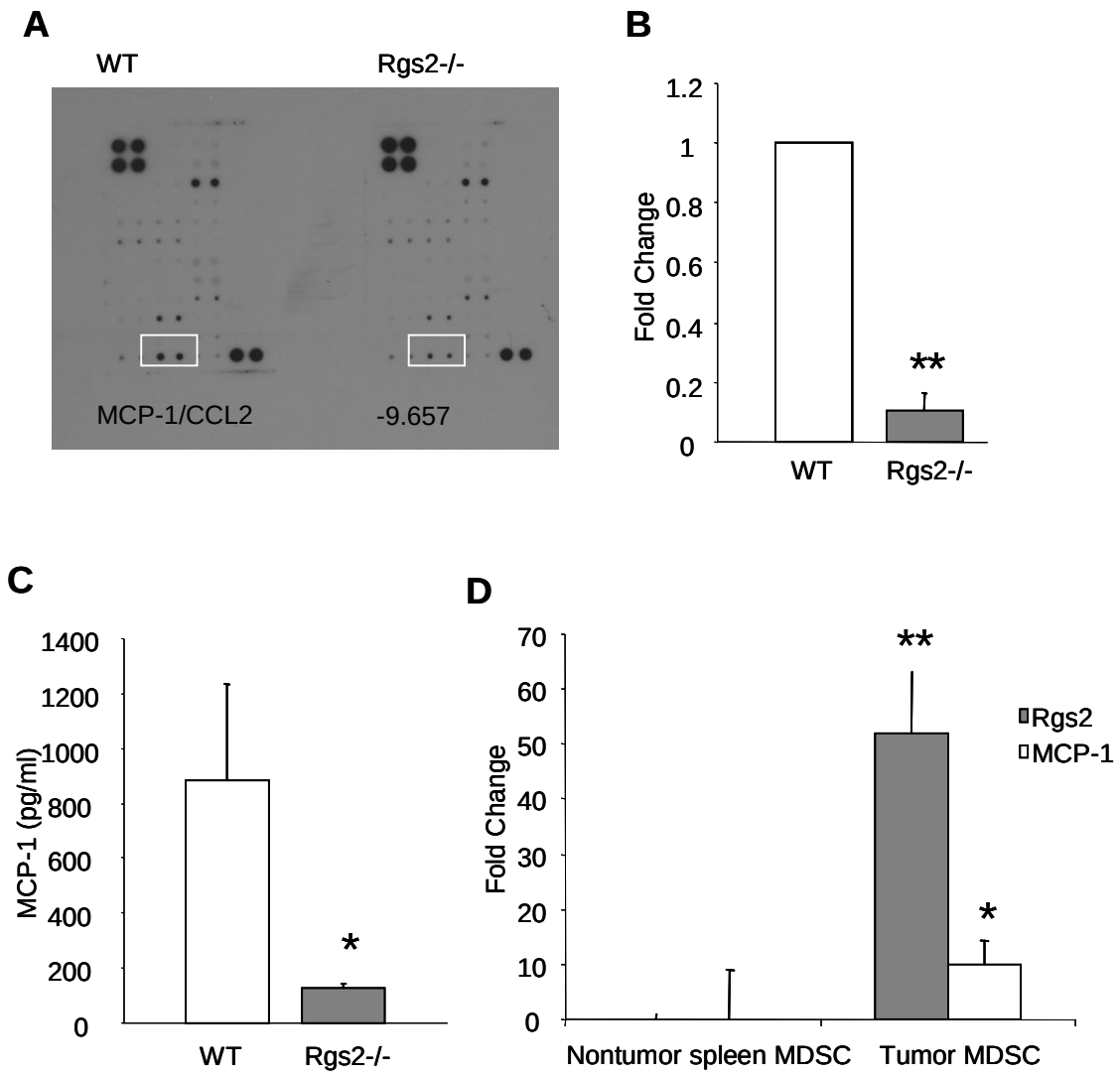

Figure 23. Rgs2 regulates MCP-1 expression in MDSCs. MDSCs were isolated from 3LL tumor tissues of wild type or Rgs2-/- mice by magnetic sorting after digestion of the tissues with hyaluronidase and collagenase. (A) Protein lysates from the isolated cells were analyzed using a cytokine array. Each cytokine is detected in duplicate, and intensity was determined using ImageJ software. Positive controls provided on the array were used for normalization. This experiment was performed twice. (B) RNA was extracted from the isolated MDSCs, and analyzed by real time PCR. b-actin was used as an internal control. This experiment was repeated 3 times. ** $p<0.00005$. (C) MDSCs were isolated from tumors of wild type and Rgs2 null mice, and cultured for 48 hours. Culture medium was assayed for MCP-1 protein by ELISA. This experiment was performed in duplicate and repeated 3 times. * $p< 0.05$. (D) MDSCs were isolated from normal spleen and 3LL tumor tissues of wild type mice. Rgs2 and MCP-1 levels were measured by real time PCR. b-actin was used as an internal control. This experiment was repeated 2 times. * $p< 0.05$, ** $p<0.005$

(Figure 17A-B). To determine whether decreased production of MCP-1 in the null MDSCs is responsible for defective angiogenesis associated with the Rgs2 null condition, we performed *in vitro* angiogenic assays. MDSCs isolated from tumor tissues of wild type or Rgs2-/- mice were cultivated overnight. Endothelial cells, (HUVECs) were then cultivated in the conditioned media from the MDSCs on Matrigel, which allowed vascular tube structures to form. Branch points were counted over time. We found that significantly fewer vascular structures developed in the group treated with Rgs2-/- MDSC conditioned medium compared to those treated with wild type MDSC conditioned medium (Figure 24A-B).

Next, we performed Boyden chamber migration assays to assess the ability of endothelial cells to migrate towards wild type and null MDSCs. For this assay, MDSCs were isolated from the tumor tissues of wild type and Rgs2-/- mice and cultivated overnight. Then, HUVECs were placed in the top of a Boyden chamber and allowed to migrate toward the bottom chamber containing the MDSC for 3.5 hours. Consistent with poor vascular tube formation induced by Rgs2 null MDSC conditioned medium, a significant reduction was observed in HUVEC migration towards the null MDSCs compared to wild type cells (Figure 24C). Consistent with a role for MDSC-derived MCP-1, addition of neutralizing MCP-1 specific antibody completely blunted wild type MDSC mediated HUVEC migration. The number of migrated endothelial cells is not statistically different from the retarded migration observed toward Rgs2 null MDSCs (Figure 24C). Conversely, addition of recombinant MCP-1 with the Rgs2-/- MDSCs rescued

defective migratory function (Figure 24C). Together, these data suggest that MCP-1 is a major contributor responsible for Rgs2-mediated tumor angiogenesis in MDSCs.

Rgs2 deficiency in MDSCs affects T cell response

MCP-1 is also a potent regulator of immune responses, as it regulates migration and polarization of T helper cells. Several studies have demonstrated that the presence of MCP-1 leads to decreased IFNγ but increased IL-4 production by T helper cells. So, we first treated plate-coated anti-CD3-stimulated splenocytes with 10 ng/ml recombinant MCP-1 for 72 hours, and upon re-stimulation with plate-coated anti-CD3, secreted IFNγ and IL-4 were assayed by ELISA. The data revealed that MCP-1 treatment led to decreased IFNγ and increased IL-4 levels compared to untreated splenocytes, confirming published data (Figure 25A).

To examine the role of Rgs2 and MCP-1 in the immune response to tumors, a co-culture polarization experiment was performed. MDSCs were isolated from tumor tissue of wild type and Rgs2-/- mice; overnight cultures were then plated with splenocytes from wild type mice concurrently stimulated with plate-coated anti-CD3. After re-stimulation, culture supernatants were assayed by ELISA for IFNγ and IL-4 to determine whether Rgs2 expression within MDSCs regulates T helper cell polarization toward T_H1 or T_H2 responses. As expected, splenocytes co-cultivated with wild type MDSCs mediated a T_H2 response as evidenced by higher IL-4 and lower IFNγ secretion in the co-culture (Figure 25B).

