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ROLE OF ACID SPHINGOMYELINASE IN THE TUMOUR MICROENVIRONMENT

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INDEX

SUMMARY 3
INTRODUCTION 5
Chapter 1: TumourMicroenvironment 5
1.1 Composition 5
1.2 Non immune components 6
1.3 Immune components 6
1.4 Role in cancer progression 18
1.5 Melanoma microenvironment 24
1.6 Cancer therapy strategies targeting the tumour and the suppressive microenvironment 28
Chapter 2: Ceramide, Acid-sphingomielinase and Tumour 31
2.1 Lipids in cell signalling 31
2.2 Sphingolipids in tumours 36
AIM OF THE STUDY 44
MATERIALS AND METHODS 45
Materials 45
Methods 45
Animals 45
Cell Culture and Treatments 45
Immunofluorescence of Human Tissue Arrays 46
Immunofluorescence quantification 46
Analysis of A-SMase in murine tumours 47
Reverse Transcription-PCR and Quantitative Real Time-PCR (qPCR) 47
Primer pairs designed for qPCR analysis 48
B16-W6_pSIL10 and B16-F1_B1A cell line generation 48
Western blotting 50
Acid sphingomyelinase activity 51
Flow Cytometric Analysis of Tumours 52
Bone marrow derived macrophage cells preparation 52
Transwell Co-cultures of B16-F10 Melanoma/macrophages 52
Tumour growth *in vivo* 53
Statistical analysis 53

RESULTS 54
Acid sphingomyelinase decreases during tumour progression in different human cancers 54
Acid sphingomyelinase decreases during tumour progression in different murine cancers 56
Acid sphingomyelinase downregulation in melanoma could be driven by immune cells-released factors 58
TAM-induced A-SMase downregulation is dependent on Ap2α transcription factor 60
A-SMase downregulation in melanoma cells correlates to increased MDSC accumulation 61
A-SMase-related MDSC accumulation determines immunosuppression 62
Restoring A-SMase expression reduces melanoma growth 66
Restoring A-SMase expression switches tumour microenvironment from a pro- to an anti-tumoural phenotype 67
DISCUSSION 72
BIBLIOGRAPHY 76

SUMMARY

Defective apoptosis represents one of the major causative factors in the development and progression of cancer. The ability of tumour cells to evade engagement of apoptosis can play a significant role in their resistance to conventional therapeutic regimens. In the last few years, preclinical and clinical studies have indicated ceramide and the enzymes of its metabolism, in particular Acid Sphingomyelinase (A-SMase) which hydrolyzes sphingomyelin to ceramide and phosphocoline, as key players in tumour physiopathology. Different cancers have been shown to have reduced ceramide levels and, of interest, in our previous work we showed that A-SMase down-regulation was a key event in melanoma progression. This event is crucial for the tumours to become more aggressive, but the mechanisms responsible of it haven't been investigated yet.

Taking into account that there is a complex crosstalk between tumour cells and its immunological microenvironment, in this work we first investigated its possible role in A-SMase downregulation in a melanoma model.

To this purpose we performed *in vivo* and *in vitro* experiments which led us to identify tumour associate macrophages (TAM) as possible responsible of A-SMase downregulation through the Ap2-α transcription factor. Moreover, we demonstrated that these molecular changes in tumour cells give, in turn, pro-tumoural and immunosuppressive features to the surrounding microenvironment, with the recruitment of Myeloid-derived suppressor (MDSCs) cells and Regulatory T lymphocytes (T_{REGS}). From these and our previous data, we clearly showed that the ability to create this immunosuppression together with the acquisition of a more aggressive phenotype, both depend on the naturally occurring A-SMase decrease in melanoma cells during tumour progression.

The broad role of A-SMase in tumour pathogenesis we identified, indicates that the enzyme is at the crossroad of key pathways in tumourigenesis. This aspects has clear potential in therapeutic perspective in which A-SMase overexpression or administration might be consider as an useful adjuvant for cancer therapy.

Here we demonstrated for the first time that restoring A-SMase expression in melanoma cells not only reduces tumour growth and immunosuppression, but moreover accounts for a high, unexpected recruitment of immune cells with an anti-tumoural function in the

tumour microenvironment, such as Dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T lymphocytes.

In conclusion our results reveal the central role of A-SMase expressed by melanoma cells in orchestrating the cross-talk with the surrounding microenvironment. These interactions are crucial for tumour fate, lying on its rejection or progression. Our observation that A-SMase overexpression "educate" tumour microenvironment against cancer cells, further encourage the use of this enzyme as an adjuvant for cancer therapy.

INTRODUCTION

Chapter 1: Tumour Microenvironment

The tumour microenvironment is composed by several important components including the tumour parenchyma cells, mesenchymal cells, fibroblasts, blood and lymph vessels, as well as tumour infiltrating immune cells, chemokines, and cytokines (Weber and Kuo 2012).These many and varied constituents fulfill the definition of a complex system, whereby the interactions between the components are multiscale, multilevel, and consist of nonlinear dynamics (Oltvai and Barabasi 2002). Each of these components can make important contributions to tumour development and progression.

1.1 Composition

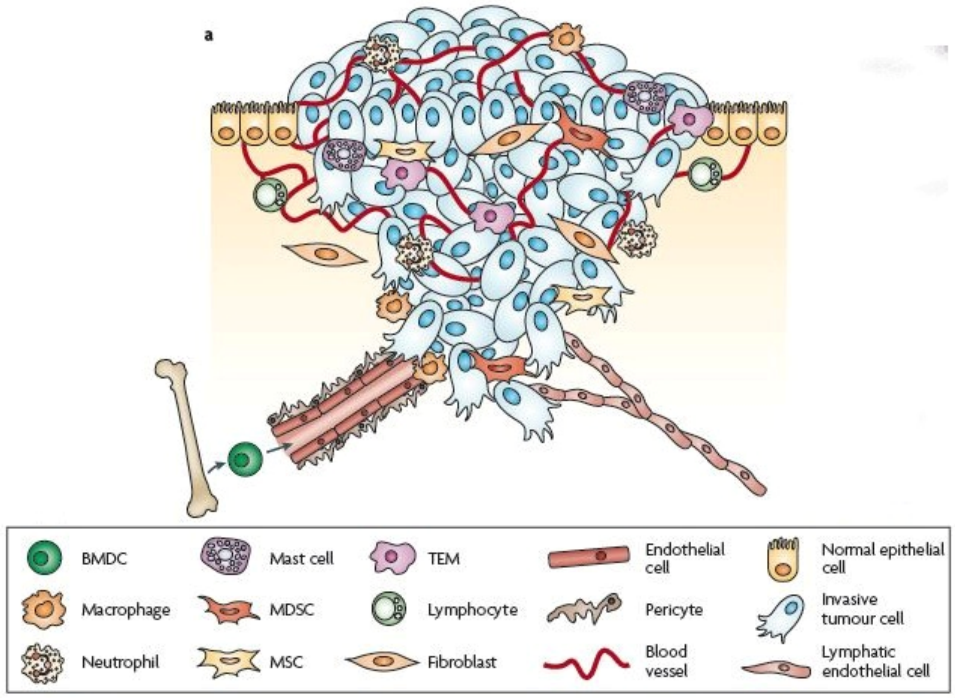

Figure 1: The primary tumour microenvironment(Joyce and Pollard 2009)
Cancer cells in primary tumours are surrounded by a complex microenvironment comprising numerous cells including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts and a variety of bone marrow-derived cells (BMDCs) including macrophages, myeloid-derived suppressor cells (MDSCs), TIE2-expressing monocytes (TEMs) and mesenchymal stem cells (MSCs).

1.2 Non immune components

Among the non immune components, tumour associated fibroblasts are responsible for the formation and remodelling of the extracellular matrix and constitute a source of factors which promote the growth of carcinoma cells (Bhowmick, Neilson et al. 2004). The formation of new vessels is critical for tumour progression as the mass grows bigger (Carmeliet and Jain 2000), while existing blood and lymphatic vessels may act as routes for local invasion and distant metastasis (Sleeman and Thiele 2009; Weis and Cheresh 2011). Many studies have shown that the density of blood vessels and the production of factors that stimulate blood vessel formation, including vascular endothelial growth factor(VEGF), platelet-derived growth factor (PDGF), and matrix metalloproteinases (MMPs), contribute to the spread of tumour cells and predict poor patient survival (Weis and Cheresh 2011). Other host cell lineages including mesenchymal stem cells not only form new carcinoma cells, but are also able to differentiate into the various cell types required to drive angiogenesis during cancer progression (Mohseny and Hogendoorn 2011).

1.3 Immune components

The immune components of tumour microenvironment have gained attention in the last decades for their critical role in tumourigenesis and tumour control. Tumour-infiltrating immune cells including myeloid-derived suppressor cells (MDSC), tumour-associated macrophages (TAM), and cytotoxic lymphocytes are critical determinants of cancer outcomes. Several studies have shown that increased densities of MDSC and TAM promote tumour progression via multiple suppressive mechanisms (Mantovani, Schioppa et al. 2006; Ostrand-Rosenberg and Sinha 2009). In contrast, the presence of cytotoxic lymphocytes within the tumour microenvironment is associated with a good prognosis in many cancers (Pages, Galon et al. 2010; Tosolini, Kirilovsky et al. 2011; Chew, Chen et al. 2012).

Other immune components of the tumour microenvironment, including chemokines and cytokines, may also alter the local balance of pro-regulatory and anti-tumourimmune responses (Wilson and Balkwill 2002; Balkwill 2004). Danger signals such as heat shock proteins, nucleic acids, and HMGB1 released from transformed, dying, or dead tumour cells in the microenvironment tare sensed by innate immunity components such as the

toll-like receptors (TLRs) and can activate antitumour immune responses (Matzinger 2002; Sims, Rowe et al. 2010).

Macrophages.

Macrophages represent a significant portion of the tumour mass, and they certainly operate as fundamental actors in various types of solid cancers (Hao, Lu et al. 2012) facilitating tumour progression via both immunological and non immunological mechanisms. They form a continuous spectrum of cells that range in phenotype from M1-like or classically activated macrophages to M2-like or alternatively-activated macrophages (Mantovani, Sica et al. 2009). M1-like macrophages are typically activated by IFNγ, and lipopolysaccharide (LPS), and are characterised by their high expression of IL-12 and low expression of IL-10. This cytokine profile promotes the development of a type 1 T cell response which facilitates anti-tumour immunity. In addition, M1-like macrophages can be tumouricidal. In contrast, M2-like macrophages are activated byIL-4, IL-13, IL-10, and glucocorticoid hormones, produce high levels of IL-10 and low levels of IL-12, and promote tumour progression. Macrophages phenotype is driven by their local tissue microenvironment and the tumour microenvironment has been shown to strongly polarise macrophages towards an M2-like phenotype, giving rise to so-called "tumour associated macrophages" (TAMs) (Ostrand-Rosenberg, Sinha et al. 2012), even if recent studies have identified up to seven phenotypically different macrophage subpopulations within tumours, demonstrating the complexity of this cell population (Movahedi, Laoui et al. 2010). Analysing TAM phenotype from a molecular point of view, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Hypoxia-inducible factor 1-alpha(HIF-1) have been identified as master regulators of TAM's transcriptional programs and, at the same time, as central regulators of tumour progression and metastasis (Sica and Bronte 2007).

TAM products can influence many aspects of tumour growth and progression (Mantovani, Allavena et al. 2008). In particular, they can regulate senescence; interact with and contribute to extracellular matrix remodelling; promote cancer cell proliferation, invasion and metastasis (Lin, Li et al. 2006); sustain angiogenesis and lymphangiogenesis (Lin, Li et al. 2006; Clear, Lee et al. 2010). TAMs express low levels of the major histocompatibility complex class II and reduced antimicrobial and tumouricidal activity. Finally, they suppress anti-tumoural adaptive immunity.

TAMs produce enzymes and proteases, which regulate the digestion of components of the extracellular matrix (ECM), such as matrix metalloproteases (MMPs), plasmin, urokinase-type plasminogen activator (uPA) and the uPA receptor (Nagakawa, Aoki et al. 2002). MMP expression has been implicated in tumour progression through enhancing angiogenesis, tumour invasion and metastasis (Naylor, Stamp et al. 1994; Wang, So et al. 2005). TAMs have been reported to correlate with the metastatic potential of a variety of human cancers (Lin, Li et al. 2011; Wei, Fang et al. 2012) and they have also been shown to be a major source of MMP-9 (Wang, Zhang et al. 2011).

Moreover, TAMs have been shown to promote angiogenesis and lymphangiogenesis by releasing proangiogenic growth factors such as TGF-β, VEGF-A, VEGF-C, EGF, thymidine phosporylase (TP) and chemokines, such as CCL2/MCP-1 and CXCL8/IL-8 (Hotchkiss, Ashton et al. 2003; Lin, Li et al. 2006; Murdoch, Muthana et al. 2008; Granata, Frattini et al. 2010; Schmidt and Carmeliet 2010) favouring subsequent dissemination of cancer cells In this perspective, macrophages act as "bridge cells" or "cellular chaperones" that guide the fusion of endothelial tip cells (vascular anastomosis) and facilitate vascular sprouting (Fantin, Vieira et al. 2010; Schmidt and Carmeliet 2010).

An additional feature of TAMs consists in their ability to suppress the adaptive immune response through their poor antigen-presenting activity and inhibition of T cell proliferation (Sica, Schioppa et al. 2006). Tumour-specific microenvironment can alter the mechanisms by which TAMs can exert their immunosuppressive functions. For instance, in murine models of breast and lung tumours, $CD8^+$ T cell suppression is dependent, at least in part, on TAM metabolic activities via arginase-1 (ARG-1) or inducible Nitric Oxide Synthase (i-NOS) (Doedens, Stockmann et al. 2010; Movahedi, Laoui et al. 2010). In contrast, in human tumours, TAM-mediated $CD8^+$ T cell suppression occurs regardless of l-arginine metabolism (Kryczek, Zou et al. 2006), but it depends on B7-H1 in hepatocellular carcinoma (Kuang, Zhao et al. 2009) and B7-H4 in ovarian cancer (Kryczek, Zou et al. 2006).

In spite of several evidences on the pro-tumoural role of TAMs in several tumours, there is evidence pointing in the opposite direction. In large B-cell lymphoma patients, Hasselblom et al. did not find any statistically significant correlation between the number of $CD68^+$ cells and any clinical aspect, including progression-free survival and overall survival. In high-grade osteosarcoma patients (Hasselblom, Hansson et al. 2008), it has been found a statistically significant correlation between $CD68^+$ cells and better survival (Buddingh, Kuijjer et al. 2011). In colorectal cancer (CRC), the role of TAM in tumour

progression seems to be controversial. Indeed, even if some studies indicated a role of TAMs in CRC progression, evidences indicate a strong anti-tumoural activity of TAMs and their association with improved disease-free survival (Forssell, Oberg et al. 2007).

T lymphocytes.

Both preclinical and clinical studies suggest a strict correlation between the presence of tumour-infiltrating lymphocytes (TILs) with a favourable prognosis in a wide number of solid tumours (Galon, Costes et al. 2006; Fridman, Pages et al. 2012; Senovilla, Vacchelli et al. 2012), although not all lymphocyte types are endowed with anti-tumour activity (Vesely, Kershaw et al. 2011). CTL play a relevant role in the process of tumour rejection. They are defined as $CD8^+$T cells producing massive amounts of IFN-γ and other molecules necessary for their cytotoxic activity, such as granzyme B and perforin. The key role of CTL both in immune surveillance against rising malignancies and in contrasting the metastatic expansion has been demonstrated in mouse models exploiting UV-induced skin cancers (Ward, Koeppen et al. 1990), chemically induced papilloma (Yusuf, Nasti et al. 2008), and in the *ret* oncogene transgenic model of spontaneous melanoma (Eyles, Puaux et al. 2010). High frequencies of circulating $CD8^+$T lymphocytes were detected in patients with metastatic melanoma, specific for Melan-A/MART-1, MAGE-10, and Ny-Eso-1, and CTL have also been found infiltrating melanoma metastases (Clark, Elder et al. 1989; Clemente, Mihm et al. 1996; van Houdt, Sluijter et al. 2008; Fuertes, Kacha et al. 2011). On this basis, adoptive transfer of CTL is therapeutically effective for mouse tumour models, although, unfortounately ,to a minor extent for cancer patients (Mempel and Bauer 2009). These T cells, attracted by chemotactic stimuli secreted by the tumour mass, acquire the ability to migrate toward it. CTL do not require integrin to interact with neoplastic cells and are endowed with the ability to shift between components of tumour mass by means of ameboid locomotion (Weigelin, Krause et al. 2011). Time-lapse microscopy in a mouse melanoma model illustrated that melanoma-specific CTL effectively traffic to the melanoma site, where they engage contact with the B16-F10 cells via TCR/MHC-peptide interactions (Weigelin, Krause et al. 2011). Subsequently, tumour cells undergo apoptotic cell death induced by cytotoxic activity of CTL. These forming apoptotic bodies are made available for tumour-infiltrating dendritic cells (DC) that are thus allowed to sustain the systemic tumour-specific immune surveillance by migrating to tumour-draining lymph nodes and other distant lymphoid organs, such as spleen, in order to present the processed peptides

to naïve T cells. Moreover, it is also widely documented that the presence of TILs, including CTLs, is correlated with a favourable prognosis for several types of cancers, such as colon, breast, lung, ovarian, and oesophagus cancer, just to cite a few (Naito, Saito et al. 1998; Schumacher, Haensch et al. 2001; Zhang, Conejo-Garcia et al. 2003; Sato, Olson et al. 2005; Alexe, Dalgin et al. 2007; Al-Shibli, Donnem et al. 2008; Fridman, Pages et al. 2012).

The killing efficiency of target tumour cells by CTL is dependent on several factors, such as the cytokine/chemokine patterns produced by the tumour microenvironment, the molecular plasticity of tumour cells to evade CTL-induced killing and the strength of TCR/MHC-peptide interactions. In this regard, melanoma cells have been shown to down-regulate the MHC-I surface expression, thus bypassing the interaction with CTL via TCR (Mempel and Bauer 2009). Of importance, it has been demonstrated that induction of co-stimulatory molecule expression in human melanoma cell surface led to stimulation of CTL activity by Natural killer (NK) cells (Tarazona, Casado et al. 2004).

On the other hand, a significant fraction of TILs is composed by the regulatory T (T_{REG}) cells, endowed with potent suppressive activity that counteract anti-tumour responses and favour tumour escape and progression. T_{REG} cells were reported to infiltrate a wide range of mouse and human tumours, such as melanoma, lung adenocarcinoma, breast cancer, and gastrointestinal tumours (Quezada, Peggs et al. 2011; Wang, Ma et al. 2012). Elevated frequencies of T_{RE} cells in peripheral blood and at the tumour site of cancer patients correlate with poor prognosis and reduced survival. However the mechanisms driving T_{REG} cell expansion, accumulation, and migration to the tumour site are currently unknown. It is likely, that factors relevant within the tumour microenvironment, such as the presence of TGF-β and low antigen stimulation, may play a role in the induction of T_{REG} cells *in vivo* (Quezada, Peggs et al. 2011). Through the secretion of inhibitory cytokines and the expression of surface markers, T_{REG} cells inhibit the effector function of most immune cells, including T and B cells, DC, macrophages, and NK cells(Wang, Ma et al. 2012). $FoxP3^+$ T_{REG} cells within tumour burden express elevated levels of multiple suppressive receptors such as programmed cell death protein 1(PD-1), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), T cell membrane protein 3 (TIM-3), Lymphocyte-activation gene 3 (LAG-3), and Glucocorticoid-induced TNFR family related gene (GITR) (Sakuishi, Apetoh et al. 2010; Park, Kusnadi et al. 2012). The identification of these receptors has gained interest for the development of targeted anti-tumour strategies aimed at selectively depleting T_{REG} cells at the tumour site (Menetrier-Caux, Curiel et al.

2012; Menetrier-Caux, Faget et al. 2012; Wang, Ma et al. 2012). For example, simultaneous blockade of CTLA-4 and PD-1 was shown to reduce the frequencies of T_{REG} cells and to increase the numbers of effector TILs in mice bearing established B16-F10 melanoma, improving the efficacy of tumour vaccines (Curran, Montalvo et al. 2010). Similar encouraging results have been obtained with melanoma patients, suggesting that approaches aimed at combining T_{REG} cell depletion-based immunotherapy with current tumour treatment protocols may be promising therapeutic strategies in clinical oncology (Hodi, O'Day et al. 2010). However, since PD-1 and CTLA-4 are not uniquely expressed by T_{REG} cells, these results may be attributable to the blockade or re-activation of other T cell subsets (Badoual, Hans et al. 2013). In some cases T_{REG} cells may also contrast tumour progression. Indeed, T_{REG} lymphocyte infiltration has been associated with favourable prognosis in several types of solid cancers, such as ovarian, bladder, head/neck, and colorectal tumours (Badoual, Hans et al. 2009; Fridman, Pages et al. 2012). The mechanisms by which T_{REG} cells exert opposite function, depending on the tumour type, are still under investigation, although it is plausible to hypothesise that the phenotypical uniqueness of each cancer microenvironment may elicit the recruitment of different T_{REG} cell subsets (Fridman, Pages et al. 2012).

Dendritic cells.

The major function of dendritic cells (DC) is to process and present antigen for the activation of $CD4^+$ and $CD8^+$ T cells. Endocytosis of antigen by immature DC drives DC maturation and the subsequent presentation of antigen to T cells. However, the tumour microenvironment systemically perturbs this process by increase in the accumulation of immature DC and decreasing DC maturation (Gabrilovich 2004). As a result, DC fail to activate tumour-reactive T cells and/or become tolerogenic. Defective dendritic cell function has been found in many patients with a variety of cancers, as well as inmost mice with transplanted or spontaneous tumours (Pinzon-Charry, Maxwell et al. 2005). Multiple conditions and factors within the tumour microenvironment, including hypoxia, lactic acid build-up, and adenosine accumulation, cause DC abnormalities (Gabrilovich, Ostrand-Rosenberg et al. 2012). In this respect, DC may be viewed as a double-edged sword at the tumour site affecting either positively or negatively the anti-tumour response, depending on the composition of the tumour microenvironment (Schiavoni, Gabriele et al. 2013).