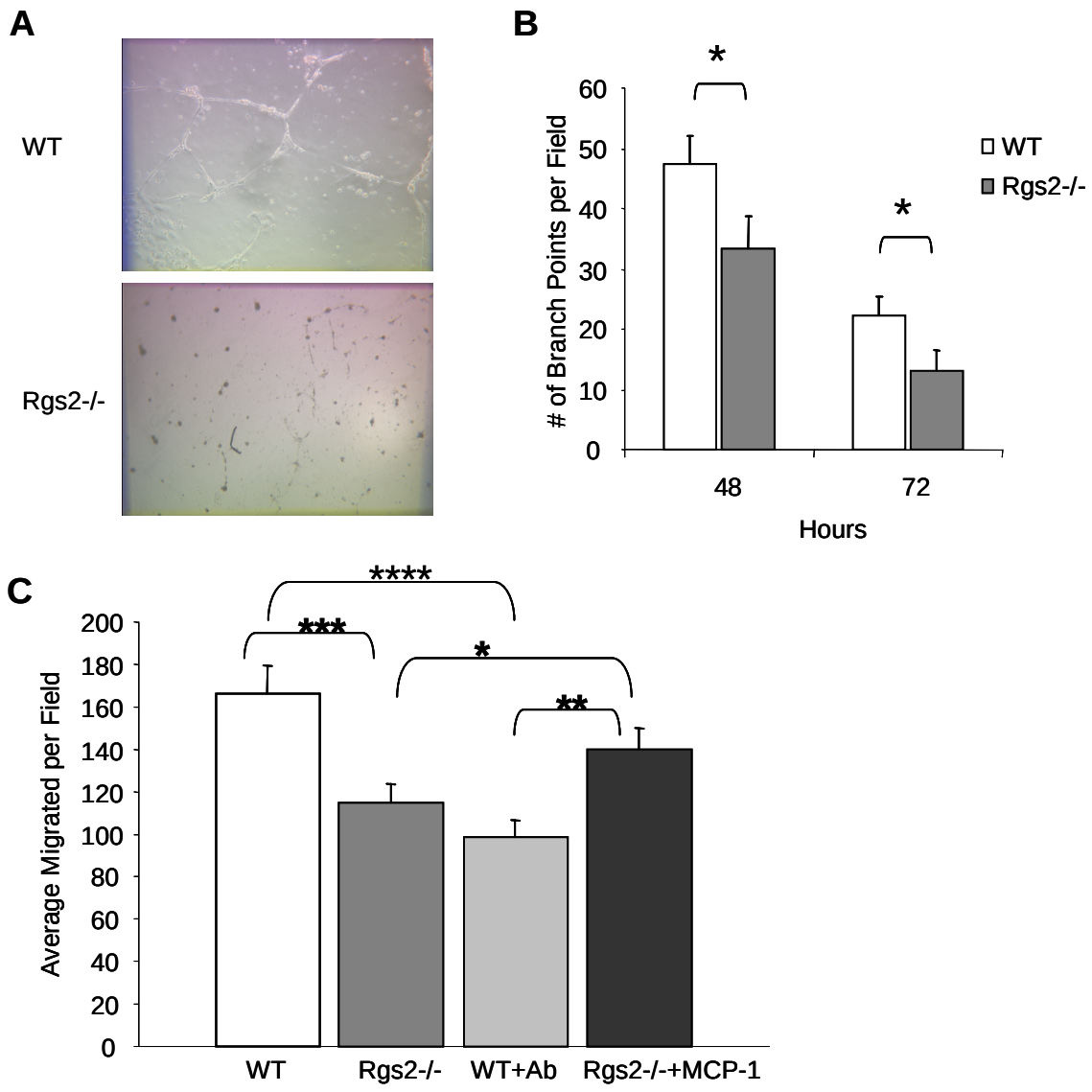

Figure 24. Angiogenic function of Rgs2 in MDSCs is mediated through MCP-1. (A) and (B) Wild type and Rgs2-/- MDSCs were isolated from 3LL tumor tissues by magnetic sorting, and incubated overnight at 37°C. $8x10^4$ HUVECs were plated in each well of a 48-well plate on top of Matrigel in the conditioned medium derived from the isolated MDSCs. Representative images are shown at 72 hours, and vascular network branch points were scored at the times indicated. This experiment was performed in duplicate and repeated twice. (C) MDSCs were isolated from tumors of Rgs2-/- and wild type mice by magnetic sorting, and incubated overnight. Transwells containing $1x10^5$ HUVECs in the top chamber were added and allowed to migrate for 3.5 hours. MCP-1 neutralizing antibody (Ab) at 1 ug/ml was added to wild type cells, or 1 ng/ml of recombinant MCP-1 was added to Rgs2-/- cells. This experiment was performed 3 times in duplicate. * $p \leq 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.00005$.