As explained above, tumour-infiltrating DC (TIDC) are present in different types of solid cancers, but the largest number of TIDC has been found in melanoma (Furumoto, Soares et al. 2004). These TIDC are composed by myeloid and to a lesser extent plasmacytoid DC (Mattei, Schiavoni et al. 2012). The latter display an immature phenotype *in situ*, but retain the ability to mature into fully competent APC following dissociation from the tumour bulk, without the need of cytokine or bacterial product exposure (Preynat-Seauve, Schuler et al. 2006). In this regard, dying tumour cells are thought to provide maturation signals. Therefore, DC spontaneously infiltrate melanoma and other types of solid cancers, and are potentially endowed with the capability to process a soluble tumour-associated antigen (Preynat-Seauve, Schuler et al. 2006). These TIDC then migrate toward draining lymph nodes in order to activate both naïve $CD4^+$and $CD8^+$T lymphocytes (Fuertes, Kacha et al. 2011; Gerlini, Di Gennaro et al. 2012; Gerlini, Sestini et al. 2013). Therefore, newly generated cytotoxic $CD8^+$T cells may further contribute to tumour rejection by migrating toward the tumour site. The extent of tumour infiltration by mature DC has been often correlated with favourable prognosis in a wide array of clinical cancers (Cox, North et al. 2005; Ladanyi, Kiss et al. 2007; Park, Lee et al. 2012). In a mouse model of melanoma, it was recently shown that host immunodeficiency results in poor tumour infiltration by effector immune cells, such as T cells and DC, and closely associates with melanoma progression (Mattei, Schiavoni et al. 2012). In this model, melanoma phenotype was shown to be shaped directly by cells of the immune system through release of soluble factors within the tumour microenvironment (Businaro, De Ninno et al. 2013).

Natural Killer cells.

NK cells are large granular lymphocytes acting by their cytotoxic capacity and massive cytokine production. Human NK cells share with macrophages the surface expression of CD16 (FcyRIII), but are diversified from them by expression of CD7, CD56, and CD57 (Milush, Long et al. 2009; Yasuda, Aritaka et al. 2011; Senovilla, Vacchelli et al. 2012) and by mechanisms of pathogen killing. Indeed, whereas macrophages kill target cells by phagocytosis, NK cells mediate target cell lysis by secretion of perforin- and granzyme B-containing granules (Pardo, Aguilo et al. 2009; Afonina, Cullen et al. 2010). A recent report demonstrated that NK cells are endowed with a potent ability to secrete calcium ions, and that this function allow these cells to increase their killing ability (Schwarz, Qu et al. 2013). For their killing activity, NK cells cover an important role in immune

responses against tumours. Another interesting function of NK cells is the so called “DC editing.” This term specifies the ability of activated NK cells to interact with autologous DC and kill those cells that are not fully mature. Through this process, NK cells contribute to maintain the reservoir of immunogenic DC by killing potentially tolerogenic DC thus optimizing effector anti-tumour responses (Moretta 2002; Moretta, Ferlazzo et al. 2006; Morandi, Mortara et al. 2012).

The functionality of NK cells is fundamental for contrasting the growth and metastatic process of several types of cancers. For example, several reports have elucidated the role of NK cells in killing cancer cells in murine models of melanoma, colon cancer, lung cancer, and breast cancer (Azogui, Avril et al. 1991; Pham-Nguyen, Yang et al. 1999; Velthuis, Stitzinger et al. 2003; Carrega, Pezzino et al. 2009; Frings, Van Elssen et al. 2011; Kim, Kim et al. 2011; Takeda, Nakayama et al. 2011; Vesely, Kershaw et al. 2011; Roberti, Mordoh et al. 2012; Srivastava, Sharma et al. 2012). There are several NK receptors, such as DNAM-1, CD155, CD16, CD69, NKp30, and NKp46, whose surface expression is fundamental for maintaining cancer immune surveillance (Clausen, Vergeiner et al. 2003; Garcia-Iglesias, Del Toro-Arreola et al. 2009; Lakshmikanth, Burke et al. 2009; Chan, Andrews et al. 2010; Levy, Roberti et al. 2011; Pfeiffer, Seitz et al. 2011; Gleason, Verneris et al. 2012; Park, Park et al. 2013). In this regard, some groups investigated the phenotypic profile and functions of intratumoural NK cells in primary human tumours of non-small cell lung carcinoma (NSCLC) by using the NK cell marker NKp46. The data showed that these intratumoural NK cells display a deeply altered phenotype that strongly contributed to the cancer progression in patients (Platonova, Cherfils-Vicini et al. 2011). These results strengthen the critical role of the cancer microenvironment and its composition, and identify NK cells as important predictive biomarkers of neoplastic disease progression. A recent study performed in a mouse tumour model elucidated the importance of NK cells in a vaccination strategy against lung cancer. A survivin-based vaccination, coupled to the use of novel form of the 4-1BBL co-stimulatory molecule as an adjuvant has been effective in completely suppressing Lewis lung carcinoma (3LL)progression. The vaccine efficacy was correlated with potent killing responses of NK cells (Srivastava, Sharma et al. 2012). Another report highlighted the role of NK cells of lung tissue during the generation of lung metastases from melanoma, elucidating that IFN-γ production by these lung-resident NK cells markedly repressed the formation of metastases (Takeda, Nakayama et al. 2011).

Defects in NK cell number or in their phenotype are events that dictate the fate of neoplastic diseases other than lung carcinoma. Many investigations defined the key role of NK cell receptors in melanoma and several studies reported possible mechanisms by which melanoma cells escape killing activity of NK cells (Balsamo, Pietra et al. 2012; Pietra, Vitale et al. 2012; Wang, Cui et al. 2012). Nevertheless, strategies aimed at promoting and sustaining the melanoma-specific killing activity of NK cells are only at early stages or poorly effective. Recently it has been shown an active role of the chemotherapeutic drug dacarbazine, largely used for this type of neoplastic disease, in activating the expression of NK1G receptor on NK cell surface, thus restoring the killing activity of NK cells toward melanoma cells (Hervieu, Rebe et al. 2013). Similar encouraging approaches have been recently started for breast cancer by using the chemotherapeutic agent Cetuximab. This drug was shown to be effective in promoting NK cell killing activity in high relapse rate, triple negative breast cancer patients. Indeed, Cetuximab restored IL-2/IL-15-mediated NK cell killing activity, thus markedly improving the outcome of these patients (Roberti, Rocca et al. 2012). Taken together, these data strongly support a key role of NK cells in tumour progression. Indeed, when activated, NK cells fight malignant cells inside the microenvironment by direct killing as well as by contrasting the generation of metastatic foci.

Myeloid Derived Suppressor Cells.

Myeloid Derived Suppressor Cells (MDSCs) were originally identified in tumour-bearing mice as cells that co-express CD11b and Gr1, however their phenotype in cancer is rather different (Peranzoni, Zilio et al. 2010; Greten, Manns et al. 2011). Currently, two main MDSC subtypes have been reported, granulocytic and monocytic MDSC (G- and M-MDSC) . The G-MDSC are defined by the phenotype $CD11b^{+}Gr\text{-}1^{hi}Ly\text{-}6G^{+}Ly\text{-}6C^{lo}CD49d^{-}$, whereas the M-MDSC are characterised by the phenotype $CD11b^{+}Gr\text{-}1^{hi}Ly\text{-}6G^{-}Ly\text{-}6C^{hi}CD49d^{+}$ (Gabrilovich, Ostrand-Rosenberg et al. 2012).In humans the situation is even more complex, but M-MDSC are predominantly $CD14^{+}$and G-MDSC are $CD15^{+}$, both being $CD33^{+}HLA\text{-}DR^{-}$ (Schmielau and Finn 2001; Zea, Rodriguez et al. 2005; Mirza, Fishman et al. 2006; Filipazzi, Valenti et al. 2007; Mandruzzato, Solito et al. 2009; Poschke, Mougiakakos et al. 2010; Gowda, Godder et al. 2011). In both mice and humans the G-MDSC represent the major subset of circulating and expanding MDSC (Gabrilovich, Ostrand-Rosenberg et al. 2012). Almost all patients and animal models with cancer displayed a higher amount of G-MDSC compared with M-MDSC (approximately

75% vs. approximately 25%) (Schmielau and Finn 2001; Kusmartsev, Nefedova et al. 2004; Youn, Nagaraj et al. 2008; Rodriguez, Ernstoff et al. 2009). Increased production of intra-tumoural granulocyte (G-CSF) or granulocyte–macrophage (GM-CSF) colony-stimulating factors may account for the difference observed in G-MDSC and M-MDSC levels (Dolcetti, Peranzoni et al. 2010; Meyer, Sevko et al. 2011). Inappropriate levels of G-CSF have been reported for many different human tumours, including pancreatic, oesophageal, gastric and glioma (Gabitass, Annels et al. 2011; Raychaudhuri, Rayman et al. 2011). Waight *et al*. (Waight, Hu et al. 2011) identified tumour-derived G-CSF as a key driver of G-MDSC accumulation in mice. Despite this, the frequency of each MDSC subset seems to be influenced by the type of cancer patients with renal cancer have immunosuppressive PMN-MDSCs(Rodriguez, Ernstoff et al. 2009), while M-MDSCs circulate in the blood of patients with melanoma, multiple myeloma, prostate cancer, hepatocellular carcinoma, and head and neck cancer (Serafini, Meckel et al. 2006; Filipazzi, Valenti et al. 2007; Hoechst, Ormandy et al. 2008; Mandruzzato, Solito et al. 2009; Vuk-Pavlovic, Bulur et al. 2010).

Both M-MDSC and G-MDSC use antigen-specific and antigen non-specific mechanisms to regulate immune responses. Interestingly, G-MDSC and M-MDSC can inhibit T-effector cells through different modes of action, although these mechanisms are not exclusively applied by one of the two subtypes (Gabrilovich and Nagaraj 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012). Production of ROS is mainly used by G-MDSC, whereas the generation of NO and secretion of ARG-1 has been predominantly established for M-MDSC (Movahedi, Guilliams et al. 2008; Youn, Nagaraj et al. 2008; Dolcetti, Peranzoni et al. 2010). Production of ROS by MDSC is mediated through the increased activity of NADPH oxidase (NOX) 2; and the up-regulation of ROS by Gr-1^{+}CD11b^{+}MDSC isolated from seven different tumour models and by CD11b^{+}CD14^{-}CD33^{+}MDSC in patients with head and neck cancer has been already identified. These MDSC showed significantly higher expression of the NOX2 subunits p47*phox* and gp91*phox* compared with immature myeloid cells from tumour-free mice (Corzo, Cotter et al. 2009).In the absence of NOX2 activity, MDSC lost the ability to control T-cell hyporesponsiveness and differentiated into mature DC. Indeed, MDSC from gp$91^{-/-}$mice are not able to induce T-cell tolerance, confirming the role of ROS in T-cell suppression (Nagaraj, Gupta et al. 2007). In addition the cooperative activity of ROS with NO forms peroxynitrite (Kusmartsev, Nefedova et al. 2004; Gabrilovich and Nagaraj 2009; Gabrilovich, Ostrand-Rosenberg et al. 2012) that leads to the nitration of

tyrosines in the T-cell receptor (TCR)–CD8 complex. This reaction might affect the conformational flexibility of TCR-CD8 and its interaction with peptide-loaded MHCI, rendering the $CD8^+$T cells (cytotoxic T lymphocytes; CTL) unresponsive to antigen-specific stimulation (Nagaraj, Gupta et al. 2007). Indeed, nitration inhibits the binding of processed peptides to tumour cell-associated MHC, and as a result, tumour cells become resistant to antigen-specific TIL (Lu, Ramakrishnan et al. 2011). Furthermore, peroxynitrite leads to the nitration of CCL2 chemokines thereby inhibiting TIL trafficking into the tumour, resulting in trapping of antigen-specific CTL in the tumour-surrounding stroma (Molon, Ugel et al. 2011).Another mechanism by which MDSC can interfere with T-cell trafficking is the expression of the disintegrin and metalloproteinase domain (ADAM) 17, which decreases CD62 ligand expression and renders T cells immobile (Gabrilovich, Ostrand-Rosenberg et al. 2012).

The suppressive activity of ARG-1 is based on its fundamental role in the hepatic urea cycle, metabolizingl-arginine tol-ornithine. Expression of ARG-1 has been reported to down-regulate TCR cell surface expression by decreasing CD3 ζ-chain biosynthesis(Rodriguez, Zea et al. 2002). This induces an arrest of T cells in the G0–G1 phase of the cell cycle, associated with a deficiency of protein kinase complexes that are important for G1 phase progression(Rodriguez, Ernstoff et al. 2009).*In vivo*, the depletion of $CD14^-CD15^+$G-MDSC re-established CD3ζ-chain biosynthesis and T-cell growth; further emphasizing the detrimental role that these MDSC play in cancer (Zea, Rodriguez et al. 2005). Cancer-expanded MDSC can also induce anergy in NK cells through membrane bound TGF-β, signal transducer and activator of transcription 5 (STAT5) activity, ARG-1 or via the NKp30 receptor (Liu, Yu et al. 2007; Hoechst, Voigtlaender et al. 2009; Oberlies, Watzl et al. 2009; Alizadeh, Zhang et al. 2010). The MDSC can suppress NK cell cytotoxicity by inhibiting NKG2D and interferon-γ (IFN-γ) production in models of glioma (Alizadeh, Zhang et al. 2010).

Another type of immunosuppression modulated by MDSC is the activation and expansion of T_{REG} cells. Mouse studies *in vivo* suggested that MDSC support the *de novo* development of T_{REG} cells through TGF-β-dependent(Huang, Pan et al. 2006; Yang, Cai et al. 2006)and -independent pathways (Serafini, Mgebroff et al. 2008). Yang *et al.* reported that suppression of $Gr\text{-}1^+CD11b^+$MDSC isolated from ovarian-carcinoma-bearing mice was dependent on the presence of CD80 on the MDSC and involved $CD4^+CD25^+T_{REG}$ cells and CD152, suggesting a relationship between MDSC and T_{REG} cells (Yang, Cai et al. 2006). In a mouse colon carcinoma model, IFN-γ activated Gr-

$1^{+}CD115^{+}$M-MDSC were shown to up-regulate MHCII and produce IL-10 and TGF-β to mediate the development of tumour-induced $CD4^{+}CD25^{+}T_{REG}$ cells. The production of NO by Gr-1^{+} (Poschke, Mao et al. 2012)dispensable for T_{REG}-cell induction (Huang, Pan et al. 2006). These data exemplify the relationship between MDSC and T_{REG} cells.

Finally MDSCs have been demonstrated to exert their immunosuppressive functions trough DCs impairment. In many cancer patients the numbers of mature DC are reduced and DC function is deficient. Although multiple factors are likely to contribute to DC dysfunction, evidence is accumulating that MDSC–DC cross-talk may at least be partially responsible. In vitro studies in which mouse MDSC were differentiated from $ckit^{+}$ bone marrow progenitor cells in the presence of IL-4, GM-CSF, and PGE2demonstrated that the numbers of mature DC decreased proportionately to the increasing numbers of MDSC (Sinha, Clements et al. 2007). The differentiation of murine DC was similarly reduced when mixtures of murine myeloid cells were treated with LPS and IFNγ (Greifenberg, Ribechini et al. 2009). IL-10 produced by hepatocellular carcinoma-induced MDSC has also been shown to decrease DC production of IL-12 (Hu, Gan et al. 2011). Since MDSC and DC share a common progenitor cell, the reduction in mature DC observed in cancer patients may be due to the skewing of the common MDSC/DC progenitor towards the preferential differentiation of MDSC at the expense of DC.

Recent in vitro studies assessing the effects of MDSC obtained from cancer patients on DC differentiation further support a role for MDSC–DC cross-talk. Studies with MDSC from melanoma patients demonstrated that MDSC impaired DC maturation by reducing antigen uptake, preventing migration of immature and mature DC, blocking the ability of DC to induce IFNγ-producing T cells, and altering DC cytokine production towards an anti-inflammatory phenotype (Poschke, Mao et al. 2012). DC production of the pro-inflammatory cytokine IL-23 and its downstream induction of Th17 cells may contribute to the effects of MDSC on DC. Further studies are needed to clarify the role of MDSC in regulating DC function, and the complexity of the tumour microenvironment with respect to the milieu of cytokines and chemokines is likely to make this task difficult.

Collectively, the data show that MDSC can employ a diverse set of distinct mechanisms to affect tumour cells, endothelial cells and immune cells to create a local environment that sustains tumour growth and survival while suppressing anti-tumour immune responses.

There are many evidences that the immunosuppressive role of MDSC is dependent on STAT transcription factors. By using $STAT6^{-/-}$mice models transplanted with 4T1 cancer

cells (Sinha, Clements et al. 2005)it has been shown that MDSC render 4T1 mammary tumours poorly immunogenic by suppressing the activation of $CD4^+$and $CD8^+$T cells. The involvement of STAT signalling in the immunosuppressive activity of MDSC in cancer has been further highlighted in studies on human head and neck squamous cell carcinoma and breast cancer, suggesting a pivotal role of STAT3 and STAT1 pathways, respectively, for MDSC suppressive activity within cancer microenvironment (Hix, Karavitis et al. 2013; Vasquez-Dunddel, Pan et al. 2013).

1.4 Role in cancer progression

Immune cell infiltration within a solid tumour is a naturally occurring event, when cells belonging to immune system enter within the tumour microenvironment by means of tumour-forming blood vessels and/or extravasation. This event can sometimes lead to blocking of cancer progression and thus limit or even prevent the generation of metastasis (Vesely, Kershaw et al. 2011).

Multiple lineages of immune cells are involved in antitumour responses. It has long been established that NK cells are able to kill tumour cells in various cancer models (Pross and Lotzova 1993; Stojanovic and Cerwenka 2011). Similarly, cytotoxic T lymphocytes can detect “abnormal” tumour antigens expressed on carcinoma cells and target those cells for destruction (Weigelin, Krause et al. 2011). Antigen-presenting cells, and dendritic cells in particular, process and present tumour derived antigens in the context of MHC class I molecules to activate $CD8^+$ T cells via a mechanism known as cross presentation (Flinsenberg, Compeer et al. 2011). Apart from direct killing of carcinoma cells, the activated $CD8^+$ T cells may also inhibit angiogenesis by secreting IFN-γ (Qin, Schwartzkopff et al. 2003). B cell activation and differentiation into antibody-producing plasma cells as well as T- and B cell memory are each important components of long lasting immune surveillance in cancer vaccines (DiLillo, Yanaba et al. 2010).

In general, all these events occur when tumour progression is at early stages as a result of host immune surveillance. At this point, if the organism is able to generate a prompt tumour-specific immune response, cancer progression is contrasted by immune forces and sometimes inhibited. In many cases, unfortunately, the host is unable to generate an effector immune response toward the tumour, due to its ability to activate immunosuppressive mechanisms that circumvent or dampen immunity, thus resulting in to tumour escape from host immune surveillance. In this scenario, the balance of immune populations endowed with effector *vs.* suppressive activities at the tumour site, is a critical parameter predicting cancer progression (Schiavoni, Gabriele et al. 2013) (Figure 2

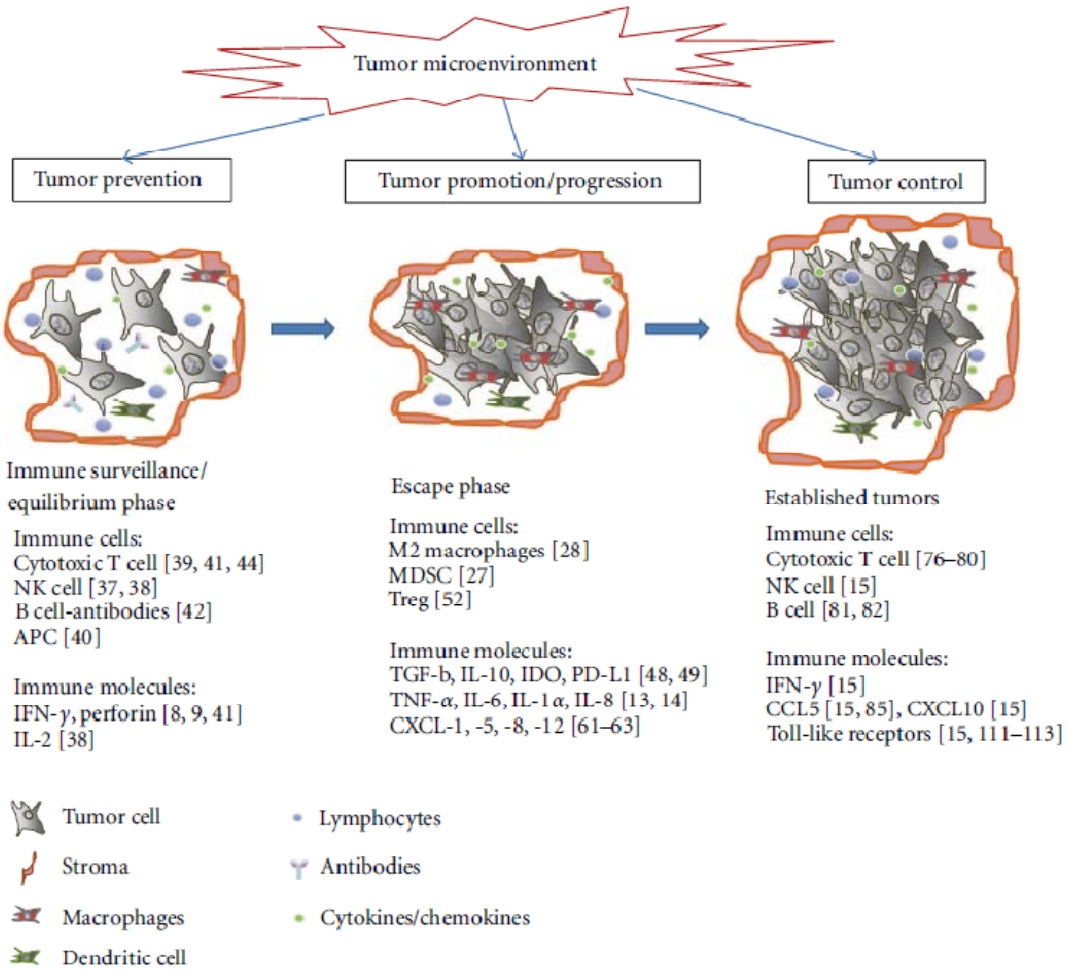

Figure 2: Multiple roles of the immune microenvironment during tumour development(Chew, Toh et al. 2012).

The immune system initially eliminates tumour cells via cytotoxic T cell and NK cell killing mechanisms (immune surveillance). This is obtained with the help of antigen-presenting cells (APC) such as the

dendritic cells, antibodies expressed by B cells, and inflammatory cytokines including IFN-γ and IL-2 which activate the local immune response. However, with the progressive accumulation of tumour cell mutations and modifications to the microenvironment, the tumour cells can eventually "escape" from immunesurveillance. Multiple lineages of immune cells including myeloid-derived suppressor cells (MDSC), tumour-associated macrophages (TAM), and regulatory T cells (T_{REG}), as well as various immune mediators such as TNF-α, IL-6, CXCL-1, CXCL-5, VEGF, and MMP, are responsible for modelling a favourable microenvironment for tumour growth. Recent findings also show that the immune response continues to play an important role in established tumours via mechanisms that involve cytotoxic T cells and NK cells, as well as IFN-γ, CCL5, CXCL10, and toll-like receptors.

Initial Elimination/Equilibrium Phase of immune response

The period during which tumour cells are constantly being killed and controlled by the immune system is referred to as the "equilibrium phase." The tumour can stay dormant for long periods of time until the microenvironment becomes permissive for growth (Almog 2010). This tumour dormancy is effectively explained in a spontaneous melanoma mouse model wherein tumour cells disseminate early but remain dormant at remote metastatic sites (Eyles, Puaux et al. 2010). Dormancy is partly controlled by cytotoxic $CD8^+$ T cells, since depletion of $CD8^+$ T cells results in faster outgrowth of visceral metastases (Eyles, Puaux et al. 2010). However, the continuous control of tumour cells exerts a selective pressure which eventually favours the more aggressive tumour cells. For example, these tumour cell variants may mutate surface antigens in order to become less immunogenic via a process termed "immunoediting." The equilibrium phase will thus eventually reach a state of exhaustion when the more aggressive and less immunogenic tumour cells are able to "escape" immune surveillance (Chew, Toh et al. 2012).

The "Escape" Phase

Tumour development and progression are influenced by modifications to tumour parenchymal cells or their microenvironment. One important mechanism of escape from immune surveillance is the selection of poorly immunogenic tumour cells (Browning and Bodmer 1992). Alternatively, modification of the microenvironment may also result in the acquisition of a "pro-cancer" profile that encourages tumour outgrowth. These pro-cancer modifications include the expression of anti-apoptotic molecules which prevent tumour cell death (Reed 1999); growth factors which stimulate tumour growth (Zhang, Nie et al. 2010), and immunosuppressive mediators such as VEGF, transforming growth factor-β (TGF-β), interleukin (IL)-10, indoleamine 2,3-dioxygenase (IDO), and programmed cell death-ligand 1 (PD-L1) which suppress antitumour immunity (Ben-

Baruch 2006; Gajewski, Meng et al. 2006). TLR pathways such as TLR4 activation on tumour cells have also been shown to directly stimulate tumour growth (Huang, Zhao et al. 2005). Furthermore, as a result of imbalances between pro- and antiangiogenic factors, the microvasculature formed within the tumour microenvironment is often leaky and dysfunctional (Goel, Duda et al. 2011), thus limiting T-cell infiltration and drug diffusion into the tumour. The tumour microenvironment is further shaped by resident leukocytes and the ongoing recruitment of different immune cell subsets. For example, the recruitment of T_{REG} and MDSC contributes to immunosuppression within the tumour microenvironment (Zou 2006; Ostrand-Rosenberg and Sinha 2009). MDSC for instance act on multiple levels to inhibit naïve T-cell proliferation and differentiation, to block T-effector cell functions, and to induce T_{REG} via the expression of IL-10 and TGF-β (Ostrand-Rosenberg and Sinha 2009). TAM (with a unique M2-like phenotype) have similarly been shown to correlate with poor prognosis in various cancers due to their immunosuppressive and angiogenic or lymphangiogenic properties (Mantovani, Schioppa et al. 2006). The contribution of other leukocyte subsets to shaping the tumour microenvironment is less clear. For istance, while the role for Th17 cells in cancer is rather controversial (Middleton, Annels et al. 2012), investigators have reported that these cells are associated with a poor prognosis in colorectal cancer (Tosolini, Kirilovsky et al. 2011). Previous reports have even implicated B cells in enhanced tumour metastasis (Staquicini, Tandle et al. 2008; Olkhanud, Damdinsuren et al. 2011). On the other hand, the tumour microenvironment has been reported to prevent DC maturation hence making them incapable of functioning as effective APC to trigger antitumour immunity (Yang and Carbone 2004). Inflammation has been implicated in the development of cancers since the seminal observation made by Virchow in 1863 (Balkwill and Mantovani 2001; Aggarwal, Shishodia et al. 2006) that chronic inflammation creates a microenvironment conducive to tumourigenesis. The inflammation associated with chronic infections such as Helicobacter pylori or hepatitis B virus promotes the respective development of gastric and liver cancers (Chemin and Zoulim 2009; Hatakeyama 2009). Chronic inflammation associated mechanisms of tumourigenesis include cellular transformation, proliferation, invasion, angiogenesis, chemoresistance, metastasis, and inhibition of apoptosis (Aggarwal, Shishodia et al. 2006; Mantovani 2010). Proinflammatory cytokines such as IL-6, IL-1α, and IL-8, as well as various chemokines, are known to favour tumour growth and progression (Balkwill and Mantovani 2001; Aggarwal, Shishodia et al. 2006). The inappropriately named tumour necrosis factor (TNF)-α has also been linked to several

aspects of tumourigenesis including cellular transformation, proliferation, invasion, and metastasis (Aggarwal, Shishodia et al. 2006). The role of IL-6 and STAT-3 as anti-apoptotic factors in various cancers is also well recognized (Hodge, Hurt et al. 2005). Chemokines such as CXCL1 and CXCL8 are able to enhance tumour cell proliferation (Payne and Cornelius 2002); CXCL5 and CXCL12 attract neutrophils and MDSC (Yang, Huang et al. 2008), while CXCL12 promotes the migration of tumour cells that express the cognate receptor CXCR4 (Balkwill 2004). Many of these immunological mediators are regulated by transcription factor NF-κB, which is constitutively active in many cancers and is inducible by various carcinogens including viruses (Aggarwal, Shishodia et al. 2006; Ben-Neriah and Karin 2011).

Tumour metastasis is the primary cause of cancer-related death (Jemal, Siegel et al. 2008). Epithelial-to-mesenchymal transition (EMT) of cancer cells is associated with enhanced cell migration, local invasion and distant metastasis, and expression of EMT markers correlates with poor prognosis (Micalizzi and Ford 2009). EMT is a common process in early embryogenesis and carcinoma progression (Thiery, Acloque et al. 2009). During EMT, the carcinoma cells undergo morphological changes that confer enhanced motility and reduced intercellular adhesion which enable local invasion and distant metastasis (Savagner 2010). A recent study in a spontaneous melanoma model showed that tumour recruitment of MDSC promotes EMT (Toh, Wang et al. 2011). In particular, it has been found that granulocytic (G)-MDSC induce EMT in vitro and in vivo via multiple pathways involving TGF-β1, epidermal growth factor (EGF), and hepatocyte growth factor (HGF) (Toh, Wang et al. 2011). Other immune cells such as activated $CD8^+$ T cells (Santisteban, Reiman et al. 2009) and macrophages (Bonde, Tischler et al. 2012) have also been shown to stimulate EMT in tumour-bearing mice. Together, these data emphasize the intimate relationship between host immune responses and the microenvironment in modelling tumour development and progression.

Established Tumours

At the same time as the immune system fails to control tumour formation, the immune response within the microenvironment of established tumours remains an important factor in determining the outcome of cancer. Regression of established liver tumours by induction of $CD8^+$ T-cell responses with peptide-based immunotherapy was reported in several mouse models (Qian, Li et al. 2001; Belnoue, Guettier et al. 2004). Recent genomics studies in various human tumours including breast cancer have identified

immunological parameters as important determinants of disease outcome (Chew, Tow et al. 2010; Hsu, Kim et al. 2010; Suzuki, Kachala et al. 2011). Several studies have underlined the importance of the tumour microenvironment on the clinical evolution of Hepatocellular Carcinoma (HCC) (Xu, Shen et al. 2008; Chew, Tow et al. 2010). Chew et al. discovered an intriguing association between the expression of intratumoural pro-inflammatory genes and patient survival (Chew, Tow et al. 2010; Chew, Chen et al. 2012). In 172 HCC patients, they demonstrated that a 14-gene immunological signature is predictive of patient survival, especially at the early stages of the disease (Chew, Chen et al. 2012). These 14 immune genes encode chemokines CXCL10, CCL5, and CCL2; cytokines IFNG, TNF, and IL6; pattern recognition receptors TLR3 and TLR4; T cell markers CD8A and TBX21, and NK cell marker NCR3. Of importance, in this study IFN-γ and TLR3 ligand-induced intra-tumour chemokine expression was shown to promote infiltration by cytotoxic T cells and NK cells to enhance tumour cell apoptosis and reduce their proliferation (Chew, Chen et al. 2012). The immune microenvironment of noncancerous hepatic tissues has also been shown to impact on the development of venous metastases in HCC patients (Budhu, Forgues et al. 2006). A pro-inflammatory phenotype combined with tumour infiltration by cytotoxic lymphocytes is associated with a better prognosis in various cancers (Pages, Galon et al. 2010; Chew, Chen et al. 2012). Tumour infiltration by T cells has now been linked with favourable prognosis in colorectal cancer (Galon, Costes et al. 2006), melanoma (Clemente, Mihm et al. 1996), breast cancer (Menegaz, Michelin et al. 2008), ovarian cancer (Zhang, Conejo-Garcia et al. 2003), and lung cancer (Dieu-Nosjean, Antoine et al. 2008). Recent studies in liver and breast cancers have identified an important correlation between the densities and distribution of T and B cells with a favourable prognosis (Martinet, Garrido et al. 2011; Schneider, Teufel et al. 2012). Chew et al. revealed that in HCC there is a correlation between patient survival and the intratumour densities of T cells and NK cells. It is important to consider hat tumour infiltration by cytotoxic lymphocytes is often orchestrated by chemokines expressed within the tumour microenvironment. In HCC, it has been demonstrated that stimulation with cytokines in conjunction with TLR activation can promote inflammation and chemokine production in tumours (Chew, Chen et al. 2012). Chemokine mediated tumour infiltration by cytotoxic lymphocytes has also been demonstrated by several investigators (Hirano, Iwashita et al. 2007; Harlin, Meng et al. 2009). In a cutaneous melanoma model, it has been further shown that chemotherapy might induce intra-tumour expression of chemokines that favoured T-cell infiltration and

tumour control (Hong, Puaux et al. 2011). In contrast, several studies have evidenced the key role played by chemokines during metastasis, particularly among tumour cells that express chemokines receptors CXCR3 and CXCR4 (Koizumi, Hojo et al. 2007; Fulton 2009). The role of the proinflammatory microenvironment in tumour control therefore appears to be context dependent and will require further detailed investigation.

1.5 Melanoma microenvironment

Malignant melanoma is characterised by a rapid progression, metastasis to distant organs, and resistance to chemo- and radiotherapy. The definition of the immunogenic capacities of melanoma cells should allow a successful application of different immunotherapeutic strategies of this type of cancer. However, the overall results of immunotherapeutic clinical studies are not satisfactory. These paradoxical observations are supposed to be due to the profound immunosuppression mediated by different mechanisms dealing with alterations in tumour and surrounding stroma cells. This complex immunosuppressive network is supposed to be stimulated by chronic inflammatory conditions developing in the tumour microenvironment (Figure3) (Umansky and Sevko 2012). Indeed, chronic inflammation has been demonstrated to correlate with the tumour onset and progression (Grivennikov, Greten et al. 2010; Mantovani 2010).

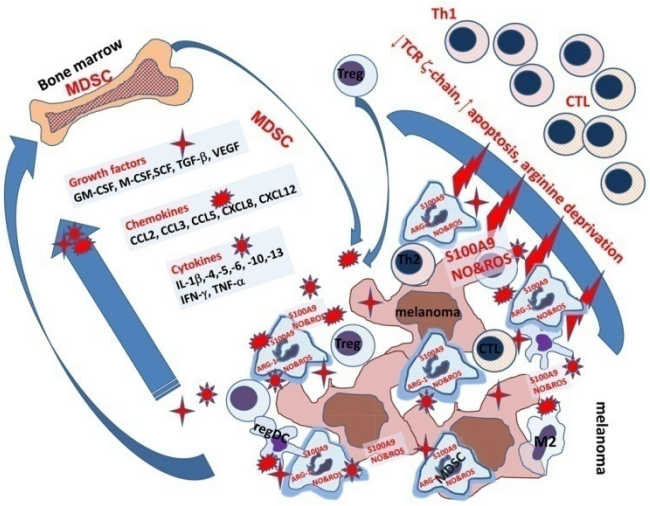

Figure 3: Melanoma microenvironment (Umansky and Sevko 2012)
Soluble mediators of chronic inflammation secreted by the melanoma and host cells that include various cytokines (like IL-1β, IL-6, IFN-γ, TNF-α etc.), chemokines (such as CCL2, CCL3, CXCL8 etc.), and growth factors (e.g., TGF- _, VEGF GM-CSF etc.) can induce the migration in tumour lesions and activation of many immunosuppressive leucocytes such as myeloid-derived suppressor cells (MDSC),

regulatory T cells (T_{REG}), M2 polarized macrophages (TAM), regulatory dendritic cells (regDC), and Th2 lymphocytes. Activated MDSC, producing high amounts of nitric oxide (NO) and reactive oxygen species (ROS) as well as expressing elevated levels of arginase (ARG)-1 considerably contribute to the inhibition of antitumour responses mediated by effector CD4 (Th1) and CD8T cells by inducing a down-regulation of the TCRζ-chain, an arginine deprivation, and apoptosis.

The role of chronic inflammation in melanoma development

Chronic infection and inflammation have long been considered to be strongly related to cancer progression; however, only during the last decade this complex association has commenced to be deciphered (Tan and Coussens 2007; Mantovani 2010; Rook and Dalgleish 2011). Although human malignant skin melanoma is not generally associated with apparent inflammation, recent publications have highlighted the critical importance of a chronic inflammatory microenvironment, especially the role of particular cytokines and chemokines, for melanoma initiation and progression (Navarini-Meury and Conrad 2009). For istance, it has been observed that patients with advanced metastatic melanoma display a Th2 pattern of immune homeostasis (mirroring a state of chronic inflammation) as reflected by the accumulation of the cytokines IL-4, IL-5, IL-10 and IL-13 as well as the chemokines CCL5 (RANTES), CCL11 (Eotaxin) and CXCL10 (IP-10) in patients' plasma (Nevala, Vachon et al. 2009). In contrast, healthy donors or patients with completely resected melanoma were characterised by Th1 biased immune homeostasis. The reprogramming of immunity towards the dominance of Th2 cytokines could be mediated by tumour-derived VEGF leading to tumour progression and metastasis (Nevala, Vachon et al. 2009). Moreover, such Th2 bias has been also observed in patients with renal cell carcinoma (Tatsumi, Kierstead et al. 2002):of interest, both these malignancies were characterised by increased levels of VEGF production (Mahabeleshwar and Byzova 2007; Milella and Felici 2011). Melanoma cells have been demonstrated to produce a variety of chemokines including CCL2, CCL5, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL10 (Richmond, Yang et al. 2009). Although at the beginning chemokines were described just with respect to the leucocyte migration, it became soon quite clear that chemokines are produced by different cell types. Chemokines released by melanoma cells have been found to induce tumour growth, angiogenesis, metastasis and alterations in cellular components of the tumour microenvironment (Murakami, Cardones et al. 2004; Navarini-Meury and Conrad 2009; Talmadge 2011). Furthermore, melanoma cells were able to influence fibroblasts and macrophages in the tumour stroma to produce a number of chemokines such as CCL2,

CXCL8 and CXCL12 as well as growth factors like VEGF, TGF-β or tumour necrosis factor (TNF)-α and cytokines (IL-1, IL-6 or IL-8). On the other hand, stromal cells can further stimulate the chemokine production by tumour cells creating thereby an autocrine and paracrine loop of the tumour growth stimulation (Labrousse, Ntayi et al. 2004; Raman, Baugher et al. 2007; Somasundaram and Herlyn 2009). Of importance, chemokine expression was found to be steadily enhanced during melanoma progression by the activation of the NF-kB family of transcription factors, whereas blocking NF-kB signalling might strongly reduce the chemokine concentration and suppress melanoma progression in different mouse models (Richmond, Yang et al. 2009; Yang, Splittgerber et al. 2010). Moreover, the discovery of other transcription factors such as activator protein 1(AP-1)and STAT3 that together with the NF-kB can induce the production of chemokines and other proinflammatory mediators like TNF-α, IL-1, IL-6, IL-8, cyclooxygenase-2 (COX-2), matrix metalloproteases, VEGF have provided the molecular basis for the link between chronic inflammation and cancer. This cross-talk is indicated by (i) the induction of tumour development under chronic inflammatory conditions and (ii) the formation of chronic inflammatory microenvironment in tumour lesions, which strongly stimulates tumour growth and metastasis (Baniyash 2006; Grivennikov, Greten et al. 2010; Mantovani 2010; Allavena, Germano et al. 2011; Ben-Neriah and Karin 2011; Rook and Dalgleish 2011). Moreover, recent publications indicated that several oncogenes are able to promote uncontrolled cell proliferation and stimulate their resistance to apoptosis as well as to activate a cascade of proinflammatory molecules. In particular, components of the RAS-RAF signalling pathway have been demonstrated to trigger NF-kB activation and the following enhanced production of proinflammatory cytokines and chemokines (Sparmann and Bar-Sagi 2004; Sumimoto, Imabayashi et al. 2006; Haluska, Pemberton et al. 2007). This can results into a never ending chronic inflammatory process as reflected by the constant release of cytokines, chemokines and growth factors, by the recruitment and activation of myeloid immunosuppressive cells like MDSCs and TAMs as well as by the stimulation of pathways promoting melanoma growth and metastasis (Baniyash 2006; Sawanobori, Ueha et al. 2008; Sica, Larghi et al. 2008; Allavena, Germano et al. 2011).

MDSC in the inflammatory melanoma microenvironment

The number and activity of $Gr1^+CD11b^+$ MDSCs in melanoma lesions (skin tumours and lymph node metastases) as well as in the spleen and bone marrow it has been already

investigated (Meyer, Sevko et al. 2011). A remarkable accumulation of MDSCs among tumour infiltrating leucocytes has been demonstrated and significantly correlate with the increasing weight of these primary tumours. Furthermore, quickly growing tumours showed elevated MDSC frequencies within infiltrating leucocytes. Significantly high percentages of MDSCs were detected also in metastatic lymph nodes as well as in the spleen and bone marrow of melanoma-bearing mice in the ret transgenic spontaneous model(Meyer, Sevko et al. 2011). Similar observations on MDSCs recruitment have been also demonstrated in different mouse models of tumour transplantation and in cancer patients (Filipazzi, Valenti et al. 2007; Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg 2010; Peranzoni, Zilio et al. 2010; Poschke, Mougiakakos et al. 2010). These data indicate that an observed enhanced production of numerous inflammatory mediators during melanoma progression in ret transgenic mice and in other systems may attract MDSCs into tumour lesions. Furthermore, MDSC accumulation in the site of chronic inflammation has been previously described in a mouse chronic inflammatory model (Baniyash 2006). One of the important consequences of the increased MDSC proportion in tumour-bearing hosts might be diminished numbers of mature myeloid cells like DCs (Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg 2010). Indeed, it has recently observed a significant reduction in numbers of mature DCs in melanoma lesions and lymphoid organs from ret transgenic mice (Zhao, Falk et al. 2009). Enriched in the tumour microenvironment MDSCs are able to display a high level of activation reflected by intensive NO production, and ARG-1 expression associated with their strong capacity to suppress T cell activities *in vitro* assays (Rodriguez and Ochoa 2006; Serafini, Borrello et al. 2006; Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg and Sinha 2009; Meyer, Sevko et al. 2011). One of the most important mechanisms of MDSC-mediated blocking of T-cell functions is associated with a remarkable decrease in the expression of T-cell receptor ζ-chain (Rodriguez, Zea et al. 2002; Ezernitchi, Vaknin et al. 2006), which plays a critical role in coupling the TCR-mediated antigen recognition to diverse signal transduction pathways (Baniyash 2004). In ret melanoma model, a profound down-regulation of TCR ζ-chain expression has been detected in T lymphocytes infiltrating primary and metastatic lesions as well as in cells localised in lymphoid organs (Meyer, Sevko et al. 2011). Moreover, a decrease of ζ-chain levels has been reported not only in T cells from patients with different tumour types (Nakagomi, Petersson et al. 1993; Whiteside 2004), but also in mice with chronic inflammation (Baniyash 2004; Ezernitchi, Vaknin et al. 2006), suggesting again an amazing resemblance of both pathological

processes. Furthermore, a direct MDSC inhibitory effect on TCR ζ-chain expression has been documented i*n vitro* co-culture of MDSCs isolated from tumour-bearing mice or animals under chronic inflammatory conditions with normal T lymphocytes (Baniyash 2004; Ezernitchi, Vaknin et al. 2006; Meyer, Sevko et al. 2011).. Taken together, all above mentioned findings suggest a strong linkage among developing tumours, chronic inflammation, and immunosuppression.

1.6 Cancer therapy strategies targeting the tumour and the suppressive microenvironment

Given the complex roles of the immunological microenvironment in tumourimmunity (Figure 1), developing methods for targeting the relevant effector molecules or pathways for cancer treatment remains challenging. Indeed, the limited success of cancer immunotherapy to date can primarily be attributed to three main factors: (1) poor host responses towards tumour antigens, (2) low infiltration of effector cells into solid tumours, and (3) the intrinsically immunosuppressive tumour microenvironment. Reversing the immune responses from tumour protection towards tumour rejection seems to be the key for effective cancer immunotherapy (Mantovani, Romero et al. 2008; Quezada, Peggs et al. 2011; Gajewski 2012). Manipulation of the tumour microenvironment will therefore be an important consideration for achieving optimal antitumour responses with future treatments.