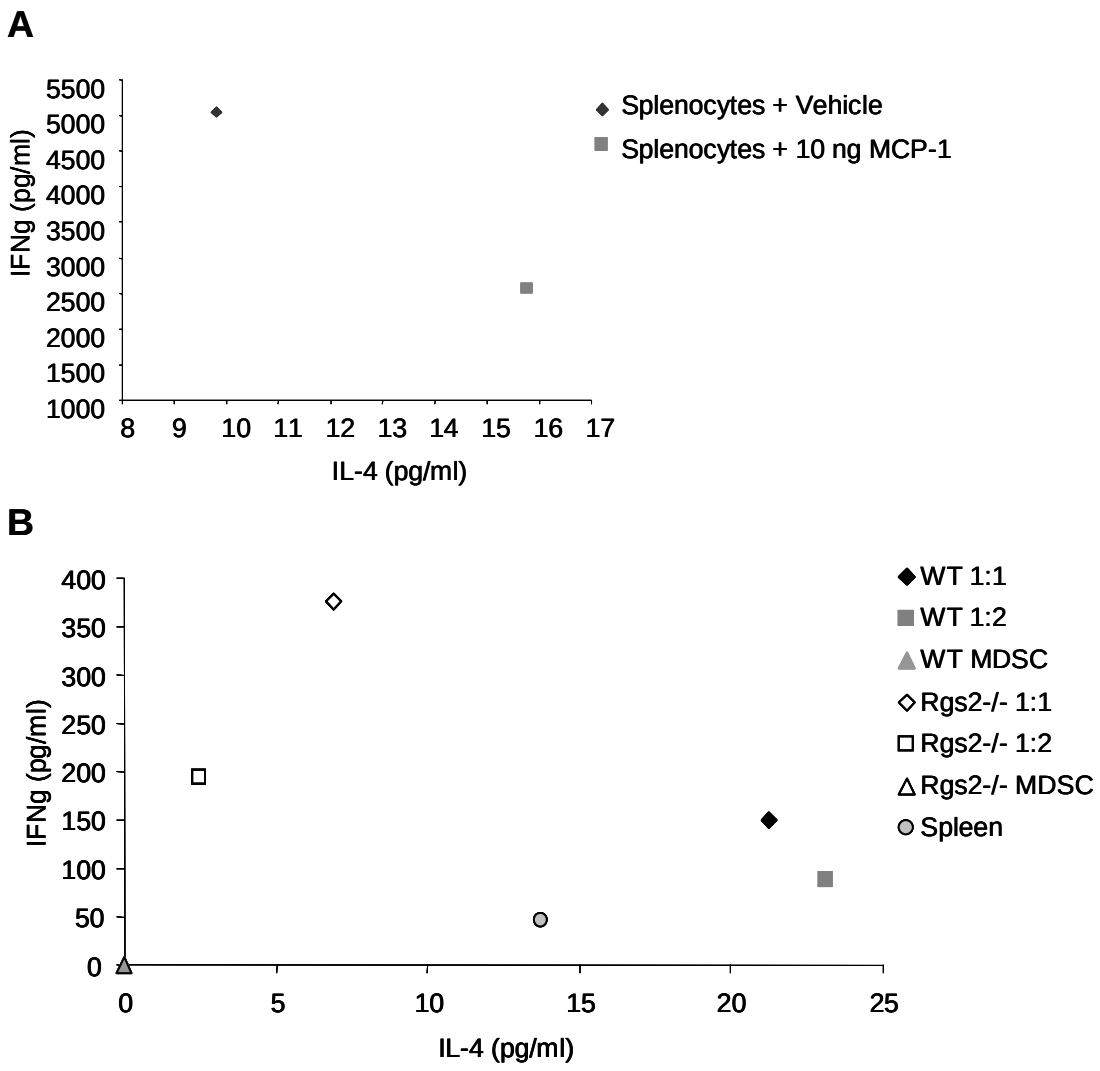

Figure 25. Rgs2-/- MDSCs promote a shift in anti-tumor immune response. (A) Splenocytes were isolated from wild type mice, and seeded onto anti-CD3-coated plates, incubated for 24 hours, then transferred to an uncoated plate and cultured for two more days. The splenocytes were re-stimulated by transferring them to a new anti-CD3-coated plate. Culture supernatants were assayed 48 hours later by ELISA for IFNγ and IL-4. (B) Wild type and null MDSCs were isolated from tumors of mice and incubated overnight. Splenocytes were isolated from wild type mice and seeded onto anti-CD3-coated plates. MDSCs were added to the stimulated splenocytes. The co-culture was incubated for 24 hours and then transferred to an uncoated plate and cultured. After two more days, the cultures were re-stimulated by transferring them to a new anti-CD3-coated plate. Culture supernatants were assayed 48 hours later by ELISA for IFNγ and IL-4. This experiment was performed twice in triplicate. At 1:1 for IFNγ $p < 0.01$, and at 1:2 for IFNγ $p < 0.05$. At 1:1 for IL-4, $p \leq 0.05$.

Notwithstanding, the Rgs2-/- MDSCs induced higher IFNγ and lower IL-4 from anti-CD3-stimulated splenocytes, indicating a switch from a T_H2 response, normally observed with wild type MDSCs, to a T_H1 response. These data identify Rgs2 as a critical regulator of the immune suppressive function of MDSCs.

Discussion

MDSCs play a major role in tumor progression. They promote tumor growth at the primary site, as well as enhance metastasis (Yang et al., 2004; Yang et al., 2008). They have been linked to resistance to therapy in cancer (Crawford and Ferrara, 2009; Rodriguez and Ochoa, 2008; Shojaei et al., 2007a). Efforts focused at differentiating or attenuating the function of MDSCs have been promising, leading to improved immune response against the tumor and a better prognosis for the patient (Gabrilovich and Nagaraj, 2009). Clearly, identifying molecular mediators important for MDSC function will enhance our ability to better target these cells for cancer treatment.

MDSCs utilize different mechanisms to promote tumor growth and progression, two of which are promotion of tumor angiogenesis and modulation of host immune response. With critical relevance to this role of MDSCs, we discovered a single regulatory molecule Rgs2, which controls tumor neoangiogenesis and polarizes T helper cell responses toward pro-tumor conditions, both of which are requisites for promotion of tumor progression. Thus, in this study, we found that tumor conditions upregulate Rgs2 expression in MDSCs, and MDSCs lacking Rgs2 were no longer capable of promoting tumor

growth. Tumors in Rgs2-/- mice grew more slowly, and had less vascular density and increased cell death. We also found that the tumors in the null mice had more infiltrating immune cells. These data suggest that Rgs2-/- MDSCs create a different environment from wild type MDSCs, in which the immune response is likely less tolerogenic, and more anti-tumor, than that normally seen in tumors (Balkwill et al., 2005; Johansson et al., 2008; Noonan et al., 2008; Sinha et al., 2005).

Furthermore, we found that Rgs2-/- MDSCs isolated from tumors produce drastically reduced levels of MCP-1 compared to wild type. While MCP-1 has been reported to promote migration of MDSCs (Huang et al., 2007a; Sawanobori et al., 2008), production of MCP-1 by MDSCs has previously not been well studied. MCP-1 is a potent angiogenic factor. It promotes angiogenesis through indirect effects, such as through monocyte migration or induction of molecules such as VEGF and MMP9 (Goede et al., 1999; Raman et al., 2007; Varney et al., 2005), or by directly functioning on endothelial cells (Salcedo et al., 2000). In this study, we show that wild type MDSCs secrete MCP-1, which promotes endothelial cell migration and vascular tube formation. Rgs2-/- MDSCs secrete much lower levels of MCP-1, which leads to reduced angiogenesis in tumors from Rgs2-/- mice.

While MCP-1 is known to mediate cell migration, in particular migration of monocytes, studies also report an important role of MCP-1 in modulating immune response. Mice deficient in MCP-1 are not able to mount a T_H2 response (Gu et al., 2000). In contrast, mice deficient in the receptor for MCP-1, CCR2, exhibit

both reduced T_H1 and T_H2 responses, and are considered to be defective in T_H1 immunity (Boring et al., 1997; Conti and Rollins, 2004; Warmington et al., 1999). Blocking or knocking out MCP-1 in several models led to the decrease of T_H2 mediators, particularly IL-4 (Gonzalo et al., 1998; Gu et al., 2000; Lu et al., 1998), and induction or addition of MCP-1 led to decreased T_H1 and increased T_H2 responses (Karpus et al., 1998; Karpus et al., 1997). Our data indicate that MCP-1 produced by MDSCs affects the immune response mounted towards the tumor. Splenocytes cultured with Rgs2-/- MDSCs possessed a T_H1 cytokine profile with regard to IL-4 and IFNγ production, compared to splenocytes cultured with wild type MDSCs, which had a T_H2 cytokine profile.

Our data show that tumor conditions, such as hypoxia and likely secreted factors, upregulate Rgs2 expression in MDSCs, which leads to production of MCP-1. The MCP-1 then mediates both the angiogenic effects and the response of T helper cells. While it is uncertain how Rgs2 modulates MCP-1 levels, Rgs2 was predicted to encode a basic helix-loop-helix protein (Siderovski et al., 1990; Siderovski et al., 1994). Nuclear expression of Rgs2 was reported in several studies including our unpublished data. These findings point to a function of Rgs2 in gene transcription.

A well studied mediator of MCP-1 upregulation is NF-κB (Melgarejo et al., 2009). Alternatively, MCP-1 production has been linked to Stat3 signaling, which has a major role in MDSC expansion and myeloid cell-dependent angiogenesis (Gabrilovich and Nagaraj, 2009; Kujawski et al., 2008). In addition to functioning as a GAP for Gα proteins, Rgs2 can also directly inhibit several isoforms of

adenylyl cyclase, thereby affecting cAMP levels (Roy et al., 2006a; Salim et al., 2003; Sinnarajah et al., 2001). In human airway smooth muscle cells, stimulating adenylyl cyclase, which leads to PKA activation through increasing cAMP levels, led to a decrease in MCP-1 (Wuyts et al., 2003). In cultured rat mesangial cells, decreased MCP-1 was observed with increased cAMP levels (Iwamoto et al., 2003). However, other studies indicate a role for increased cAMP and active PKA in MCP-1 upregulation in monocytic cells (Fietta et al., 2002; Hirano et al., 2007). The adenylyl cyclase/cAMP/PKA pathway activates the transcription factor CREB, and CREB shares coactivators with NF-κB, such as CBP. These coactivators are present in limited numbers, creating a situation where there is competition (Almawi and Melemedjian, 2002; Kim et al., 2003; Takahashi et al., 2002). Therefore, Rgs2 might be increasing MCP-1 levels through NF-κB by inhibiting adenylyl cyclase. Future studies will be necessary to elucidate the mechanism involved.