Several cases of spontaneous regression associated with specific antitumour immune responses have been reported in various cancers (Bodey 2002; Saleh, Renno et al. 2005; Kalialis, Drzewiecki et al. 2009). Efforts to activate local adaptive immune responses in tumours have obtained some success, and cell-based therapies such as adoptive T-cell transfer have shown convincing signs of efficacy in treating metastatic melanoma patients (Rosenberg and Dudley 2009). DC-based vaccines represents a new and promising immunotherapeutic approach, explored in pre-clinical and clinical studies, for treatment of advanced cancer as well as for prevention of cancer (Perrotta, Falcone et al. 2004; Perrotta, Bizzozero et al. 2007; Palucka and Banchereau 2013).Recent developments in cancer immunotherapies have now also begun to explore the use of NK cells (Lee and Gasser 2010; Gillgrass and Ashkar 2011). Cancer vaccines aim to induce immune responses against tumour-associated antigens and several such vaccines are currently under development to treat various cancers (Schreiber, Raez et al. 2010; Slingluff 2011). However, the low immunogenicity of most tumour antigens represents a major difficulty

in developing potent cancer vaccines. Intensive research will be needed to improve the specificity and effectiveness of these type of therapy. Furthermore, the immunosuppressive tumour microenvironment limits the effectiveness of the antitumour immune responses induced by these cancer vaccines (Slingluff 2011). Therefore, manipulation of the tumour microenvironment either by enhancing the antitumour activity or blocking the immunosuppressive pathways is among the strategies pursued to obtain a more effective tumour therapy. Alternatively, vaccines that aim to control the chronic inflammation may serve as effective tumour preventing measures (Carcinoma 2010; Kane 2010). One such example is the vaccination against hepatitis B which has successfully diminished the incidence of liver cancer in Taiwan since being introduced in 1984 (Chang 2011). Vaccines against oncogenic human papilloma viruses (HPV) achieved similar success in preventing cervical cancer (Roden, Monie et al. 2006; Albers, Sinikovic et al. 2009).

Other cancer immunotherapies included the administration of immunostimulatory cytokines such as IL-2 and IFN-α (Antony and Dudek 2010; Moschella, Proietti et al. 2010), as well as antibodies against tumour antigens (Grillo-Lopez, White et al. 2000; Hortobagyi 2005; de Cerio, Zabalegui et al. 2007), as adjuvants in combination with chemotherapy or cancer vaccines. The use of toll like receptor (TLR) ligands can trigger effective innate immune responses within tumours (Garland 2003; Meyer and Stockfleth 2008; Adams 2009). For instance, some success has been achieved with the application of TLR7 agonists in the treatment of skin carcinoma (Garland 2003; Urosevic and Dummer 2004). As intra-tumour expression of chemokines correlates with increased lymphocyte infiltration, transfection of chemokine cDNAs in murine tumour cells has shown promising tumour rejection in preclinical models (Braun, Chen et al. 2000). These recent advances in immunotherapy confirm that boosting the activity of tumour-infiltrating lymphocytes, which are reported to be exhausted in many cancers (Baitsch, Baumgaertner et al. 2011; Klebanoff, Acquavella et al. 2011), will be the key to the development of the most effective treatments. Such strategies may include the blockade of immunosuppressive pathways including PD/PDL (Hirano, Kaneko et al. 2005; Dotti 2009), CTLA-4 (Lipson and Drake 2011; Prieto, Yang et al. 2012) and Cox 2 (Dempke, Rie et al. 2001; DeLong, Tanaka et al. 2003), T_{REG} depletion prior to vaccination (Dannull, Su et al. 2005; Morse, Hobeika et al. 2008), or activation of the TLR pathway (Adams 2009; Woller, Knocke et al. 2011). For example, Ipilimumab, an antibody generated against CTLA-4, key negative regulator of T cell responses, has been recently

approved by the Food and Drug Administration (FDA) for the treatment of metastatic melanoma (Lipson and Drake 2011; Prieto, Yang et al. 2012). These interventions enhance the effectiveness of therapies by pushing the immunological balance towards antitumour responses within the microenvironment of cancers (Mantovani, Romero et al. 2008; Zitvogel, Apetoh et al. 2008; Gajewski 2012). Some cancer drugs that were initially developed to induce carcinoma cell death were later found to act on the tumour microenvironment. One such example is Imatinibmesylate (Gleevec), a tyrosine kinase inhibitor which was developed to inhibit tyrosine kinase BCR-ABL in chronic myeloid leukemia (CML). Gleevec has been recently approved for the treatment of gastrointestinal stromal tumours (GIST) which exhibit a c-kit tyrosine kinase mutation. However, it has been shown later that clinical responses to Imatinib correlated with the inhibition of immunosuppressive enzyme Indoleamine 2,3 dioxygenase (IDO) and hence enhanced levels of T cell activation (Balachandran, Cavnar et al. 2011). Of interest, the class of small molecules that inhibit mTOR has recently been shown to exert antitumour activity by stimulating homeostatic proliferation of memory $CD8^+$ T cells (Li, Rao et al. 2011). In addition, cytotoxic or genotoxic agents which induce cellular stress or DNA damage could release danger signals that are sensed by TLRs and activate innate immune responses (Gasser and Raulet 2006). Chemotherapeutic drugs have also been found to activate the immune system despite the prevailing view that these agents induce immunosuppressive effects. For example, low doses of cyclophosphamide inhibit T_{REG}, and gemcitabine or 5-fluorouracil eliminate MDSC (Zitvogel, Kepp et al. 2011). Cyclophosphamide, paclitaxel, doxorubicin, and vinblastine given at regular intervals normalize the tumour-associated vasculature, thereby facilitating the delivery of drugs and recruitment of T lymphocytes (Bocci, Nicolaou et al. 2002). Gemcitabine can activate both the adaptive and humoral immunity to elicit meaningful antitumour responses in animal models (Lake and Robinson 2005). In melanoma patients responding to dacarbazine, we also found that chemotherapy is able to induce intra-tumour expression of T cell and NK cell-attracting chemokines CXCL9, CXCL10, and CCL5, which was associated with improved survival (Hong, Puaux et al. 2011). Therefore it will be important to develop future cancer drugs in the context of potential effects on the tumour microenvironment.

Chapter 2: Ceramide, Acid-sphingomielinase and Tumour

2.1 Lipids in cell signalling

The high organization of biological systems involves communication between cells.
The cells of a multi-cellular organism use released molecules to communicate with each other, and single-celled organisms respond to these messengers in their environment. Moreover, the organelles and the intracellular compartments inside the cell must communicate with each other. Among the molecules used in cellular communication, in the last decade the attention has been focused on lipids. Cell membranes constitute lipid barriers that must be traversed by signal transduction pathways but membranes are also the source of the lipids involved in signal transduction.
Membrane lipids participate as components of signal transduction pathways and as docking sites for cytoplasm signalling proteins, and they give rise to cleavage products that act as ligands or substrates for other signalling molecules. Non-membrane lipids have a role in signal transduction as well; lipids serve as ligands, and posttranslational lipid modifications provide a means for proteins to associate intimately with the membrane.

The sphingolipids

The role of lipids in cell signalling has been first suggested with the discovery of the phosphoinositides cycle (Berridge and Irvine 1984).The subsequent identification of other lipid second messengers and intracellular mediators such as the aracidonic acid, phosphatidic acid and phosphatidil-inositol 3,4,5 tridphosphate further confirmed the importance of lipid molecules in cell signalling.
More recently another class of lipids has been identified as important signalling molecules: the sphingolipids.All sphingolipidsare composed by a long-chain sphingoid base backbone (e.g. sphingosine), an amide-linked, long-chain fatty acid and one of various polar head groups. The structure of these head groups defines the various classes of sphingolipid subtypes, with a hydroxyl group found in ceramide, phosphorylcholine in sphingomyelin (SM) and carbohydrates in glycosphingolipids. Sphingolipids are mainly present at the level of the plasma membrane, where they have a fundamental role in defining its physical and chemical properties. In addition to the structural role of the sphingolipids in the membrane bi-layer some of the intermediate molecules of its metabolism (ceramide, glucosyl-ceramide, and sphingosine-1-phosphate) have important

functions in cellular functions, such as growth, differentiation and apoptosis (Bleicher and Cabot 2002; Payne, Milstien et al. 2002; Hannun and Luberto 2004).

The mechanisms of sphingolipid-mediated signal transduction are characterised by an extreme complexity (Riboni, Prinetti et al. 1995; Huwiler, Kolter et al. 2000). There are a number of metabolic processes, which regulate the levels of one type of sphingolipid. Furthermore there is a strict interaction between the different pathways, as for example the connection between ceramide and sphingosine-1-phosphate (S1P) metabolism(Van Brocklyn and Williams 2012).

Enzymes in the sphingomyelin cycle

Sphingomyelin is an important structural component of the cell membrane and of lipid rafts. It has an fundamental functional role as well as it is the parent compound of several lipid mediators (Figure 4) (Cremesti, Goni et al. 2002). Sphingomyelin is produced by sphingomyelin synthase (phosphatidylcholine/ceramide-PChtransferase) through the transfer of a phosphocholine head group from phosphatidylcholine to ceramide, after its transport to the cisternae of the Golgi. Sphingomyelin synthase is an integral membrane protein with membrane-bound ceramide and phosphatidylcholine as its substrates. The major site of sphingomyelin synthesis is not yet clear, because greater quantities of the enzyme reside in the plasma membrane than in the Golgi. However, the rate-limiting factor in the Golgi seems to be the availability of ceramide substrate. Inhibitors of glucosylceramide synthase stimulate synthesis of sphingomyelin, suggesting that sphingomyelin and ganglioside synthases compete for the same pool of substrate ceramide. An alternative pathway to the formation of sphingomyelin exists through the addition of phosphoethanolamine to ceramide followed by single methyl transfer to form the choline head group.

The main component of sphingomyelin cycle is ceramide that can be synthesised either de novo by the sequential action of serine palmitoyltransferase, (dehydro) ceramide synthase, and (dehydro)- ceramidedesaturase, or through the breakdown of sphingomyelin by the activation of the catabolic enzymes sphingomyelinases. Sphingomyelinases are the most heavily studied enzymes in sphingolipid metabolism. Currently, five distinct enzymes have been identified based upon their pH optima, cellular localization, and cation dependence. The neutral membrane-bound $Mg2^{+}$-independent sphingomyelinase (N-SMase) and the lysosomal acid pH optima sphingomyelinase (A-SMase) have been the best studied for their roles in ceramide generation.

An increase in N-SMase activity, a corresponding decrease in SM, and an increase in ceramide have been demonstrated in response to TNF, Fas ligand, 1α,25-dihydroxyvitamin D3,γ-interferon, chemotherapeutic agents, heat stress, ischemia/reperfusion, and interleukin-1 (Pettus, Chalfant et al. 2002). The factor associated with N-SMase activation (FAN) was recently identified as an adapter protein that associates with the intracellular domain of the TNF receptor, acting upstream of N-SMase. In addition, both arachidonic acid and glutathione depletion have been shown to activate this enzyme.

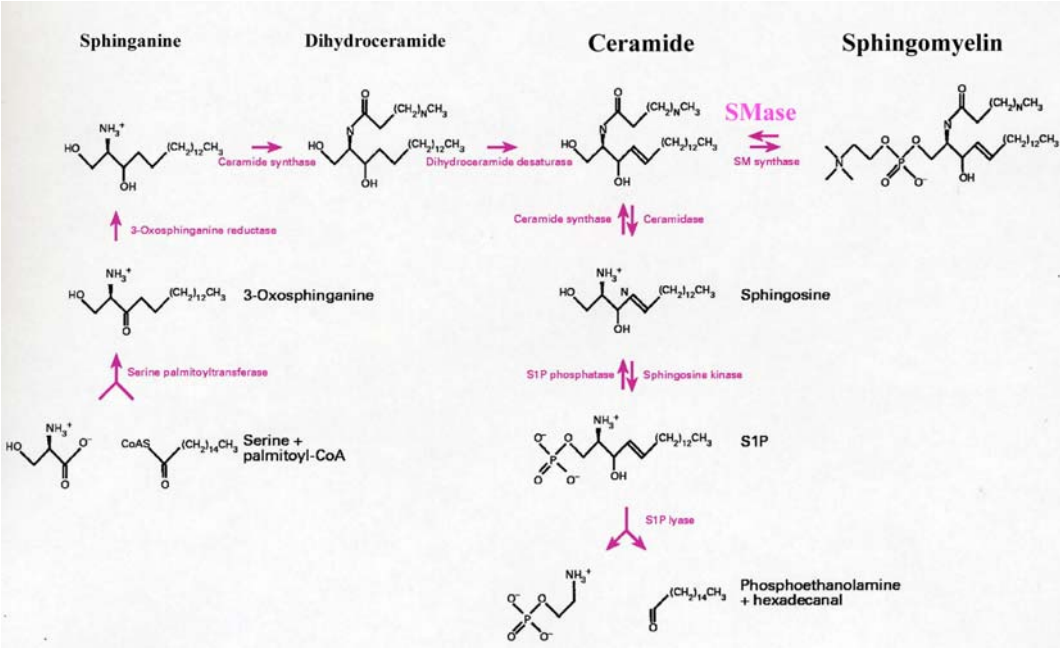

Figure 4: Sphingomyelin cycle enzymes

Acid sphingomyelinase

A-SMase was originally identified as a cation-independent hydrolase contributing to the catabolism of sphingomyelin in lysosomes, and responsible for the onset of Niemann–Pick disease (NPD) (type A and B),determined by the deficiency of the enzyme that leads to lysosomal accumulation of Sphingomyelin (Horinouchi, Erlich et al. 1995). The enzyme is a 258 amino acid sequence that contains 11 cysteine residues at the C terminal, 10 of which are forming disulphide bonds (C221-C226, C227-C250, C385-C431, C584-C588, C594-C607), while at the N-activating terminal there are only 3 (C120-C131, C89-C165, C92-C157) (Lansmann, Schuette et al. 2003)(Figure 5). At the N-terminus of the protein there is a signal peptide that directs the translation product into the endoplasmic reticulum (Hurwitz, Ferlinz et al. 1994). The murine enzyme has six potential N-glycosylation sites, which prevent the enzyme form proteolytic degradation in the lysosome (Newrzella and Stoffel 1996). Additionally, A-SMase has a mannose 6-phosphate (M6-P) residue that is required for lysosomal targeting of the enzyme via the M6-Preceptor (Newrzella and Stoffel 1992). However, recent studies have indicated that its localization is in fact not limited to the interior of lysosomes. Indeed, A-SMase is also secreted from vascular endothelial cells and macrophages through the Golgi secretory pathway, as a Zn^{2+}-dependent enzyme (Marathe, Schissel et al. 1998). The Zn^{2+} dependency apparently results from the respective intracellular trafficking pathway; in the lysosomal pathway the enzyme is exposed to cellular pools of Zn^{2+} for full saturation, but in the secretory pathway there is no such exposure (Schissel, Jiang et al. 1998).

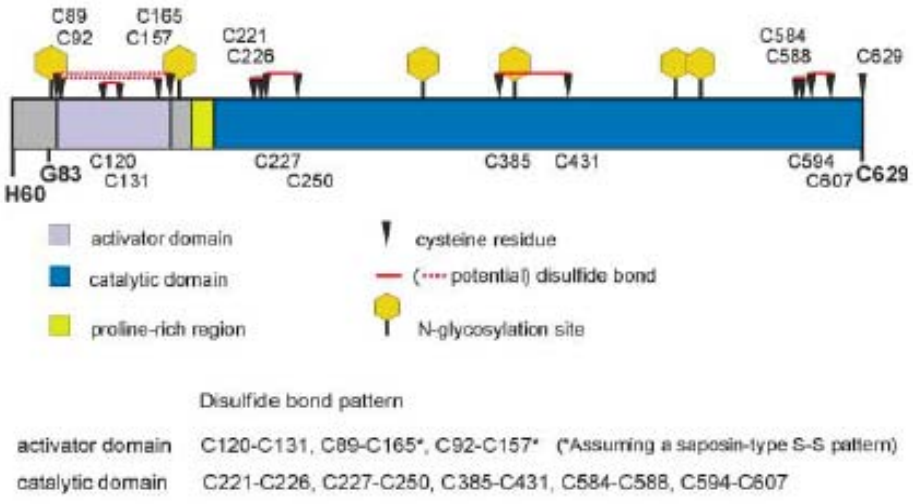

Figure 8: A-SMase structure from (Lansmann, Schuette et al. 2003)

The identified disulfide bond structure of haSMase is presented. The N-terminus of native haSMase from human placenta is Gly83 referring to the open reading frame of the haSMase-cDNA (His60: N-terminus of mature bc-haSMase expressed in SF21 cells using the baculovirus expression vector system. Contrary to position N503 in the native human protein, glycosylation site N503 of bc-haSMase is glycosylated.

Studies carried out in COS-1 cells transfected with A-SMasecDNA, have identified three major forms of the protein of 75, 72, and 57 kDa that are generated from the same nascent polypeptide derived from a single processed transcript (Ferlinz, Hurwitz et al. 1994). After deglycosilation they are reduced in size of 64, 61 and 47 kDa respectively. The 75 kDa form is the prepolypeptide, which is found in the endoplasmic reticulum. The second form is considered the enzyme precursor and is obtained by cutting a possible signalling sequence of 4 kDa. The 72 kDa precursor is then transported to the endo/lysosomal compartments (Hurwitz, Ferlinz et al. 1994) where it is processed to its mature form of 70 kDa. The mature enzyme is degraded to an inactive form of 52 kDa.

When, upon stimulation, A-SMase is relocated from intracellular organelles to the outer leaflet of the plasma membrane, it plays a central role in the hydrolysis of SM localized within lipid raft microdomains, that are formed by dynamic clustering of sphingolipids and cholesterol and function as platforms for signal transduction and protein sorting (Simons and Ikonen 1997). Cell surface lipid microdomains are thought to be specific sites for Cer generation in response to various agonists and stress signals. Grassme et al. showed that stimulation with CD95 (Fas) ligand rapidly induces the translocation of A-

SMase from intracellular compartments to the outer leaflet of the plasma membrane, and that Cer generation by the A-SMase within lipid microdomains is essential for CD95 signalling and the induction of apoptosis (Grassme, Jekle et al. 2001; Perrotta, Bizzozero et al. 2010).Cer provides the driving force for the coalescence of lipid microdomains into larger platforms and the subsequent induction of CD95 clustering and oligomerization of the downstream effectors FADD and pro-caspase-8, events required for transmission of the CD95 death signal (Grassme, Jekle et al. 2001)(Figure 6). Furthermore, this signalling model is not restricted to CD95, as A-SMase on the cell surface is essential for the clustering of CD40 (Grassme, Jendrossek et al. 2002), Fcγ receptor II (Abdel Shakor, Kwiatkowska et al. 2004), CD20, and TRAIL receptor (Martin, Phillips et al. 2005).

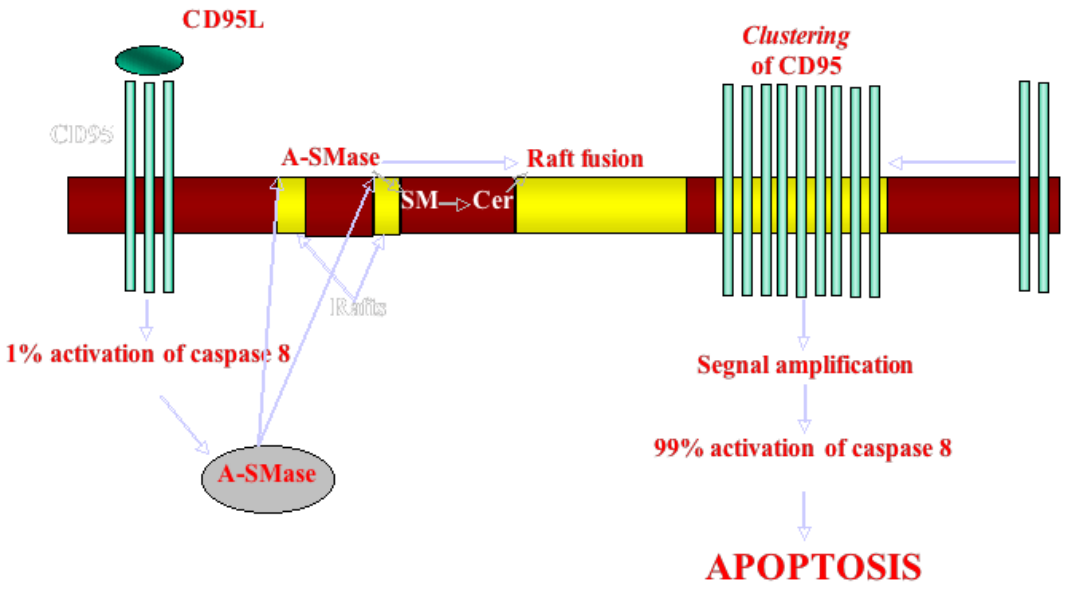

Figure 6. Acid sphingomyelinase activation, adapted from (Grassme, Jekle et al. 2001).
A-SMase and ceramide-mediated receptor clustering in membrane platforms functions as a prerequisite for receptor signalling. Stimulation via CD95 activates A-SMase and triggers a translocation of the enzyme onto the cell surface. The release of ceramide in small rafts correlates with the fusion of these small membrane domains to large platforms and the trapping of receptor molecules within ceramide-enriched membrane platforms.

2.2 Sphingolipids in tumours

Many sphingolipid-regulated functions have significant and specific links to various aspects of cancer initiation, progression and response to anticancer treatments. Ceramide in particular is intimately involved in the regulation of cancer-cell growth, differentiation, senescence and apoptosis (Hannun 1996; Hannun and Obeid 2002). Many cytokines,

anticancer drugs and other stress-causing agonists result in the increase in endogenous levels of ceramide through *de novo* synthesis and/or the hydrolysis of sphingomyelin. Reciprocally, decreased levels of endogenous ceramide caused by increased expression of glucosylceramide synthase (GCS), which clears ceramide levels by incorporating it into glucosylceramide, results in the development of a multidrug resistance phenotype in many cancer cells(Liu, Han et al. 2001).

In contrast to the actions of ceramide, S1P is emerging as a key regulator of proliferation, inflammation, vasculogenesis and resistance to apoptotic cell death(Payne, Milstien et al. 2002). Many growth factors and cytokines activate sphingosine kinase, and so lead to transient increases in S1P levels. S1P is then secreted from the cell and acts either in a paracrine or an autocrine manner to engage specific transmembrane hepta-helical G-protein-coupled receptors, termed S1P receptors (S1PRs), which were formerly known as the endothelial differentiation gene receptors (Hla 2003). Activation of S1PRs mediates signalling pathways that predominately result in increased cell motility and proliferation(Hla 2003). In addition, several studies indicate a non-receptor, intracellular action of S1P(Payne, Milstien et al. 2002), although the direct target remains elusive. S1P also exerts pro-survival functions that derive, at least in part, from its role in activating the transcription factor NF-κB(Maceyka, Payne et al. 2002)and the induction of COX-2 and its pro-survival prostaglandin products (Pettus, Bielawski et al. 2003). The regulation of COX-2 and NF-κB by S1P is important in tumourigenesis, and both of these proteins are activated in various human malignancies.

Ceramide in tumours

Ceramide is a powerful tumour suppressor, potentiating signalling events that drive apoptosis, autophagic responses and cell cycle arrest, thus its metabolism is tightly regulated in cancer cells. In order to survive, cancer cells upregulate the enzymes that metabolise ceramide, thus resulting in apoptotic responses and/or in the promotion of mitogenicity, depending on the routes by which ceramide is metabolised (Morad and Cabot 2013) (Figure 7).

Many papers have described the role of ceramide in the extrinsic apoptosis pathway, which begins outside the cell via the engagement of pro-apoptotic receptors, as CD95, TRAILRs and TNFR1, on the cell surface. Activation of these receptors increases ceramide levels at the plasma membrane and in the nucleus through the activation of SMase and leads to caspase activation and apoptosis (Cai, Bettaieb et al. 1997; Colell,

Morales et al. 2002; Huang, Yang et al. 2004; Dumitru and Gulbins 2006; Park, Zhang et al. 2008; Park, Mitchell et al. 2010). Cancer cells have developed several mechanisms to overcome this signalling cascade. Low levels of intracellular ceramide are associated with resistance to: TRAIL-induced death in breast (Zhang, Yoshida et al. 2009)and colon cancer cells (Voelkel-Johnson, Hannun et al. 2005; White-Gilbertson, Mullen et al. 2009), as well as in human renal carcinoma cells (Asakuma, Sumitomo et al. 2003); and TNF-induced apoptosis in breast cancer and hepatoma cells (Cai, Bettaieb et al. 1997; Liu, Han et al. 1999; Autelli, Ullio et al. 2009).

As the failure of cancer chemotherapy partly arises from resistance to death receptor signalling pathways, therapeutic ceramide should be considered as an adjuvant therapy with drugs that target the TNF receptor superfamily (Morad and Cabot 2013).

Ceramide is also involved in the intrinsic apoptotic pathway as it can induce mitochondrial outer membrane permeabilization (MOMP), a key event in apoptotis, through the formation of ceramide channels (Morad, Levin et al. 2012).Another way that ceramide affects mitochondrial function is by inducing the translocation to the mitochondria and the activation of protein kinase Cδ (PKCδ), which in turn promotes cytochrome *c* release and caspase 9 activation, as shown in prostate cancer cells(Sumitomo, Ohba et al. 2002).From the above, we should state that ceramide–mitochondria interplay is an important, but complicated, aspect of cancer biology. Of importance, it is the role of N-SMaseandA-SMase, as well as Ceramide Synthases (CERSs), to present ceramide for the initiation of mitochondrial-directed apoptotic responses. These enzymatic events are often deregulated in cancer in a cell-type specific manner, meaning that some phenotypes might harbour suppressed CERS activity, whereas others might overexpress GCS to decrease ceramide levels. The overexpression of GCS is particularly relevant to multidrug-resistant cancer(Liu, Han et al. 1999; Senchenkov, Litvak et al. 2001; Bleicher and Cabot 2002).

This relationship between ceramide and mitochondria and the consequences of this union highlight the appeal of ceramide as a therapeutic agent that can shutdown cancer cell resistance to the intrinsic apoptotic pathway.

Autophagy regulates the balance between cell survival and cell death. On the survival side, autophagy eliminates damaged proteins and organelles and maintains cellular homeostasis. On the cell death side, autophagy has a tumour suppressive role. However, in established cancers, tumour cells can use autophagy as a survival mechanism in response to chemotherapeutic or nutritional stress. Therefore, the induction of autophagy

by dihydroceramide and ceramide in cancer cells is not necessarily a good thing, unless it can be manipulated to induce apoptosis (Morad and Cabot 2013).

In various cancer models, dihydroceramide and ceramide trigger autophagy by eliciting cellular starvation through the downregulation of nutrient transporter proteins, leading also to the downregulation of the activity of mTOR, one of the most important players in autophagy (Guenther, Peralta et al. 2008; Pattingre, Bauvy et al. 2009; Pozuelo-Rubio 2011). Moreover, ceramide and dihydroceramide can block mTOR activity by inhibiting one of its regulator, phospholipase D (PLD)(Venable, Bielawska et al. 1996).

Ceramide has been shown to inhibit mTOR, leading to autophagy, by blocking AKT (Scarlatti, Bauvy et al. 2004),by activating AMP-dependent protein kinase (AMPK) (Peralta and Edinger 2009; Kim, Kundu et al. 2011), another upstream regulator of mTOR, and finally by the promotion of BNIP3 transcription(Daido, Kanzawa et al. 2004).

Finally, ceramideis also involved in cell cycle arrest through different mechanisms: by affecting an increase in p21 association with Cyclin-dependent kinase 2 (CDK2)and by activation of protein phosphatases that selectively inhibit CDK2 (Lee, Bielawska et al. 2000); by increasing the expression of p21 in a p53-dependent manner (p21 expression is induced by p53) (Struckhoff, Patel et al. 2010)and in a p53-independent manner (Pruschy, Resch et al. 1999; Kim, Kang et al. 2000), thus leading to G1 arrest; by suppressing survivin expression (Ahn and Schroeder 2010; Lechler, Renkawitz et al. 2011), and survivin is a key regulator of mitosis that is involved in regulating the G2/M transition (Lechler, Renkawitz et al. 2011).

Taking into account that the deregulation of molecular events governing the cell cycle is a hallmark of cancer and is a central issue in cancer progression, the control of cell cycle regulatory molecules by ceramide is one means of facing cancer progression and tumourigenesis, and of limiting drug resistance.

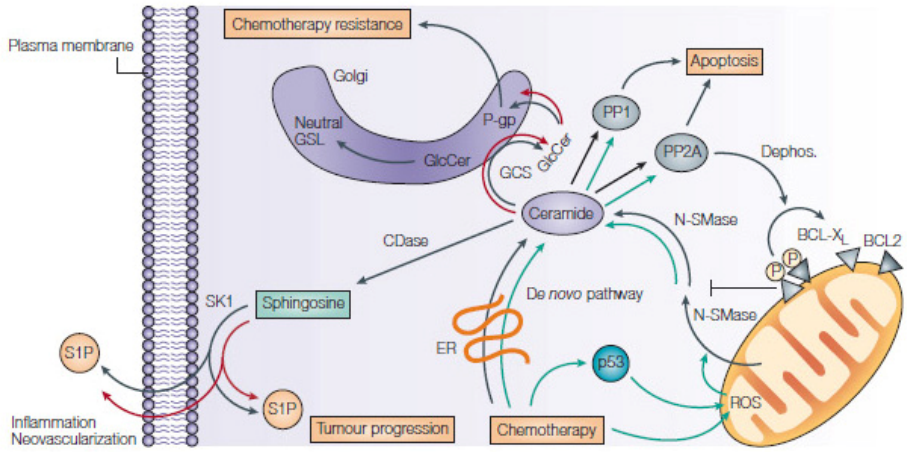

Figure 7: Roles of ceramide/sphingosine-1-phosphate in specific cancers (Ogretmen and Hannun 2004)

Levels of the candidate tumour-suppressor lipid ceramide are altered in various solid tumours, whereas the levels of the tumour promoting lipid sphingosine-1-phosphate (S1P) are increased in colon cancers and leukaemias. The red arrows show the sphingolipid pro-survival, tumourigenic and chemotherapy-resistance pathways that have been identified in tumours. In colon cancers, induction of sphingosine kinase 1 (SK1) results in the accumulation of S1P, possibly leading to neovascularization and inflammation. In breast cancer cells, attenuation of ceramide levels by the action of glucosylceramide synthase (GCS), which converts ceramide to glucosylceramide (GlcCer), results in the development of drug resistance. Expression of P-glycoprotein (P-gp) might exacerbate this situation, where as inhibition of GCS reverses drug resistance.

CDase, ceramidase; ER, endoplasmic reticulum; PP1, protein phosphatase 1; PP2A,protein phosphatase 2A; GSL, glycosphingolipid.

Acid Sphingomyelinase in tumours

The link between cancer, A-SMase, and ceramide has been extensively studied. For example, decreased ceramide has been reported in astrocytomas, ovarian, lung, and other cancers (Rylova, Somova et al. 1998; Selzner, Bielawska et al. 2001; Riboni, Campanella et al. 2002; Ogretmen and Hannun 2004).In addition, an inverse relationship has been found between ceramide levels and glioma stratification into high- and low-grade tumours (Riboni, Campanella et al. 2002). Of interest, an analysis of the microarray database Oncomine (www.oncomine.org, January 3, 2008) revealed that 12/104 matched cancer *vs*. normal tissue comparisons underexpressed the A-SMase mRNA (P<0.0005), with 4/104 comparisons that overexpressed it. In 104 comparisons with a Bonferroni corrected threshold for significance of P< 0.0005, five false positives are expected. This would predict that in at least some cancers A-SMase may be down-regulated, contributing to their reduced ceramide content and perhaps directing the cells away from apoptosis and

toward proliferation. Down-regulation of A-SMase and ceramide production also may play a role in drug resistance(Smith and Schuchman 2008).

In addition to these observations, many publications have shown that A-SMase is important in the response of cancer cells to a variety of antineoplastic treatments. For example, in the Fas-induced apoptotic response of glioma cells, A-SMase was shown to be activated, and its inhibition caused a Fas-resistant phenotype(Sawada, Nakashima et al. 2002; Perrotta, Bizzozero et al. 2010). Paclitaxel-induced apoptosis of human ovarian carcinoma cells also was found to be mediated by A-SMase-generated ceramide, and cells conditioned to be paclitaxel resistant were characterised by their lack of ceramide generation after treatment (Prinetti, Millimaggi et al. 2006). Cisplatin treatment of colon cancer cells was similarly shown to induce activation of A-SMase, changes in membrane fluidity, clustering of CD95, and ultimately apoptosis. Pharmacologic inhibition of A-SMase decreased membrane changes, CD95 clustering, and apoptosis (Lacour, Hammann et al. 2004). More recently, thesame group has demonstrated that changes in the Na^+/H^+ transporter activity (NHE1) after cisplatin treatment of these cells is responsible for acidification, contributing to A-SMase activation at the cell surface and the apoptotic response (Rebillard, Tekpli et al. 2007).In addition, the apoptotic response of neuroblastoma cells to fenretinide was ameliorated by A-SMase targeted siRNA but not by scrambled siRNA (Lovat, Di Sano et al. 2004). Thus, ASM is involved in the response of many cancers to antioncogenic treatments. Although all of these cancer treatments have other “known” mechanisms of action (*e.g*., DNA damage, disruption of microtubule assembly,*etc*.), it is becoming increasingly clear through work on sphingolipids and in other fields that secondary cell membrane changes play an important part in the action of these compounds. Clearly, A-SMase is an important component of this process.

In addition to the role that A-SMase plays in cancer cells themselves, researchers are also becoming aware of its importance in the tumour microenvironment, and some data have been published regarding its role in angiogenesis. It is well known that solid tumours cannot grow beyond a certain size without the recruitment of a vascular supply, and the development of antiangiogenetic cancer treatments is an extremely active area of research. The microvasculature of the small intestine relies on A-SMase for irradiation-induced apoptosis (Paris, Fuks et al. 2001). Endothelial cell death after irradiation in the Central Nervous System (CNS) was also found to be dependent on A-SMase through analysis of A-SMase KO mice(Pena, Fuks et al. 2000; Li, Chen et al. 2003). Perhaps the most interesting and substantial finding linking A-SMase to angiogenesis came from a

2003 study by Garcia-Barros *et al*. (Garcia-Barros, Paris et al. 2003)where, identical tumours were established in either wild-type or A-SMase KO mice and then irradiated, and it was shown that the tumour response to irradiation was dependent on the A-SMase status of the recipient mouse.

All these evidences highlight the key role of A-SMase in tumour development and in response to chemotherapeutics whenever it is expressed by host or tumour cells.. It is not still clear in particular how the host effect of A-SMase are mediated and possible roles have been proposed not only for the endothelium (Garcia-Barros, Paris et al. 2003)but also for the host immune system. Indeed, previous studies, including some in the B16 melanoma model in vivo, indicate that A-SMase is a key determinant in the function of immune cells, regulating not only their sensitivity to apoptosis but also their differentiation and response to inflammatory/differentiative stimuli (Sciorati, Rovere et al. 1997; Paolucci, Rovere et al. 2000; Paolucci, Burastero et al. 2003; Falcone, Perrotta et al. 2004; Perrotta, Bizzozero et al. 2007). The function and mechanisms of action of A-SMase in host vs. tumour interactions have not been elucidated: a thorough elucidation of these aspects may lead to identification of novel relevant molecular players and hence to significant advances in the identification of possible new therapeutic strategies to cancer therapy.

Ceramide and Acid Sphingomyelinase in Cancer Therapeutics

Recently, modulating the ceramide pathway by various approaches has been shown to successfully inhibit cancer growth both *in vivo* and *in vitro*. One example is through the inhibition of an enzyme that degrades ceramide, acid ceramidase (AC). Systemic administration of AC inhibitors has been shown to inhibit xenograph growth of both head and neck squamous cell carcinomas (Elojeimy, Liu et al. 2007) and hepatomas (Morales, Paris et al. 2007) in mice. Grammatikos et al. showed for the first time that transfection of the A-SMase cDNA into murine glioma cells can sensitize them to the chemotherapy agents doxorubicin and gemcitabine (Grammatikos, Teichgraber et al. 2007). Most recently overexpression of A-SMase has been shown to enhances the antineoplastic effects of irradiation *in vitro* and *in vivo* (Smith and Schuchman 2008). Moreover Savic et al. demonstrated that rhASM/sorafenib combination exhibited a synergistic effect on reducing the tumour volume and blood vessel density in Huh7 xenografts, despite modest activity of rhASM in these tumours, despite no significant increases in survival were observed from the rhASM/sorafenib treatment (Savic, He et al. 2013).

All these evidences suggest a possible and particularly intriguing use of A-SMase as an antioncogenic drug, even because preclinical studies performed for the development of NPD enzyme replacement therapy have shown that it can be administered at high doses into normal animals without deleterious effect. In addition, the acidic pH optimum of the enzyme favours its preferential activity within the acidic microenvironment of tumours. Good Manufacturing Practice (GMP)-approved recombinant A-SMase has already been manufactured and is currently being evaluated in clinical trials as a treatment for type B NPD.

AIM OF THE STUDY

The aim of this study is the investigation of the role of Acid Sphingomyelinase (A-SMase) expressed by cancer cells in host versus tumour interactions, in particular dissecting the possibility that a different expression of this enzyme is related to modification in the extent and type of tumour microenvironment, with the purpose to consider A-SMase as an adjuvant for cancer therapy. To this end we characterised in vivo the connection between A-SMase expression and the composition of tumour microenvironment.

All the in vitro and In vivo experiments were performed using the murine melanoma model and C57/BL6J mice.

MATERIALS AND METHODS

Materials

The following reagents were purchased as indicated: the Malignant melanoma, metastatic malignant melanoma and benign nevus tissue array ME1003, the Lung disease spectrum tissue array BC04002, the Colon cancer tissue array CO1503, the Adrenal gland disease spectrum (adrenal cancer progression) tissue array AD2081,from US Biomax, Inc.; the polyclonal antibody (Ab) against A-SMase from Areta international; the polyclonal Ab against CD11b, Gr1 from MiltenyiBiotec; the polyclonal Ab against F4/80 from BioLegend; the polyclonal Ab against CD80, CD86, CD11c, MHCII, Cd4, CD8, CD44, CD25, Foxp3 from eBiosciences; the polyclonal Abs against MycTagand the anti-rabbit Ab HRP-conjugated from Cell Signaling Technology; Clarity Western Blotting ECL substrate from Bio.Rad. Reagents for cell cultures were from Euroclone; Bicinchoninic Acid kit from Thermo Scientific; Spmd1siRNAs, p*Silencer*4.1-CMV neo kit and the scrambled controls siRNA, goat Alexa Fluor 546, LipofectamineRNAiMAX Transfection Reagent, pEF1/Myc plasmid, Trizol Reagents from Life Technologies; Fugene Transfection Reagent from Roche; RNeasy Micro kit from Qiagen; ImProm-II™ Reverse Transcription System from Promega; all other reagents from Sigma-Aldrich.

Methods

Animals

Female C57BL/6J mice (6–8 weeks old) were purchase by Charles River (Wilmington, MA, USA) and treated in accordance with the European Community guidelines and with the approval of the Institutional Ethical Committee.

Cell Culture and Treatments

MC38 murine colon cancer and LLC murine Lewis lung carcinoma cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS, glutamine (200 mM), penicillin/streptavidin (100 U/ml), pH 7.4, 3T3 mouse fibroblasts, H5V mouse endothelial cells, B16-F1 mouse melanoma cell line and all B16 subclones were cultured

in Iscove's supplemented with 10% heat-inactivated FBS, glutamine (200 mM), penicillin/streptavidin (100 U/ml), 1% Hepes 1M pH 7.4. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO2.

Immunofluorescence of Human Tissue Arrays

The tissue arrays were deparaffinized by two xylene rinses of 30 minutes each followed by two rinses with 100% ethanol for 1 minute each and a rinse in water. Antigen retrieval was done by boiling the slides in a pressure cooker in a sodium citrate buffer at a pH of 6.0. After rinsing briefly in 1xPBS, a 10 minute incubation with 0.1M glycine-PBS was used to quench tissues autofluorescence. To reduce nonspecific background staining, slides were incubated with 4% bovine serum albumin/1× PBS for 1 hour at room temperature. After permeabilization with 1xPBS/1% Triton, slides were incubated overnight at 4°C with with specific antibody for A-SMase in 4% BSA/1xPBS. For fluorescent detection appropriate secondary antibody conjugated with Alexa 546 (red) was used. Slides were examined using a Leica DMI4000 B automated inverted microscope equipped with a DCF310 digital camera (Leica Microscopy Systems, Heerbrugg, Switzerland). Image acquisition was controlled by the Leica LAS AF software.

Immunofluorescence quantification

Immunofluorescence was quantified with MacBiophotonics Image J. The RGB images were splitted to the respective red, green and blue image components. The red images were processed separately. By using five random test samples stained for every primary antibody, suitable threshold levels red were determined and kept constant for all analysis. Thresholding creates binary red positive masks in which red-positive pixels are pseudo-coloured with black, while the background is indicated with white colour. The extent of staining is calculated as integrated optical density (IOD), which is equal to the *area* × *average density* of image occupied by immunoreactivity(Konsti, Lundin et al. 2011) and represented in graph as the mean ± SEM.

To verify the reproducibility of the analysis, two blinded operators performed the same measurements for Human Melanoma Tissue Arrays using also the computer-assisted imaging analysis AxioVision Rel.4.6 (Carl Zeiss) (Kashani-Sabet, Rangel et al. 2009).

Analysis of A-SMase in murine tumours

On day 0, C57BL/6 mice received 5 × 10^4 B16-F1 cells, 2 × 10^5 LLC cells subcutaneously (*s.c.*) in the lower-right flank; and 2,5 × 10^4 MC38 cells intraperitoneally (*i.p.*).

Mice were sacrificed and tumour collected when they reached 5×5×3 mm and 10×10×8 mm for each diameters for B16-F1, RMA and LLC-derived tumours, and at day 7 and day 15 after injection for MC3-derived ones. A-SMase expression was then evaluated by quantitative real time PCR (qPCR)

Reverse Transcription-PCR and Quantitative Real Time-PCR (qPCR)

Total RNA from cells and tissues was extracted with the High Pure RNA Isolation Kit and High Pure RNA Tissue Kit (Roche), according to the manufacturer's recommended procedure. After solubilization in RNase-free water, total RNA was quantified by the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was generated from 1 μg of total RNA using ImProm-II Reverse Transcription System (Promega).

qPCRwas performed using LightCycler 480 SYBR Green I Master (Roche) on Roche LightCycler 480 Instrument, according to manufacturer's recommended procedure. As shown in Table below, a set of primer pairs were designed to hybridize to unique regions of the appropriate gene sequence. All reactions were run as triplicates. The melt-curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. As to control experiments, gel electrophoresis was also performed to verify the specificity and size of the amplified qPCR products. Samples were analysed using the Roche LightCycler 480 Software (release 1.5.0) and the second derivative maximum method. The fold increase or decrease was determined relative to a control after normalizing to GAPDH (internal standard) through the use of the formula $2^{-\Delta\Delta CT}$(Livak and Schmittgen 2001; Schefe, Lehmann et al. 2006).

Primer pairs designed for qPCR analysis

AP-2α (v.1)	*tfap 2a*	NM_011547	GAAGACTGCGAGGACCGTC	GAAGTCGGCATTAGGGGTGTG	126	PrimerBank*
AP-2α (v.1,2)	*tfap 2a*	NM_011547, 001122948	CGTCCCGCACGTAGAAGAC	CGCCACCGAAGAGGTTGTC	137	PrimerBank*
A-SMase	*smpd1*	NM_011421	TGGGACTCCTTTGGATGGG	CGGCGCTATGGCACTGAAT	134	PrimerBank*
GAPDH	*gapdh*	NM_008084	ACCCAGAAGACTGTGGATGG	ACACATTGGGGGTAGGAACA	172	RTPrimerDB#

*http://pga.mgh.harvard.edu/primerbank/

#http://medgen.ugent.be/rtprimerdb/index.php

B16-W6_pSIL10 and B16-F1_B1A cell line generation

Two stable transfected cell lines expressing respectively a shRNA sequence silencing ASMase expression and pEF1/Myc vector containing cDNA for A-SMase were generated from B16 F1 cell line.