This study links the tumor promoting roles of Rgs2 in MDSCs to a secreted molecule, MCP-1. MCP-1 is produced mainly under pathological conditions, and is expressed by several cancer types, suggesting that it could be a good target for therapy (Conti and Rollins, 2004; Raman et al., 2007). Indeed, neutralizing antibodies and several CCR2 antagonists have been used *in vitro* and *in vivo* in rodents to show that blocking MCP-1/CCR2 signaling ameliorates symptoms of diseases associated with increased MCP-1 levels, including inflammatory arthritis, a model of multiple sclerosis, and renal fibrosis (Brodmerkel et al., 2005; Buntinx et al., 2008; Furuichi et al., 2003; Kitagawa et

al., 2004; Shin et al., 2009; Tominaga et al., 2009). In addition, in a mouse model of breast cancer, treatment with neutralizing antibodies against MCP-1 enhanced survival and decreased metastasis (Salcedo et al., 2000). Selective targeting of this pathway in human disease will likely prove beneficial, specifically, as our data suggest, in cancer.

In summary, our studies indicate that Rgs2 and MCP-1 are important molecules for the effector functions of MDSCs. Lack of Rgs2 leads to decreased levels of MCP-1, along with slower tumor progression and decreased vascular density in tumors. We show that these phenotypes are directly linked to the deficiency in MCP-1 seen in Rgs2-/- mice. Targeting Rgs2 and MCP-1 signaling may have important clinical implications, through inhibiting tumor growth and increasing host immune response, thus leading to a better prognosis for cancer patients.

CHAPTER IV

CONCLUSION

Summary

The current body of work has attempted to elucidate the role of Rgs2 in hematopoiesis and in tumor myeloid derived suppressor cell biology. In Chapter II, we provide evidence that Rgs2 does not greatly affect hematopoiesis. We show that although Rgs2 levels do increase as myeloid cells are differentiated, this molecule does not appear to play a role directly in differentiation of these cells. We show this *in vivo*, using flow cytometry to examine cell populations, as well as *in vitro*, using a granulocytic differentiation assay and a methocellulose assay. Whereas the Rgs2 deficient mice do show a level of increased sensitivity to serial 5-FU treatment, we find no major defects in hematopoietic stem function or multipotent progenitor development. Our data also indicate that although Rgs2 has been implicated to play an important role in Flt3-ITD mutation signaling in leukemia (Schwable et al., 2005), it does not appear to have a major role in normal Flt3 signaling. This suggests that targeting Rgs2 in this signaling pathway in leukemias with this mutation may be beneficial to the patient, as normal signaling in non-cancerous blood cells should not be affected.

In Chapter III, we present evidence that Rgs2 plays a major role in MDSC function. Gr-1+CD11b+ cells isolated from spleens of tumor bearing mice

produce significantly more Rgs2 mRNA than those from spleens of non-tumor bearing mice. Furthermore, Rgs2 mRNA is increased in cells treated with tumor conditioned medium or placed under hypoxic conditions, which are commonly found in tumors. Tumors in mice deficient in Rgs2 grow significantly slower compared to those in wild type mice, and a reconstitution assay shows that this is due to the lack of Rgs2 in MDSCs. In addition, the tumors from Rgs2 -/- mice exhibit less vascular density, increased tumor cell death, and increased leukocyte infiltration, including Gr-1+ cells, one of the markers for MDSCs. MDSC production is not altered in Rgs2 deficient mice, nor is the morphological or cell surface molecule phenotype, suggesting that the defect is in the function of MDSCs. We found that Rgs2 null MDSCs isolated from tumor tissue produce significantly reduced levels of MCP-1 compared to wild type MDSCs, and this translates functionally into angiogenic and immunological defects within the null MDSCs. In sum, these data point to a pathway where Rgs2 is upregulated under tumor conditions in MDSCs, which leads to upregulation of production and secretion of MCP-1. The secreted MCP-1 can then function in both pro-angiogenic and anti-inflammatory manners, leading to a pro-tumor environment (Figure 26).

Taken together, our findings point to novel pathway to target for cancer therapy. We show that Rgs2 does not have a major role in hematopoietic cell production or differentiation, suggesting that targeting this pathway would not affect normal hematopoietic cell development, but could target the ability of MDSCs to promote tumor progression in cancer patients. In targeting this

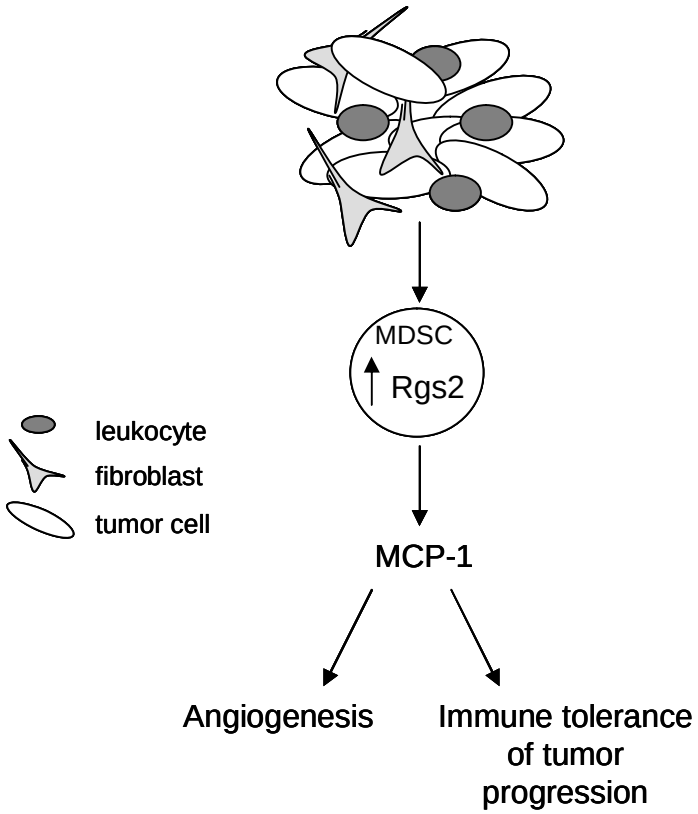

Figure 26. Proposed model of Rgs2 function in MDSCs. Tumor conditions, including hypoxia, upregulate Rgs2 in MDSCs, which leads to increased MCP-1 production into the tumor microenvironment. The increased MCP-1 mediates tumor vascularization and immune tolerance of the tumor.

pathway, we would be able to inhibit the pro-tumor functions of MDSCs, both angiogenesis and suppression of immune function.

Preliminary data and future directions

While we have convincing data that Rgs2-driven MCP-1 production is important in the immune modulatory role of MDSCs (Figure 25), further work is necessary to fully elucidate the mechanisms involved. We have *in vitro* data, but would like to move into *in vivo* models, as well. For example, we could use flow cytometry to examine the subpopulations of T cells within the tumors of wild type and Rgs2-/- mice to determine if there is a skewing of immune response *in vivo*, as would be suggested by our *in vitro* data, by observing the cytokines produced using intracellular staining. We may even find that *in vivo* we have a stronger shift from T_H2 to T_H1 responses. A couple of studies have reported data indicating that MCP-1 stimulation reduces IL-12 production in monocytes and macrophages, and neutralizing MCP-1 leads to increased IL-12 production by macrophages (Braun et al., 2000; Chensue et al., 1996), suggesting that the decreased MCP-1 production in MDSCs of Rgs2-/- mice may contribute to increased levels of M1 macrophages. As such, we could examine markers of tumor associated macrophages to ascertain whether these cells have a type 1 or a type 2 profile. Additionally, as technology improves for culturing and transducing MDSCs, we could perform experiments where we “rescue” the Rgs2-/- phenotype by tranducing MCP-1 into Rgs2-/- MDSCs and examining the resulting immune and angiogenic responses *in vitro* and *in vivo*.

We found that Rgs2 mediates MCP-1 production, but the mechanism is unknown. Inflammatory factors, such as TNFα and IL-1β, have previously been shown to induce MCP-1 expression, suggesting that MCP-1 is downstream of NF-κB activity (Melgarejo et al., 2009; Proost et al., 1996). We isolated MDSCs from spleens of tumor bearing wild type and Rgs2 deficient mice, spun them onto slides, and stained them for NF-κB p65 and p50 molecules. We did not see a difference in p65 (data not shown); however, we found that Rgs2 -/- tumor derived MDSCs had higher levels of p50, and increased nuclear p50, seen by immunofluorescent staining (Figure 27). The p50 subunit can either be activating or inhibitory (Pereira and Oakley, 2008), depending on the other molecules that it is complexed with. As such, we cannot speculate on its role in these cells fully. We are limited in the assays we are capable of performing to study signaling within MDSCs, as isolating even small numbers of the cells is quite expensive. As technology improves for isolating, or even culturing these cells, we would like to perform electromobility shift assays (EMSA) or chromatin immunoprecipitation assays (ChIP) in order to determine the other subunits/binding partners present, as well as observe if p50 or other NF-κB subunits are at the promoter region of MCP-1 with modulation of Rgs2 levels.

The loss of MCP-1 expression coupled to Rgs2 deficiency suggests that Rgs2 is somehow promoting MCP-1 expression. However, Rgs2, as a regulator of G protein signaling, promotes cessation of signaling downstream of GPCRs. In addition to functioning as a GAP for Gα proteins, Rgs2 can also affect cAMP

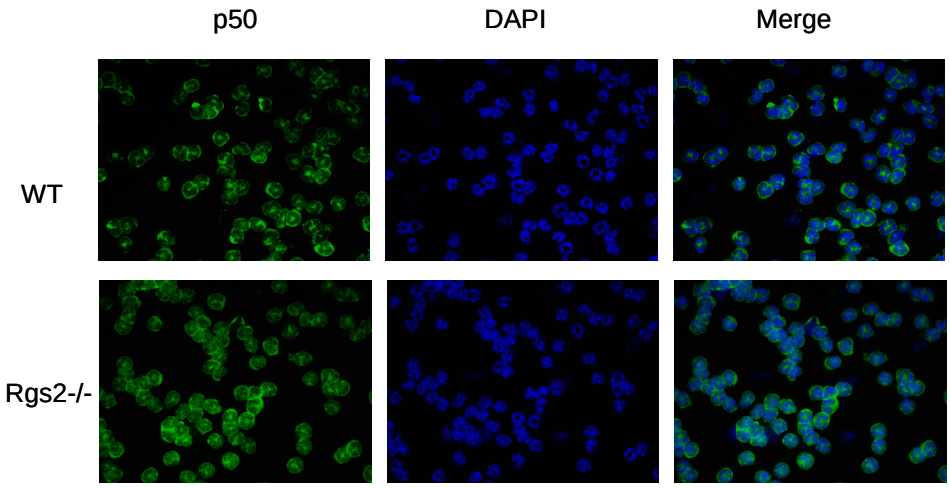

Figure 27. Rgs2 deficiency increases the level of p50 in the cell, particularly in the nucleus. MDSCs were isolated from spleens of tumor bearing wild type and Rgs2-/- mice by MACS using anti-CD11b and anti-Gr-1 antibodies. The MDSCs were placed on slides using a cytospin centrifuge, fixed, and then stained with a p50 antibody (sc-114) and DAPI. Merging of the images was performed in Adobe Photoshop.

levels by directly inhibiting some isoforms of adenylyl cyclase (Roy et al., 2006a; Salim et al., 2003; Sinnarajah et al., 2001). Studies indicate that active adenylyl cyclase leading to increased cAMP levels, and subsequent activation of PKA, mediates a decrease in MCP-1 levels in smooth muscle cells (Iwamoto et al., 2003; Wuyts et al., 2003). However, in myeloid/monocytic cells, other studies point to a role for increased cAMP and active PKA in upregulation of MCP-1 (Fietta et al., 2002; Hirano et al., 2007). The adenylyl cyclase/cAMP/PKA pathway activates the transcription factor CREB, which shares coactivators with NF-κB. Competition for the small pools of coactivators, such as CBP, is one way cells control transcription (Almawi and Melemedjian, 2002; Kim et al., 2003; Takahashi et al., 2002). Therefore, Rgs2 might be increasing MCP-1 levels through NF-κB by inhibiting adenylyl cyclase.

In order to determine if and how Rgs2 enhances signaling through the NF-κB pathway, we performed luciferase reporter experiments. We overexpressed Rgs2 or vector control along with a NF-κB driven luciferase reporter in 293 cells, and found that luciferase activity increased two-fold with Rgs2 expression (Figure 28). This increase in luciferase activity was present even when we used a mutant of Rgs2 that has lost its GAP function (RGS2N149A) or a mutant that cannot inhibit adenylyl cyclase (RGS2NT1), the two major known functions of Rgs2 (Salim et al., 2003). Rgs2 was originally predicted to encode a basic helix-loop-helix protein (Siderovski et al., 1990; Siderovski et al., 1994), and it has been widely published that it is mainly expressed in the nucleus. These data suggest that there may be an unknown role for Rgs2 within the nucleus, perhaps in

regulating gene transcription. However, further study is necessary, as the fold increase in luciferase activity is comparatively low. This may be due to endogenous Rgs2 activity in 293 cells. We have observed that transfection itself can induce Rgs2 mRNA, consistent with Rgs2 being a stress response gene (data not shown). To circumvent this issue, we attempted to perform this assay in bone marrow cells isolated from Rgs2-/- mice. While we were able to detect upregulation of Rgs2 mRNA, the cells did not survive long enough to perform the luciferase assay (data not shown).

Alternatively, MCP-1 production has been linked to Stat3 signaling, which has a major role in MDSC expansion and myeloid cell-dependent angiogenesis (Gabrilovich and Nagaraj, 2009; Kujawski et al., 2008; Schroer et al., 2011). In hepatocytes, it has been reported that increased levels of Rgs2 can inhibit the phosphorylation, and therefore activity, of Stat3 (Han et al., 2008). However, several studies have reported roles for unphosphorylated Stat3 (and Stat1) in transcription under pathological conditions (Chatterjee-Kishore et al., 2000; Yang et al., 2005a; Yue et al., 2010), suggesting that accumulation of Rgs2 in MDSCs could lead to increased levels of unphosphorylated Stat3 driven transcription. Interestingly, increased unphosphorylated Stat3 has been linked to abnormal Rgs2 expression (Yue et al., 2010). Further studies are necessary in order to determine if Stat3, phosphorylated or unphosphorylated, could be involved in the loss of MCP-1 observed in the Rgs2-/- mice, or in Rgs2 expression in tumor derived MDSCs.