Transfection of the shRNA vector was performed in B16-F1 cells using Fugene transfection reagent. Briefly, cells seeded in 100-mm petri dish one day prior to transfection. were transfected with 5 μg vector DNA at 30-40% confluence. After 36 hour incubation in the appropriate growth medium, cells were splitted at 40% confluence in medium containing 500 μg/ml of G418, previously identified as the optimal antibiotic concentration for the seclection, and selection was performed for 7-10 days. Approximately 30 resistant cell colonies were tested for A-SMase silencing through FACS analysis. The selected clone (clone B16-W6_psil10) showed the highest decrease in A-SMase expression comparing to the control cell line (Figure 1).

Transfection of the plasmide pEF1/Myc containing A-SMase cDNA was performed in B16-F1 cells using Fugene transfection reagent according to the manufacturer's protocol. Approximately 20 colonies resistant to the antibiotic G418 (500 μg/ml) were tested for Myc expression through WB analysis and only 5 showed positivity to it (Figure 2B).

Among this five clones, we chose B1A which showed the highest increase in A-SMase expression both at mRNA (Figure 2A) and protein (Figure 2B) levels comparing to B16-F1 mock transfected cell line. Of importance, this clone also showed the highest A-SMase activity (Figure 2C)

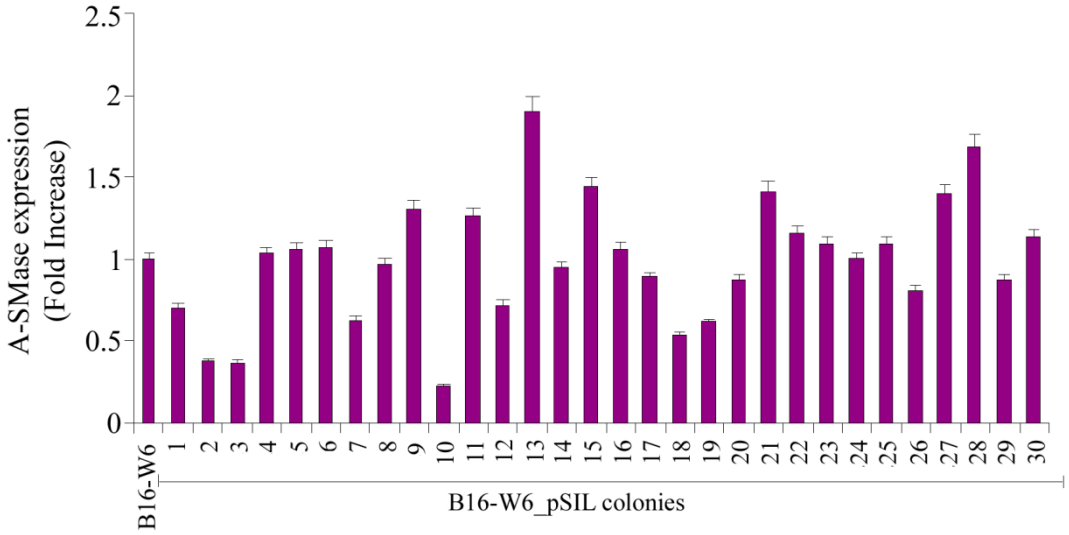

Figure 1: B16-W6_pSIL colonies A-SMase expression screening

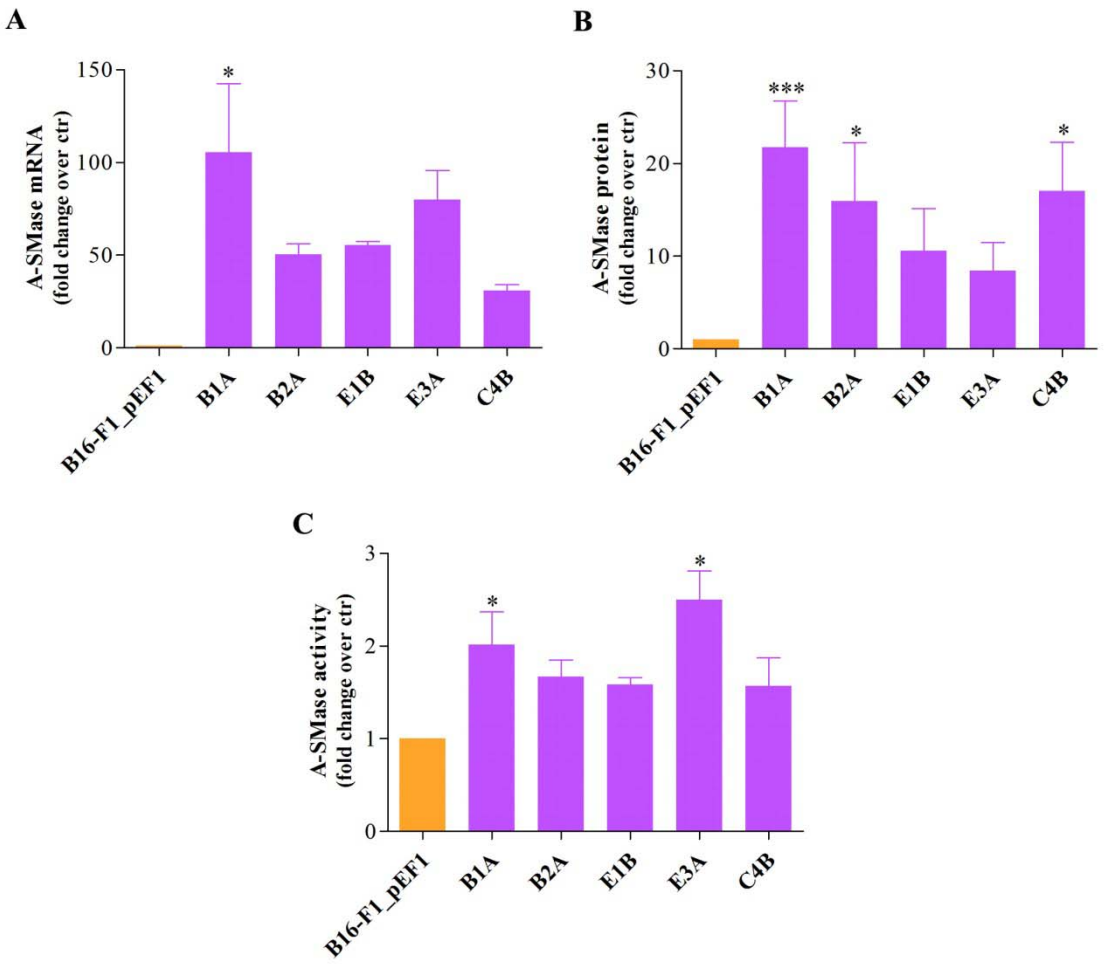

Figure 2: Stably trasfection with A-SMase accounts for higher protein expression and activity in B16-F1 clones.

qPCR analysis of A-SMase expression in B16-F1_pEF1 and different clones(**A**). Values are expressed as fold change over control (ctr) (n = 3). Western blotting analysis of A-SMase on cell lysates. The graph shows the densitometric values normalized on TBP. Values are expressed as fold change over control (ctr) (*n* = 3) (**B**). A-SMase activity on cell lysates measured as sphingomyelin hydrolysis to phosphorylcholine at pH 5.5. Values are expressed as fold change over control (ctr) (*n* = 3) (**C**).

Asterisks indicate statistical significance *vs*control (mock-trasfected B16-F1 cells). **p* < 0.05; ****p* < 0.001.

Western blotting

Cells were washed twice with ice cold PBS lysed in RIPA lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.25% Na-deoxycholate) containing a cocktail of proteases and phosphatases inhibitors. After 30 min of incubation on ice, the cell lysates were centrifuged 5 min at 1500 x g in order to pellet cell debris and the supernatant was transferred into new tubes.

Protein separation was performed following the standard protocol for western blotting. Samples were solubilized in Laemmli buffer, boiled 5 min at 100 °C. and loaded on g 7,5

% polyacrylamide precast gels (Mini-PROTEAN TGX™; Bio-Rad) immersed in running buffer (Tris-base, SDS, glycin) (Gels were run at 300V). Proteins separated in the gel were transferred onto a nitrocellulose membrane using a Bio-Rad's Trans-Blot Turbo System™ (7 min at 2.5 A) using Bio-Rad Transfer pack™ .

The nitrocellulose membrane was blotted with Ponceau red, to determine the quality of the protein transfer. Then it was incubated for 1 hour at room temperature in a blocking buffer (TBS-T: Tris-buffered saline-Tween 20 (Tris-HCl, NaCl, Tween 20) and non fat dry milk 5%), to block the sites for unspecific binding of the antibodies to the membrane. Then, membrane was incubated with a primary antibody, specific for the protein of interest, and, after three washes in TBS-T, with a secondary anti-rabbit horseradish peroxidase (HRP)-conjugated antibody, both diluted in Blocking buffer for 1 hour each. After these incubations the membrane was rinsed with TBS-T 3x10 min. Detection of the antigen was performed with the system of HRP/Hydrogen Peroxide catalyzed oxidation of luminol in alkaline conditions using the Clarity Western ECL Substrate by Bio-Rad. The light produced by this enhanced chemiluminescent reaction was detected using CemiDocMP Imaging System™.

Acid sphingomyelinase activity

A-SMase activity was determined by measuring conversion of sphingomyelin to phosphorylcholine in cell homogenates using the Amplex Red Sphingomyelinase Assay Kit according to the two step standard protocol. In brief, 2 x 10^6 cells were homogenated with 0.2% Triton-X100 in H_2O for 15 minutes at 4°C, sonicated, and incubated overnight at 80°C. For enzymatic activity assay 100 µg of homogenate from each sample were diluited in 100 µl50 mM sodium acetate, pH 5.0 and plated in a 96 well microplate. Similarly a negative control without the enzyme were set up. The first step reaction was started adding 10 µl of the 5 mMsphingomyelin solution to samples or negative control and incubated at 37°C for 1 hour. At this point two positive controls were prepared diluiting sphingomyelinase from *bacillus cereus* at the final concentration of 4 U/ml and H_2O_2 10 µM in 100 µl of 1x reaction buffer and adding 10µl of the 5 mM sphingomyelin solution. The second step reaction were performed adding to samples, and negative and positive controls, 100 µL of the Amplex Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase and 8 U/mL alkaline phosphatase and incubated for 30 minutes at 37°C, protected from light. The fluorescence were measured in a fluorescence microplate reader

using excitation in the range of 530–560 nm and emission detection at ~590 nm. For each point, background fluorescence was corrected by subtracting the values derived from the no-sphingomyelinase control.

Flow Cytometric Analysis of Tumours

For flow cytometric analysis, tumours were removed from euthanized mice, smashed, filtered through a 70-μm filter, and washed twice with 1x PBS. After erythrocyte lysis, Fc receptors were blocked with 1% BSA in 1xPBS for 30 min at 4°C. After one wash with PBS, cells were incubated for 15 min at room temperature with appropriate antibodies (CD4, CD8, CD25, CD44, Foxp3, CD11b, GR1, F4/80, CD11c, CD80, CD86, MHCII). Samples then were washed and resuspended in PBS. Samples were acquired using a Fluorescence-Activated Cell Sorter (FACS) (FC500 Dual Laser system Beckman Coulter, Brea, CA, USA) and analysed using FCS Express 4 Flow Research Edition software.

Bone marrow derived macrophage cells preparation

Bone marrow cell suspensions were isolated by flushing femurs and tibias of 8- to 12-week-old C57Bl/6 mice with complete MEM alpha (+10% FBS, +1% Pen/Strep, +1%l-glutamine). Aggregates were dislodged by gentle pipetting, and debris was removed by passaging the suspension through a 40-μm nylon cell strainer. Cells were washed twice with medium, and seeded on ultra-low attachment surface plates. Cells were cultured in with complete MEM alpha (+10% FBS, +1% Pen/Strep, +1%l-glutamine) supplemented with 100 ng/mL rhM-CSF and cultured in a humidified incubator at 37C and 5% CO_2 for 7 days. On days 3 and 5 cells were amplified and medium was refreshed.

Transwell Co-cultures of B16-F1 Melanoma/macrophages

Bone marrow derived macrophages (2×10^6) were plated on the upper compartment of 0.4-μm pore size transwell plates (Costar) in 0.1 ml of ISCOVE complete medium, while B16-F1 melanoma cells (0.3×10^6 in 0.6 ml) were placed on the lower chamber. Co-cultures were incubated for 24 hours at 37°C and 5%CO2, after which B16 cells were harvested and total RNA was extracted. Samples were then assayed for A-SMase expression by qRT-PCR.

Tumour growth *in vivo*

C57BL/6 mice (ten animals per group) received 5×10^4B16-F1_pEF1 and B16-F1_B1A cells *s.c.* in the lower-right flank on day 0.
Tumor growth was monitored twice a week by a caliper. Mice were sacrificed when their tumours reached 15 mm on either diameter.

Statistical analysis

Upon verification of normal distribution, statistical significance of raw data between the groups in each experiment was evaluated using unpaired the Student's *t*-test or ANOVA followed by the Bonferroni post-test. Kaplan-Meyer data were analysed with the multiple comparison survival curve method using the Log-rank (Mantel-Cox) test.Results are expressed as means ± standard error of the mean (SEM).

RESULTS

Acid sphingomyelinase decreases during tumour progression in different human cancers

A-SMase plays a key role in tumour progression and in mediating responses to chemotherapeutics (Smith and Schuchman 2008), thus we decided to analyse its expression in different human cancers.

A-SMase expression was evaluated by immunofluorescence in histological sections from human bioptic specimens of different tumours at various stages (melanoma tissue array). Interestingly, in tumours from skin, adrenal gland, lung and colonwe found an inverse correlation between tumour progression and A-SMase expression (Figure **1A-D**). Quantitative analyses of results from several samples carried out using ImageJ as analysis software, indicate that the differences in A-SMase expression between the groups of specimens are significant (Figure **1A-D**).

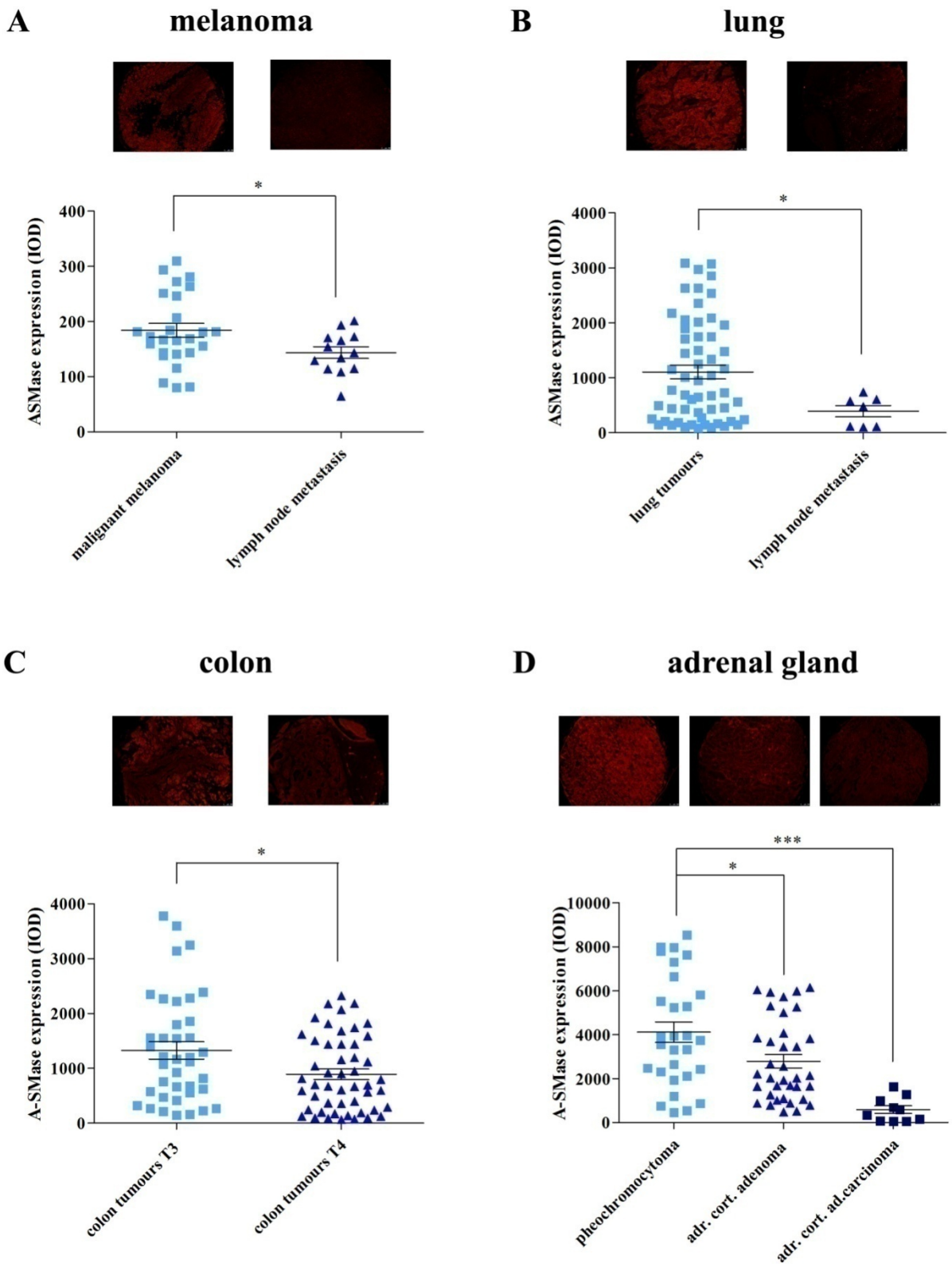

Figure 1:Acid sphingomyelinase decreases during tumour progression in different human cancers

Immunofluorescence staining of human tumours from skin (**A,** *upper panels*), adrenal gland (**B,** *upper panels*), lung C, *upper panels*) and colon (**D,** *upper panels*) at different stages with A-SMase antibody (scale bar, 110 μm).
Quantitative assessment of immunofluorescence staining of A-SMase on human samples from skin (malignant melanoma, n=26; lymph node metastasis, n=13) (**A,** *lower panel*), adrenal gland (pheochromocytoma, n=29; adrenal cortex adenoma, n=36; adrenal cortex adenocarcinoma, n=10) (**B,** *lower panel*), lung (lung tumours, n=59; lymph node metastasis n=7) (**C,** *lower panel*), colon (T3 colon tumours, n=38; T4 colon tumours n=50) (**D,** *lower panel*). Values in the graphs are expressed as mean ± SEM.
Asterisks indicate statistical significance ,*$p < 0.05$; ***$p < 0.001$.

Acid sphingomyelinase decreases during tumour progression in different murine cancers

To assess whether the same variability in A-SMase expression during tumour progression occurs also in mouse, we analysed its expression in different *in vivo* model of murine tumours: MC38, colon carcinoma, , Lewis lung carcinoma (LLC), and B16-F1melanoma.
To this end, C57B/L6 female mice were *s.c.*with5 × 10^4B16-F1 cells, 1 × 10^5 RMA cells and 2 ×10^5 LLC cells and *i.p.* with 2,5 × 10^4 MC38 cells.
Mice were sacrificed and tumour collected when they reached 5×5×3 mm (here referred to as early stage) and 10×10×8 mm (here referred to as late stage) for each diameters for B16-F1, RMA and LLC tumours, and at day 7 (early stage) and day 15 (late stage) after injection for MC38 ones.
We analysed these samples for A-SMase expression (mRNA) by qPCR and we observed that only in B16-F1 and MC38-derived tumours it decreased significantly during tumour progression (Figure **2A** and **B**).
Taken together these data indicate that in some tumours, both in human and in mouse, there is an inverse correlation between A-SMase expression and tumour malignancy. Moreover, the regulation of A-SMase levels is transcriptional, as indicated by the decrease not only of the protein expression (Figure **1**) but also of its mRNA (Figure **2A** and **B**).
These results prompted us to investigate the mechanisms responsible of this event and their biological consequences. We decided to study this phenomenon using the B16-F1melanoma model, as, together with MC38, it showed similar results both in human and mouse models.