One other possibility is that Rgs2 is acting as a transcription factor. Beyond the original study suggesting that Rgs2, then known as G0S8, was a putative basic helix-loop-helix protein based upon its sequence, no evidence has been published indicating a nuclear role for Rgs2 (Siderovski et al., 1994). However, our data suggest that Rgs2 may have a role in gene expression outside of its functions as a GAP for Gα proteins or as an adenylyl cyclase inhibitor (Figure 28). Rgs2 has been shown to passively diffuse into the nucleus, where it is likely bound by another protein or set of proteins in order to retain it in the nucleus (Heximer et al., 2001). This suggests that upon GPCR signaling, Rgs2 must be released in order to exit the nucleus. In addition, post-translational modifications of Rgs2 have been shown to reduce its GAP activity (Cunningham et al., 2001; Ni et al., 2006), indicating that there are mechanisms outside of nuclear retention which work to mediate the function of Rgs2. Therefore, it seems unlikely that the cell would go to such lengths to keep Rgs2 in the nucleus if Rgs2 did not have a nuclear function. However, whether Rgs2 can indeed bind DNA (directly or indirectly) has yet to be determined. The lack of a specific antibody for Rgs2 has contributed to the dearth of experimental evidence for, or against, a role of Rgs2 within the nucleus. With a good antibody, one could not only determine if Rgs2 binds to DNA, but also track the localization of Rgs2 with different stimuli. If Rgs2 indeed has a role within the nucleus, it is likely we would see nuclear localization with hypoxia or tumor conditioned medium treatment. While other RGS domain containing proteins have not been found bound to DNA, SRB-RGS, or Rgs3, has been found bound to the estrogen receptor (which binds

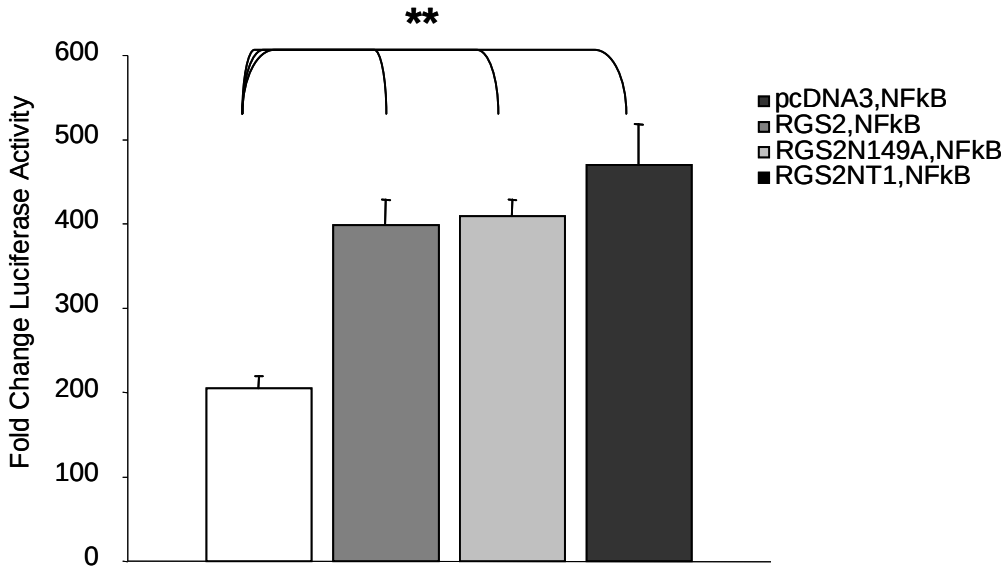

Figure 28. Rgs2 enhances NF-κB activity independently of RGS domain function or adenylyl cyclase inhibitory function. 293 cells were transfected with RGS2, RGS2N149A, RGS2NT1, or pcDNA3 control, and a luciferase reporter construct under the control of a NF-κB promoter or control vector using Lipofectamine 2000. After 24 hours, cells were lysed and assayed for luminescence using the Promega Dual Reporter Luciferase Assay Kit. Renilla luciferase was used as an internal control. RGS2N149A and RGS2NT1 were kindly provided by Dr. Carmen Dessauer. **p< 0.005

to DNA) and is suggested to inhibit transcriptional activity of genes with an estrogen response element (Ikeda et al., 2007), indicating that RGS proteins can have nuclear roles. Interestingly, Rgs3 belongs to the same subfamily as Rgs2, and can inhibit similar signaling pathways (Anger et al., 2007). Perhaps with further study and the development of the right tools, we will find that Rgs2 can also affect transcription within the nucleus.

As we observed increased leukocyte and Gr-1+ myeloid cell infiltration into tumors (Figure 18), we wondered whether Rgs2 plays a role in migration. Since Rgs2 is a negative regulator of G protein signaling, and can function on $G\alpha_i$ subunits (albeit weakly), which are normally coupled to chemokine receptors, we hypothesized that we would observe increased migration of Rgs2-/- MDSCs to FBS in a transwell assay. We isolated tumor derived splenic MDSCs using FACS, and placed the cells in the top of a transwell with FBS in the bottom chamber. After 3.5 hours, we counted migrated cells, and indeed found that more null MDSCs migrated than wild type MDSCs (Figure 29). Previous studies have indicated that Rgs2 does not play a role in migration of leukocytes (Bowman et al., 1998; Reif and Cyster, 2000); however, these studies were performed by transfecting cells with Rgs2 or vector. We have found that transfection itself induces Rgs2 mRNA (data not shown), which makes it difficult to determine differences between the experimental sample and the control sample.

Our data indicate that Rgs2 plays a role in MDSC migration, mediates production of MCP-1, enhances angiogenesis, and affects immune response to tumor cells. We began to speculate on the signaling upstream of Rgs2,

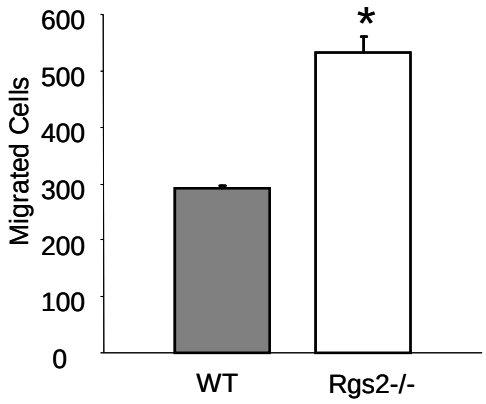

Figure 29. Rgs2 deficiency in MDSCs leads to increased migration. MDSCs were isolated from spleens of tumor bearing wild type and Rgs2-/- mice by flow cytometry using anti-CD11b and anti-Gr-1 antibodies. The MDSCs were seeded into the top wells of transwells, and allowed to migrate to medium containing 10% fetal bovine serum for 3.5 hours. The migrated cells were collected from the bottom chamber of the transwell and counted. The experiment was performed twice in duplicate. The bars indicate the average number of migrated cells per transwell, and the error bars represent standard error across experiments.

wondering what molecules could be signaling to Rgs2. One molecule, sphingosine-1-phosphate (S1P), has been linked in several studies to these functions, affecting migration, polarization of immune response, and cytokine production (Rivera et al., 2008; Rosen and Goetzl, 2005). S1P is a biologically active sphingolipid produced by nearly all cells, but released mostly by apoptotic tumor cells and cells of hematopoietic origin (Rivera et al., 2008; Rosen and Goetzl, 2005; Weigert et al., 2007). S1P is produced by first cleaving sphingomyelin to ceramide, then cleaving ceramide to sphingosine, followed by phosphorylation by sphingosine kinases (Rivera et al., 2008; Rosen and Goetzl, 2005; Spiegel and Milstien, 2003). S1P can be degraded by phosphatases or by a lyase. The enzymes involved in producing S1P, and therefore S1P, are induced in response to inflammatory factors, such as TNFα (Ledgerwood et al., 2008; Memon et al., 1998; Xia et al., 1998). S1P functions intracellularly to regulate cell survival and proliferation (Spiegel and Milstien, 2003). In addition, extracellular S1P is a ligand for a group of G protein coupled receptors (Rivera et al., 2008; Rosen and Goetzl, 2005; Spiegel and Milstien, 2003). There are 5 mammalian receptors known to date, S1P receptors 1-5 (S1P1-5), with S1P1 being the most well studied (Hla, 2003; Rivera et al., 2008; Rosen and Goetzl, 2005; Siehler and Manning, 2002; Spiegel and Milstien, 2003).