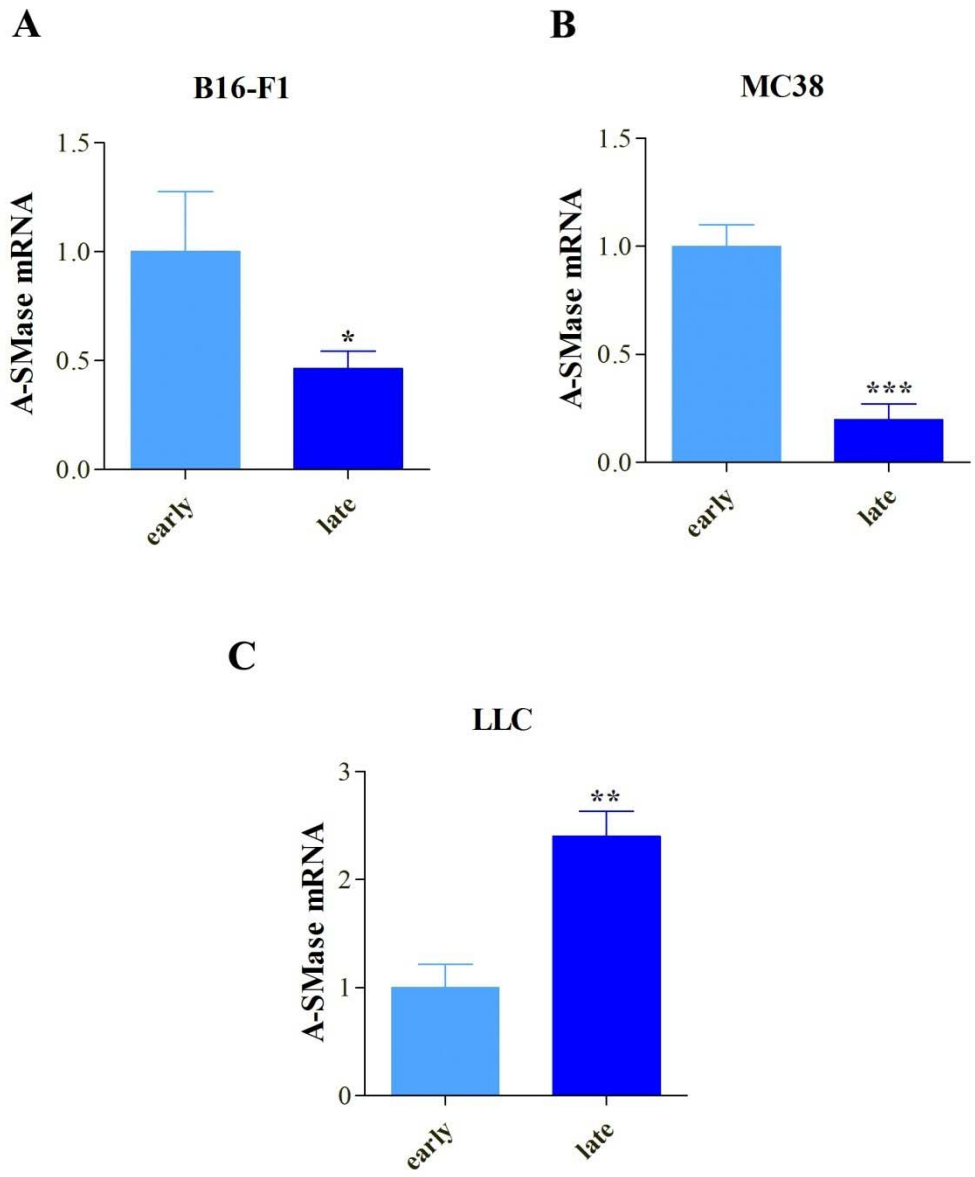

Figure 2:Acid sphingomyelinase decreases during tumour progression in different murine cancers

qPCR analysis of A-SMase expression in B16-F1 (**A**) (early, n=12, late, n=16), MC38 (early, n=10; late, n=11) (**B**) and LLC (early, n=7; late, n=6) (**C**) -derived tumours at different stages of progression. Values are expressed as fold change ± SEM.

Asterisks indicate statistical significance *vs* early stage tumours. **p*< 0.05; ***p*< 0.01; ****p* < 0.001.

Acid sphingomyelinase downregulation in melanoma could be driven by immune cells-released factors

In our previous work we have demonstrated that A-SMase downregulation during melanoma progression favours tumour growth conferring a more aggressive behaviour to melanoma cells both *in vivo* and *in vitro* (Bizzozero, Cazzato et al. 2013). Thus we decided to investigate the mechanisms responsible for this decrease.

In the last decades, it has been largely shown that a complex cross-talk exists between tumour cells and its immunological microenvironment (Yu, Kortylewski et al. 2007; Mattei, Schiavoni et al. 2012; Businaro, De Ninno et al. 2013; Schiavoni, Gabriele et al. 2013). Moreover, it has been demonstrated that citokynes released by the tumour infiltrate are able to induce molecular changes in tumour cells (Kanterman, Sade-Feldman et al. 2012), leading us to hypothesise a possible role of immune cells in the regulation of A-SMase expression in tumour cells.

As TAM have been largely shown to be the most abundant leucocytes in melanoma lesions and to be able to enhance the aggressiveness of cancer cells (Chen, Huang et al. 2011; Lin, Zheng et al. 2013), we first analysed their percentage in our tumour models. To assess unambiguously the dependence of the observed phenomena by A-SMase, we used, as control, tumours derived from a B16 clone (B16-W6_psil10) in which the enzyme is stably (Ostrand-Rosenberg, Sinha et al. 2012) silenced during the whole tumour progression as described in Bizzozero et al., 2013 (Bizzozero, Cazzato et al. 2013).

We observed that, despite changes in A-SMase levels, TAM (CD11b/F4/80 positive cells) infiltration is almost the same in B16-F1-derived tumours at early and late stage of progression as well as in B16-W6_psil10-derived ones (Figure **3B**).

The fact that TAM presence seemed not to be affected by A-SMase levels during tumour progression indicated that their recruitment is independent by A-SMase expression and suggested a possible involvement in regulating the expression of this enzyme.

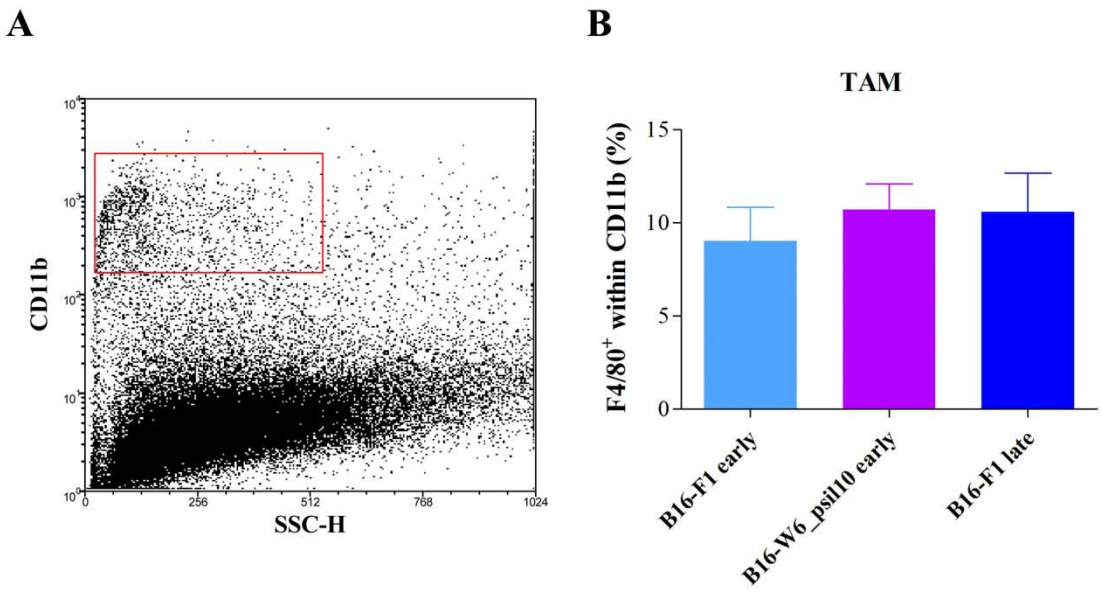

Figure 3: TAM infiltration is not affected by A-SMase levels during tumour progression in vivo

Flow cytometric analysis of tumour-infiltrating immune cells: tumour cell suspensions from different tumours were stained with anti-CD11b and anti-F4/80. Representative dot plot of gated $CD11b^+$ cells is shown (**A**). Graphs represent the percentage of $F4/80^+$ cells gated on $CD11b^+$ cells (**B**). Values are expressed as mean ± SEM (n=7 - 13).

To confirm our hypothesis, we carried out an *in vitro* experiments in which M2 bone marrow derived macrophages, the phenotype more similar to the one of TAM (Galdiero, Garlanda et al. 2013), were plated in the upper chamber of a Transwell system, while B16-F1 cells were plated in the lower one. After 24 hours of co-colture, macrophage swere able to induce a decrease of 53± SEM % of A-SMase mRNA levels. This reduction might be dependent by a specific factor released by macrophages since no changes in A-SMase expression were observed when B16 F1 cells were co-coltured with 3T3 fibroblasts or H5V endothelial cells (Figure **4**).

These data thus confirm our hypothesis about the modulation of tumoural A-SMase by TAM and underline the pro-tumoural role of TAM which modify melanoma cells conferring them a more aggressive behaviour.

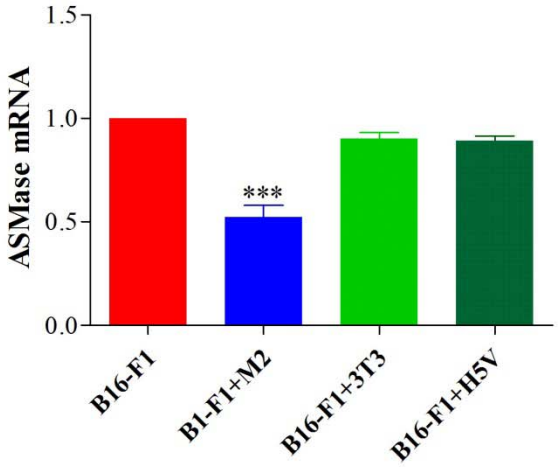

Figure 4: Macrophages derived factors downregulate A-SMase mRNA levels in melanoma cells in vitro

qPCR analysis of A-SMase expression in B16-F1 cells co-coltured for 24 hours with macrophages, 3T3 and H5V cells or cultured alone (B16-F1). Values are expressed as fold change over control (B16-F1) (n=6 - 12).

Asterisks indicate statistical significance *vs*B16-F1 coltured alone. ***$p < 0.001$.

TAM-induced A-SMase downregulation is dependent on Ap2α transcription factor

We, next, tried to elucidate the molecular mechanism leading to TAM-induced A-SMase downregulation.

It has been demonstrated that A-SMase expression is under the control of the transcription factors Sp1 and Ap2α (Langmann, Buechler et al. 1999). In addition, many papers described the down-regulation of Ap2α as a central event in melanoma progression (Melnikova, Dobroff et al. 2010; Braeuer, Zigler et al. 2011).

Starting from these data, we analysed Ap2α mRNA levels in B16-F1 cells after co-colture with macrophages or 3T3 fibroblasts or H5V endothelial cells and we observed that Ap2α mRNA decrease only in B16-F1 co-coltured with macrophages (Figure **5**) suggesting that TAM-induced A-SMase downregulation is mediated by Ap2α transcription factor through release of a soluble factor. More experiments have to be carried out to reveal which this soluble factor is.

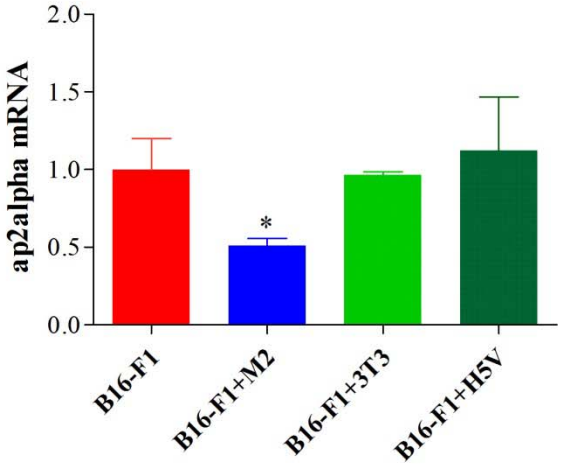

Figure 5: TAM-induced A-SMase downregulation is dependent on Ap2α transcription factor

qPCR analysis of Ap2α expression in B16-F1 cells co-coltured for 24 hours with M2-macrophages, 3T3 and H5V cells or cultured alone (B16-F1). Values are expressed as fold change over control (B16-F1) (n=4 - 2).

Asterisks indicate statistical significance *vs*B16-F1 coltured alone. *$p < 0.05$.

A-SMase downregulation in melanoma cells correlates to increased MDSC accumulation

Taking into account that there is a complex crosstalk between tumour cells and its immunological microenvironment (Yu, Kortylewski et al. 2007) and that A-SMase downregulation into melanoma cells confers them a more aggressive behaviour (Bizzozero, Cazzato et al. 2013), we decided to analyse immune cells infiltration within B16-F1 tumours at different stage of progression in order to speculate whether this event also has influences on the surrounding microenvironment. As above, even in these set of experiments B16-W6_psil10 tumours were used as control.

We first analysed the presence of MDSCs ($CD11b^+/Gr1^+$ cells), which have been shown to have a crucial role in generating the highly immunosuppressive microenvironment typical of melanoma tumours (Umansky and Sevko 2012). Interestingly we found a significant increase in $CD11b^+/Gr1^+$ MDSCs infiltrating B16-W6_pSIL10 with respect to B16-F1 at early stage of tumour growth (Figure **6,** *right panel*). Moreover, the percentage of MDSC cells in B16-F1 late stage tumours, in which A-SMase was downregulated, was similar to that of B16-W6_pSIL10 early stage (Figure **6,** *right panel*),. Thus, A-SMase downregulation seems to be crucial for MDSC recruitment at the tumour burden and might play an important role in favouring tumour immune escape.

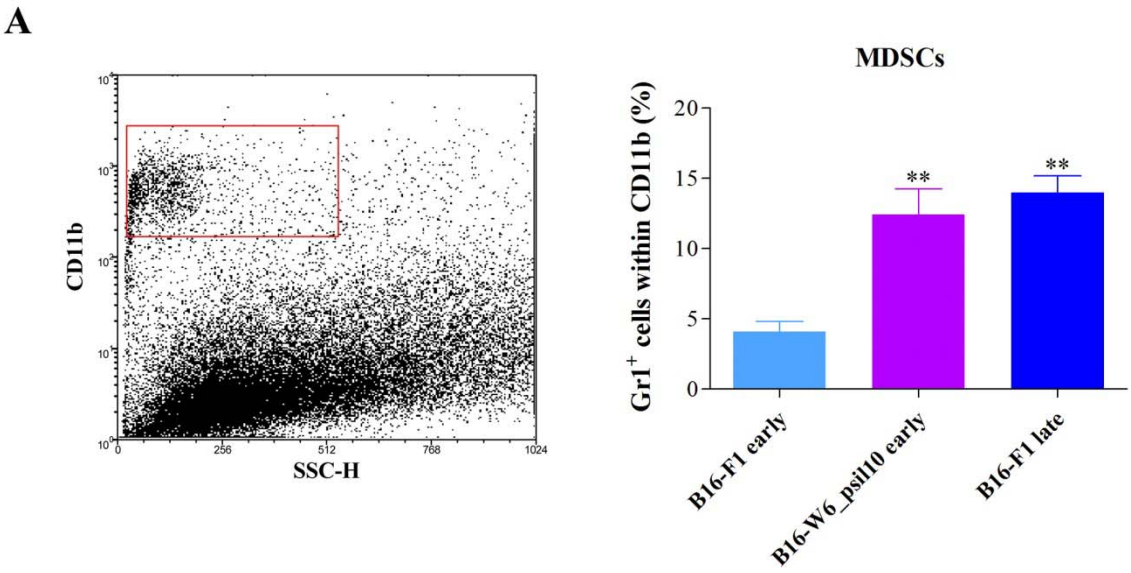

Figure 6: A-SMase downregulation in melanoma cells correlates to increase MDSCs accumulation

Flow cytometric analysis of tumour-infiltrating immune cells: tumour cell suspensions from different tumours were stained with anti-CD11b and anti-Gr1 antibodies. A representative dot plot of gated $CD11b^+$ cells is shown (**A**, *left panel*) (n = 5 - 14). Graphs represent the percentage of $Gr1^+$ cells gated on $CD11b^+$ cells (**A**, *right panel*). Values are expressed as mean ± SEM (n=5 - 14).
Asterisks indicate statistical significance *vs* B16-F1 early stage tumours. $^{**}p < 0.01$.

A-SMase-related MDSC accumulation determines immunosuppression

MDSCs exert their function through the secretion of immunosuppressive cytokines leading to the impairment of DCs and cytotoxic lymphocytes (Rodriguez, Quiceno et al. 2007; Ostrand-Rosenberg, Sinha et al. 2012; Schiavoni, Gabriele et al. 2013), and to an increased recruitment of regulatory $CD4^+/CD25^+/Foxp3^+$ T lymphocytes (T_{REGS}) (Schlecker, Stojanovic et al. 2012).

Thus, we decided to analyse these cells in our tumour models. First of all we observed a decrease in DCs number in tumour showing a downregulation of A-SMase with respect to B16-F1 early stage ones (Figure **7A,** *right panel*). Interestingly, in tumour with downregulated A-SMase, DCs are not only diminished, but were also immature and anergic as indicated by the reduction of the co-stimulatory markers CD80, CD86 and MHC II in B16-F1 late stage and B16-W6_psil10 with respect to B16-F1 early stage tumours (Figure **7B** and **C**).

Despite we didn't record any significant variation in the number of $CD8^+$ and $CD4^+$ T infiltrating lymphocytes (Figure **8A** and **B,** *right panels*), we observed higher number of T_{REGS} in B16-F1 late stage and B16-W6_psil10-derived tumours (Figure **8C**).

DCs impairment and T_{REGS} recruitment could be due to MDSC as we it correlates with their major accumulation in tumours with low A-SMase levels (Figure **6**).

These results suggested that the higher malignancy of low A-SMase-expressing tumours depends also on the instauration of an immunosuppressive microenvironment which leads to tumour immunoescape.

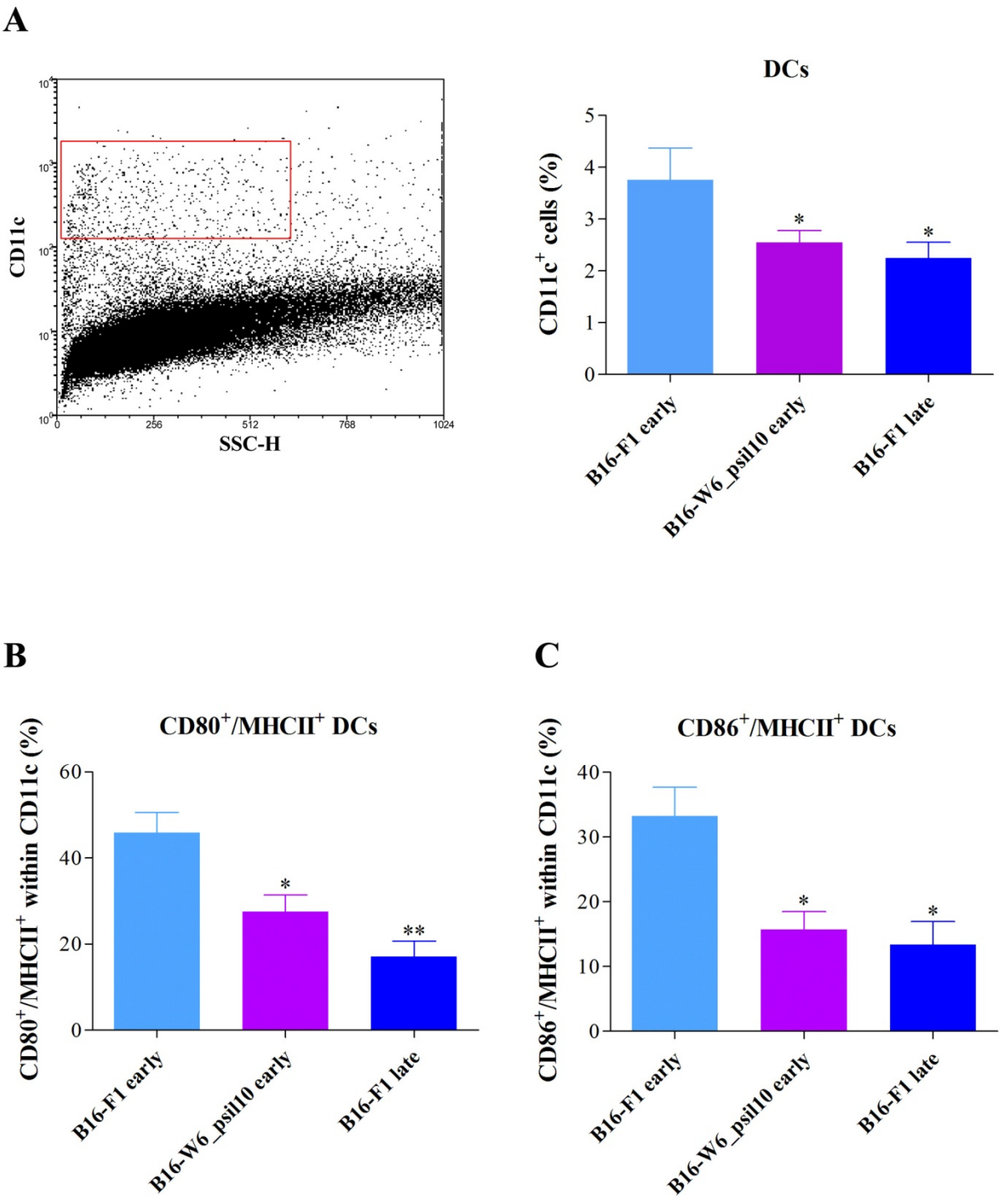

Figure 7: A-SMase-dependent MDSC accumulation accounts for DCs impairment

Flow cytometric analysis of tumour-infiltrating immune cells: tumour cell suspensions from different tumours were stained with anti CD11c, anti-CD80, anti-CD86 and anti-MHCII antibodies. A representative dot plot of gated $CD11c^+$ cells is shown (**A**, *left panel*) (n = 7 - 9). Graphs represent the percentage of:$CD11c^+$ cells (**A**, *right panel*) on total cells; $CD80^+/MHCII^+$ cells (**B**) gated on $CD11c^+$ cells; $CD86^+/MHCII^+$ cells (**C**) gated on $CD11c^+$ cells . Values are expressed as mean ± SEM (n=7 - 29). *Asterisks* in indicate statistical significance *vs* B16-F1 early stage tumours. * $p< 0.05$; ** $p< 0.01$.