S1P-S1P receptor pathways have been studied, in particular, in cells of the immune system. S1P agonist treated mature and immature dendritic cells and S1P treated macrophages showed increased type 2 and decreased type 1 immune responses (Hughes et al., 2008; Idzko et al., 2002; Jin et al., 2003;

Muller et al., 2005; Weigert et al., 2007). In macrophages, the increase in type 2 response was accompanied by decreased LPS-induced NF-κB signaling (Weigert et al., 2007). Reduction of chemotaxis and decreased levels of two of the receptors, S1P1 and S1P4, were observed with agonist treatment of dendritic cells (Muller et al., 2005). Antagonism of S1P receptors inhibits tumor progression and decreases tumor vascularization (LaMontagne et al., 2006). Hughes et al. demonstrated that S1P treatment of macrophages decreased the level of LPS induced MCP-1 (Hughes et al., 2008). S1P treatment of vascular smooth muscle cells led to an increase in Rgs2 levels (Hendriks-Balk et al., 2009). Furthermore, Rgs2 was shown to bind to S1P1 and affect migration at high ligand concentrations (Kohno and Igarashi, 2008). Since Rgs2 has been implicated in S1P signaling, and S1P treatment led to reduced MCP-1 in LPS-treated macrophages, we hypothesized that S1P was signaling through a receptor upstream of Rgs2 to produce the phenotype we observe in MDSCs.

We first determined whether MDSCs isolated from spleens of tumor bearing mice expressed receptors for S1P, and what differences there may be in Rgs2-/- MDSC expressed S1PRs, by RT-PCR. We found that there was a difference in S1P receptor 4 (S1P4) between wild type and Rgs2-/- MDSCs (Figure 30A). Rgs2-/- MDSCs have higher levels of this receptor. In addition, S1P3 and S1P5 were undetectable, S1P1 was barely detectable (but not different between groups), and a small increase in S1P2 in the Rgs2-/- MDSCs was observed. Furthermore, when we treated 32D myeloid cells with 3LL tumor conditioned medium, we observed upregulation of S1P4 (Figure 30B).

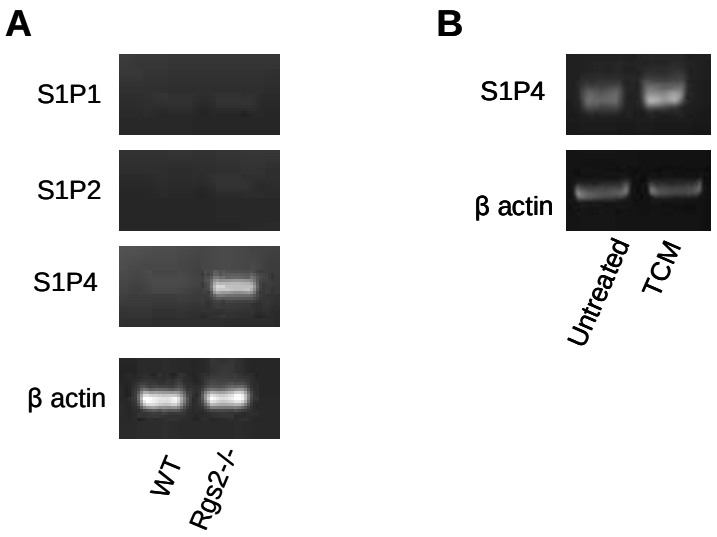

Figure 30. S1P4 is induced by tumor secreted factors, and is elevated in Rgs2-/- MDSCs. (A) MDSCs were isolated from spleens of wild type and Rgs2-/- mice by MACS. RNA was isolated, and cDNA was synthesized, followed by PCR for S1P receptors. S1P3 and S1P5 were undetectable (B) 32D cells were treated with 3LL tumor cell conditioned medium (TCM) containing 10% FBS for 24 hours. The cells were collected, and RNA was isolated. cDNA was synthesized, and PCR was run for the S1P4 receptor. β-actin was used as a control in both.

S1P4 is found specifically in lymphoid and hematopoietic tissues, and has been shown to couple to $G\alpha_i$ and $G\alpha_{12/13}$ (Graler et al., 1998; Graler et al., 2003; Kohno et al., 2003). In T lymphocytes, overexpression of S1P4 led to decreased T cell proliferation, and decreased secretion of IL-4, IL-12, and IFNγ, but increased secretion of IL-10 (Wang et al., 2005a). However, there are conflicting reports about the role of S1P4 in cell migration; Matsuyuki et al. concluded that S1P1 and S1P4 work together to mediate migration of T cells, whereas Wang et al. found that S1P4 signaling does not have an effect on T cell migration (Matsuyuki et al., 2006; Wang et al., 2005a). As signaling from some receptors feeds back positively to increase receptor levels, and in particular, on some cell types, high levels of S1P stimulation maintained S1P1 levels and function (Graeler et al., 2003), we treated 32D myeloid cells with S1P and assessed receptor levels (Figure 31A). Again, S1P5 was practically undetectable. S1P1, S1P3, and surprisingly, S1P4 levels did not change with treatment. However, S1P2 levels increased with treatment.

Consistent with data published with vascular smooth muscle cells, S1P treatment of 32D cells led to a significant increase in Rgs2 mRNA (Figure 31B), suggesting that S1P signaling may be involved in the increase of Rgs2 levels observed in MDSCs from tumor bearing mice. We also assessed migration of wild type and Rgs2-/- MDSCs isolated from spleens of tumor bearing mice with MACS to S1P. Migration to BSA was assessed as a control, as the S1P was solubilized in a solution of fatty acid free BSA. Migration to BSA was not detectable, nor was migration detectable at 10^{-8} M S1P (Figure 32). At 10^{-7} M

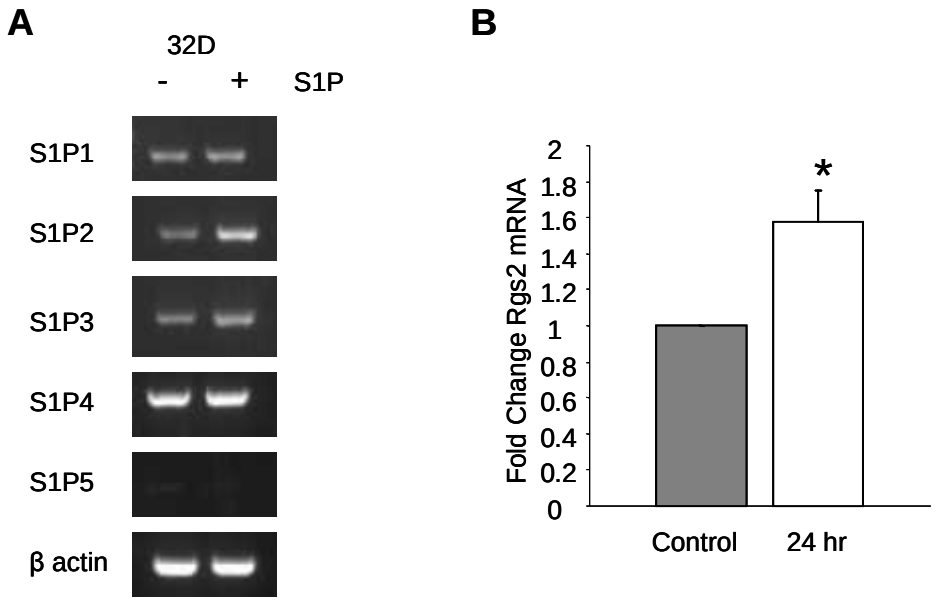

Figure 31. S1P treatment of 32D cells upregulates Rgs2 and S1P2 expression, but not S1P4 expression. 32D cells were treated with 10^{-6} M S1P for 24 hours. Cells were collected, and RNA was isolated. cDNA was synthesized. (A) RT-PCR was performed for S1P1-5, using β-actin as a control. (B) Real time PCR was performed for Rgs2, using β-actin as a control. Experiment was performed two times in duplicate. *, $p < 0.05$.

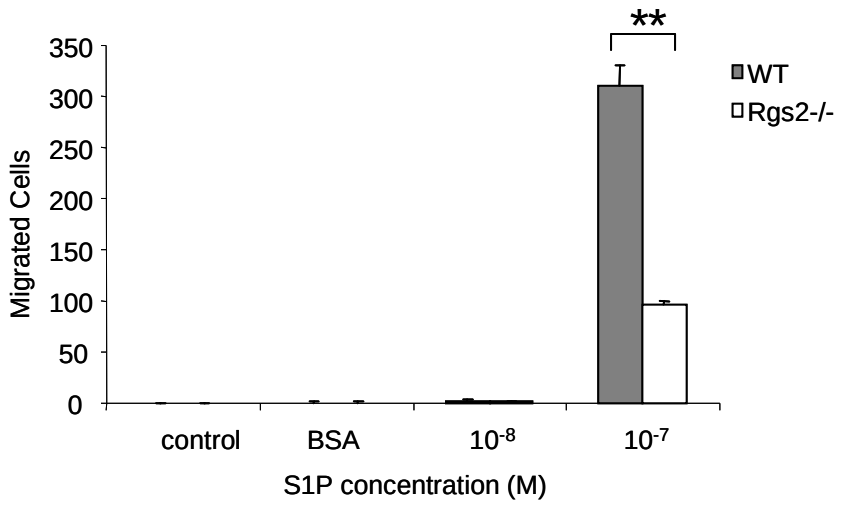

Figure 32. Rgs2-/- MDSCs exhibit decreased migration to S1P compared to wild type MDSCs. MDSCs were isolated from spleens of tumor bearing wild type and Rgs2-/- mice using MACS. $1x10^5$ MDSCs were placed in the top chamber of transwells, and allowed to migrate to different concentrations of S1P for 3.5 hours. Migrated cells were then counted, and the average was plotted. BSA solution was assessed as a control, as the S1P was solubilized in a solution of fatty acid free BSA. n=2 **, $p<0.005$.

S1P, we observed migration of wild type and Rgs2-/- MDSCs; however, we found that Rgs2-/- MDSCs showed significantly less migration to S1P compared to wild type MDSCs. These data indicate that although we see increased migration in Rgs2-/- MDSCs to FBS, S1P is likely not the factor involved. Further study is necessary to elucidate the mechanisms behind the increased migration of Rgs2-/- MDSCs.

We hypothesize that tumor secreted S1P is partly responsible for the increase in Rgs2 in MDSCs, which leads to increased MCP-1 and tumor progression, perhaps through mediating NF-κB or Stat3 function (Figure 33A). In the knockdown mice, we further posit that loss of Rgs2 leads to increased S1P4 in tumor derived MDSCs compared to wild type MDSCs, and the increase in S1P4 and its downstream signaling mediate subsequent downregulation of MCP-1 (Figure 33B). This suggests that S1P4 is mediating an inhibitory signal that Rgs2 would normally turn off. Not much is known about the signaling downstream of S1P4. Hughes et al. found that treatment of LPS treated macrophages with S1P inhibited NF-κB activity by signaling through S1P1 (Hughes et al., 2008). Perhaps a similar mechanism is employed by S1P-S1P4 signaling. We have observed increased NF-κB p50 within the nuclei of Rgs2-/- tumor derived MDSCs compared to wild type (Figure 27), and have hypothesized that this p50 is inhibitory. It is possible that S1P-S1P4 signaling is the cause of the increased nuclear NF-κB p50. More work is necessary to determine if S1P-S1P4 signaling plays a role in the loss of MCP-1 expression in Rgs2-/- mice, and to ascertain the mechanism of S1P4 upregulation due to loss of Rgs2.

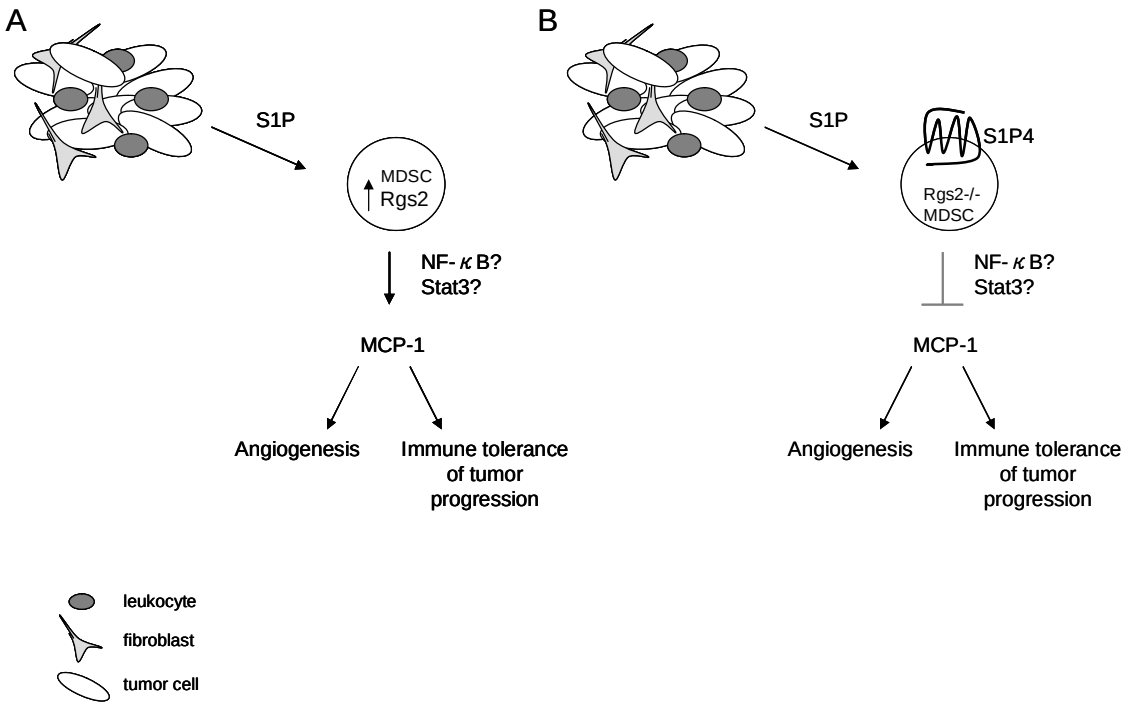

Figure 33. Proposed model of the role of S1P in the induction of Rgs2 and MCP-1. (A) The tumor cells and infiltrating leukocytes release S1P, which can then lead to upregulation of Rgs2 in MDSCs, likely through an S1P receptor. This increase in Rgs2 leads to upregulation of MCP-1 and subsequent promotion of angiogenesis, immune tolerance, and tumor progression. MCP-1 expression may be regulated by NF-κB or Stat3, or even Rgs2 itself in a proposed nuclear role. (B) In Rgs2-/- mice, S1P4 is upregulated, perhaps through lack of inhibition of signaling by Rgs2. MCP-1 is not upregulated, leading to decreased angiogenesis and immune tolerance, and consequently, slower tumor growth compared to wild type animals.

Final remarks

In conclusion, this body of work has established an important role for Rgs2 in MDSCs and cancer progression. Rgs2 does not affect production or differentiation of myeloid cells, but instead mediates pro-angiogenic and immune suppressive functions of MDSCs through regulation of MCP-1 production. Targeting this pathway in cancer treatment could promote decreased angiogenesis and increased immune response, leading to decreased tumor progression and increased patient response. Further work will elucidate the mechanisms involved in Rgs2 regulation and the role of Rgs2 in MCP-1 modulation.

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