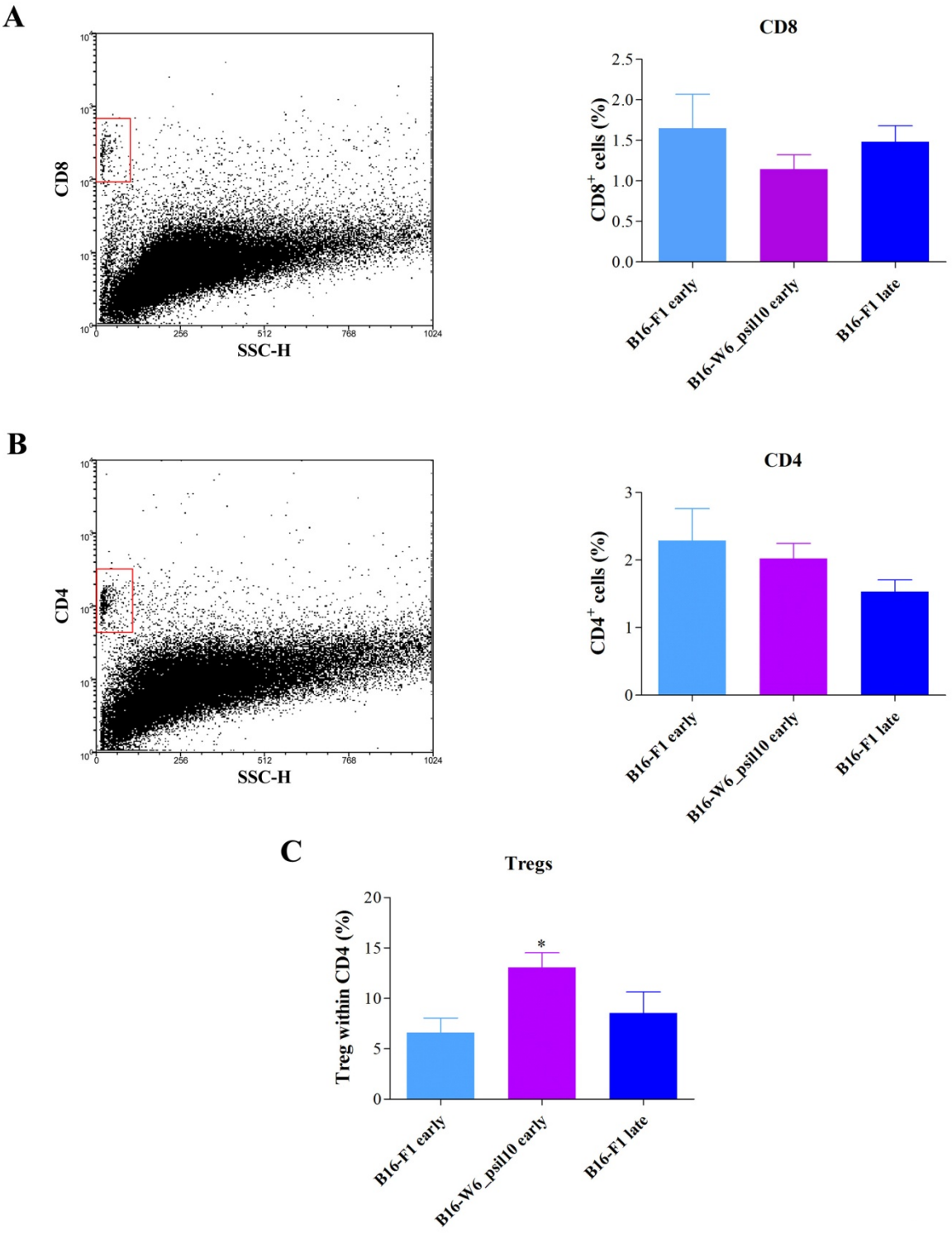

Figure 8: A-SMase-dependent MDSC accumulation accounts for T_{REGS} recruitment

Flow cytometry analysis of tumour-infiltrating immune cells: tumour cell suspensions from different tumours were stained with anti-CD4, anti-CD25 and anti-Foxp3 antibodies. Representative dot plots are shown (**A**, **B,** *left panels*) (n = 5 - 25). Graphs represent the percentage of: $CD8^+$ cells (**A**, *right panel*) and $CD4^+$ cells (**B**, *right panel*) on total cells; $CD25^+/Foxp3^+$ cells gated on $CD4^+$ cells (**C**).Values are expressed as mean ± SEM (n=5 - 25).

Asterisks indicate statistical significance *vs* B16-F1 early stage tumours. * $p< 0.05$.

Restoring A-SMase expression reduces melanoma growth

From these data and from our previous findings, we can state that the downregulation of A-SMase expression confers a double advantage to melanoma cells: on one side they acquire a more aggressive phenotype (Bizzozero, Cazzato et al. 2013), on the other they become able to create around them an immunosuppressive and pro-tumoural microenvironment. Thus, we asked whether restoring A-SMase expression in tumour cells could prevent or reduce the rising of these pro-tumoural mechanisms.

It has already been demonstrated that A-SMase overexpression or the injection of the recombinant protein (rhASM) have antineoplastic effects sensitizing subcutaneous mouse melanomas to irradiation *in vivo* (Smith and Schuchman 2008), but the consequences of restoring A-SMase levels on tumour growth and on tumour microenvironment haven't been studied yet.

To do this we generated, starting from B16-F1, a cell line (B16-F1_B1A) in which A-SMase was stably overexpressed by trasfection with the vector pEF1/myc-His containing the A-SMase gene (Figure **2**in materials and methods). Of importance, in the selected clone the increase of A-SMase mRNA (Figure **2A** in materials and methods) levels correspond with higher protein expression (Figure **2B** in materials and methods) and activity (Figure **2C** materials and methods) in respect to the control cell line. As control, we use B16-F1 cell line (B16-F1_pEF1) stably trasfected with the empty vector.

C57BL6 mice were injected *s.c.* with 5×10^4B16-F1_pEF1 and B16-F1_B1A cells and we observed that B16-F1_pEF1-derived melanomas grew significantly faster than B16-F1_B1A-derived ones (Figure **9**).

This result is in line with what we previously observed in tumours in which A-SMase was silenced (Bizzozero, Cazzato et al. 2013) and confirms the inverse correlation between A-SMase expression and tumour growth. Taking that into account together with the results regarding tumour infiltration observed in B16-F1 late stage and B16-W6_psil10 tumours, we wondered whether the slower growth of A-SMase overexpressing tumours is due also to a modification of the immunosuppressive microenvironment. .

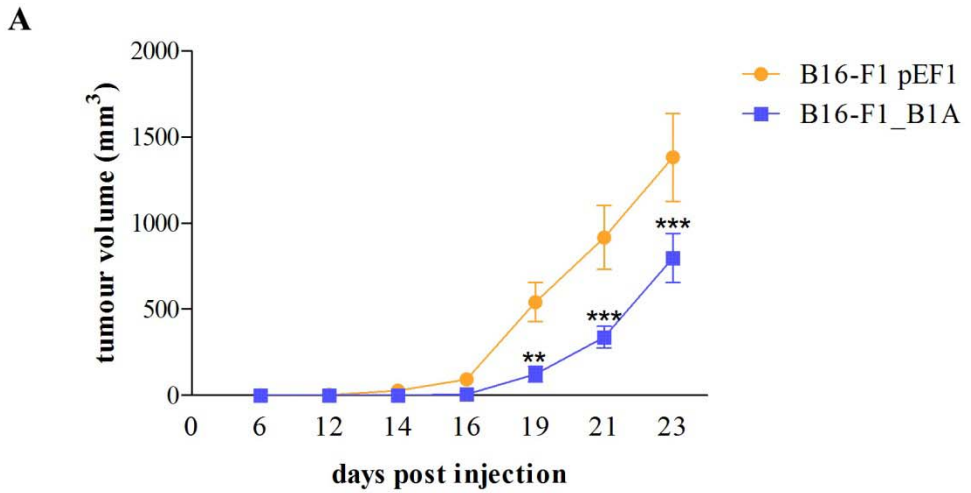

Figure 9:A-SMase overexpression reduces tumour growth
C57BL/6 mice (10 animals per group) were injected in the right flank with B16-F1_pEF1 and B16-F1_B1A (5×10^4 cells). Tumour growth was monitored by measuring tumour volume (mm^3) every 2 days. Values are expressed as mean ± SEM.
Asterisks indicate statistical significance *vs* B16-F1_pEF1 tumours. $^{**}p< 0.01$; $^{***}p< 0.001$

Restoring A-SMase expression switches tumour microenvironment from a pro- to an anti-tumoural phenotype

Because B16-F1_pEF1 and B16-F1_B1A tumours displayed different tumour growth kinetics, we addressed the immune cell infiltration along tumour progression. Thus, we explanted melanomas at two stages of progression, when tumour size was 5×5×3 mm (early stage) and 10×10×8 mm (late stage) and we proceeded with the flow cytometric analysis of tumour infiltrate.

We, first, analysed what happens to the immunosuppressive cells MDSC and T_{REGS}, that are the one affected by the downregulation of A-SMase into melanoma cells, after restoring enzyme expression and we observed that in B16-F1_B1A early stage tumours there is a 79± SEM % and a 63± SEM % decrease respectively in the number of MDSCs and T_{REGS} with respect to B16-F1_pEF1 ones (Figure **10A** and **B**, *lower panels*). Interestingly, these differences become more significant in the late stage of progression, when the percentage of MDSCs and T_{REGS} decreases respectively of 70± SEM % and 76± SEM % in B16-F1_B1A tumours with respect to B16-F1_pEF1 ones (Figure **10A** and **B**, *lower panels*).

Then, we addressed the other immune cell populations recruited at the tumour site.

Surprisingly, the percentage of TAM, which role is mainly pro-tumoural (Chen, Huang et al. 2011; Lin, Zheng et al. 2013) and did not change under A-SMase down-regulation, was significantly lower in B16-F1_B1A during tumour progression with respect to B16-F1_pEF1 ones (Figure **11A**, *lower panel*). Conversely, DCs infiltrate is higher in tumours overexpressing A-SMase (Figure **11B**, *lower panel*).Moreover, at early stage, $CD4^+$ and $CD8^+$ T lymphocytes were significantly higher in B16-F1_B1A tumours with respect to B16-F1_pEF1 ones (Figure **11C** and **D** respectively, *lower panels*). At late stage, the percentages of both $CD4^+$ and $CD8^+$ T slightly decrease, but remain however higher in B16-F1_B1A tumours with respect to B16-F1_pEF1 ones (Figure **11C** and **D** respectively, *lower panels*).

All these data demonstrate that A-SMase overexpression in melanoma cells not only reverts the effect on tumour immunosuppression following the naturally occurred down-regulation of the enzyme during tumour progression, but moreover accounts for an high unexpected recruitment of immune cells with an anti-tumoural function in the tumour microenvironment.

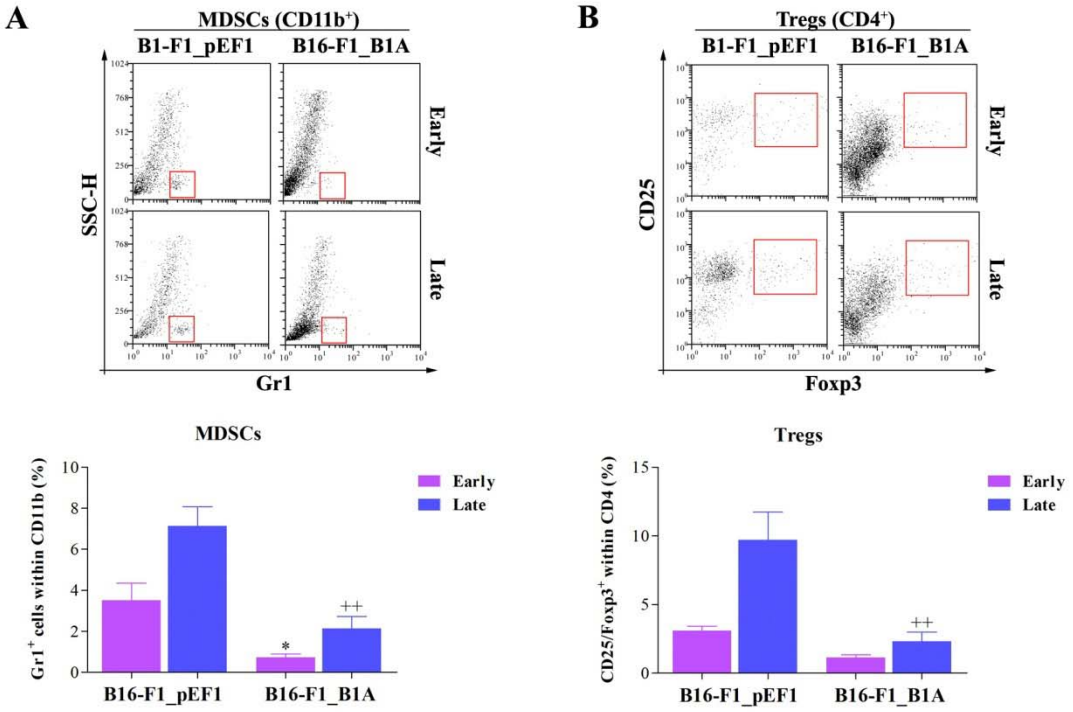

Figure 10: A-SMase overexpression reduces immunosuppression

Flow cytometric analysis of tumour-infiltrating immune cells: tumour cell suspensions from different tumours were stained with anti-CD11b, anti-Gr1, anti-CD4, anti-CD25 and anti-Foxp3. Representative dot plots of gated $Gr1^+$ (**A**, *upper panel*) and $CD25^+/Foxp3^+$ (**B**, *upper panel*) cells were shown. Graphs represent the percentage of:$Gr1^+$ cells (**A**, *lower panel*) gated on $CD11b^+$ cells and $CD25^+/Foxp3^+$ cells (**B**, *lower panel*) gated on $CD4^+$ cells. Values are expressed as mean ± SEM (n=5 - 17).

Asterisks and *cross* indicate statistical significance *vs* B16-F1 early stage and B16-F1 late stage tumours respectively. * $p < 0.05$; $^{++}p < 0.01$

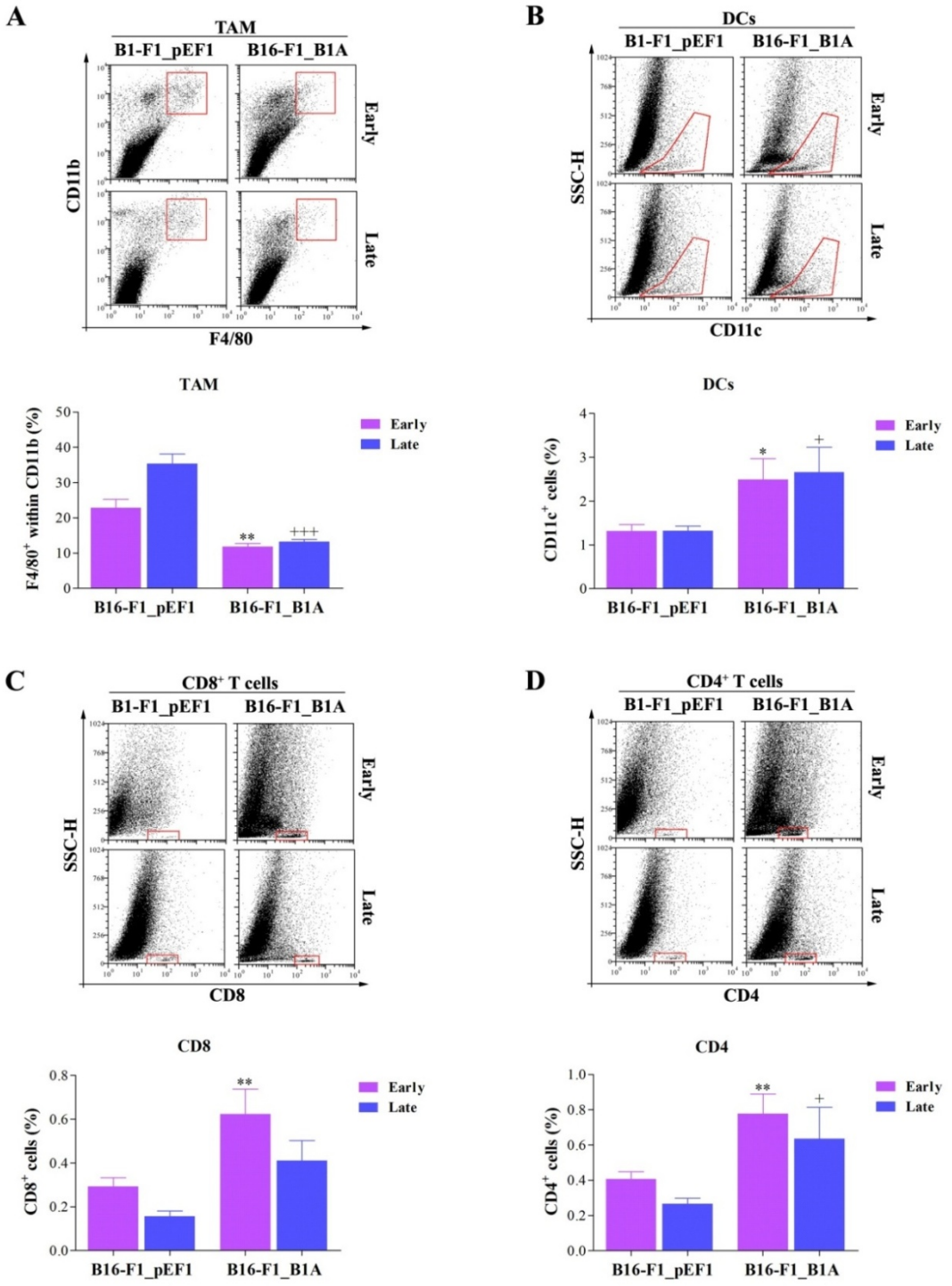

Figure 11: A-SMase overexpression increases recruitment of anti-tumoural immune cells

Flow cytometric analysis of tumour-infiltrating immune cells: tumour cell suspensions from different tumours were stained with anti-CD11b, anti-F4/80, anti-CD11c, anti-CD8 and anti-CD4. Representative dot plots of gated $CD11b^+/F4/80^+$ (**A**, *upper panel*), $CD11c^+$ (**B**, *upper panel*), $CD8^+$ (**C**, *upper panel*) and $CD4^+$ (**D**, *upper panel*) cells were shown. Graphs represent the percentage of:$CD11b^+/F480^+$(**A**, *lower panel*), $CD11c^+$(**B**, *lower panel*), $CD8^+$ (**C**, *lower panel*) and $CD4^+$ (**D**, *lower panel*) on total cells. Values are expressed as mean ± SEM (n=5 - 17).

Asterisks and *cross* indicate statistical significance *vs* B16-F1 early stage and B16-F1 late stage tumours respectively. * $p < 0.05$; ** $p < 0.01$. $^{+}p < 0.05$; $^{+++}p < 0.001$.

We did not observe differences in the maturation and activation of DCs in our tumour models at both the stages of progression.

These data showed that overexpression of A-SMase in melanoma cells contribute to the creation of an antitumoural tumour microenvironment also by inducing a decrease in MDSCs and T_{REGS}, which are the main responsible of immunosuppression in tumours.

DISCUSSION

Due to the pro-apoptotic effects of ceramide, cancer cells have developed multiple defense mechanisms to overcome its action, including reducing its production and/or enhancing its clearance, or elevating the production of the counteracting pro-survival lipid, sphingosine-1-phosphate (S1P). These defense mechanisms also may contribute to sphingolipid-mediated drug resistance (Oskouian and Saba 2010; Ponnusamy, Meyers-Needham et al. 2010). Notably, the levels of ceramide are significantly decreased in human colon cancers (Selzner, Bielawska et al. 2001), gliomas (Riboni, Campanella et al. 2002), and ovarian cancers (Rylova, Somova et al. 1998), and an inverse relationship has been found between ceramide levels and glioma stratification into high- and low-grade tumours (Riboni, Campanella et al. 2002). Thus, modulation of ceramide by cancer cell might be considered one of the mechanism of tumour to escape cell death and restoring its level by activating the enzymes of its generation a good strategy of tumour therapy.

Among these enzymes, A-SMase seems to play a crucial role in cancer physiopathology (Schuchman 2007). A-SMase is a phosphodiesterase that catalyzes the hydrolysis of membrane sphingomyelin to ceramide and phosphorylcholine. The enzyme plays important roles in pathophysiology, as it mediates the action of several apoptogenic molecules, cytokines and neurotrophins, regulating neuronal function, immunity and infections (Grassme, Jendrossek et al. 2003; Ng and Griffin 2006; Herz, Pardo et al. 2009). It also contributes significantly to the cytotoxic effects of several anticancer drugs such as cisplatin, retinoids, doxorubicin and radiation (Gulbins and Kolesnick 2003; Lacour, Hammann et al. 2004; Perrotta, Bizzozero et al. 2007; Zeidan and Hannun 2007).

In this study we showed that A-SMase levels are decreased in different human and murine tumours during their progression suggesting its involvement in malignancy development. Of interest, in melanoma and colon tumours we observed similar results both in human and mouse.

In our previous work we demonstrated that, in melanoma, A-SMase decrease favours tumour ability to grow, invade and metastasise both in mouse and in human (Bizzozero, Cazzato et al. 2013), but the mechanism responsible of this event was not investigated yet.

Here we hypothesise as a possible mechanism of this event the release of microenvironmental factors acting on melanoma cells and thus modulating A-SMase during tumour growth. Indeed, it has been largely shown that a complex cross-talk exists between tumour cells and its immunological microenvironment (Yu, Kortylewski et al. 2007; Mattei, Schiavoni et al. 2012; Businaro, De Ninno et al. 2013; Schiavoni, Gabriele et al. 2013) thus leading immune cells to release cytokines which are able to induce molecular changes in tumour cells (Kanterman, Sade-Feldman et al. 2012). Of importance for our study, TAM have been largely shown to be the most abundant leucocytes in melanoma lesions and to be able to enhance the aggressiveness of cancer cells (Chen, Huang et al. 2011; Lin, Zheng et al. 2013), evoking them as possible candidates in the regulation of A-SMase expression in tumour cells.

In support of this hypothesis we reported that TAM percentage seemed not to be affected by A-SMase levels during tumour progression indicating that their recruitment is independent from A-SMase expression and further suggesting their possible involvement in modulating the levels of this enzyme. In agreement with this, we observed that bone marrow-derived macrophages induce a significant decrease in A-SMase mRNA in B16-F1 melanoma cells after 24h of *in vitro* co-colture. Finding out the soluble factors responsible for this reduction and dissecting the pathways responsible of this event could lead to the identification of key players to be targeted to restore A-SMase levels in tumour cells.

The regulation of A-SMase expression has been poorly investigated, to date we only know that in monocytes, A-SMase promoter is under the control of Sp1 and Ap2α transcription factors (Langmann, Buechler et al. 1999). Of interest for our work, many papers described the down-regulation of Ap2α as a central event in melanoma progression (Melnikova, Dobroff et al. 2010; Braeuer, Zigler et al. 2011). Starting from these evidences, we showed that after 24h of co-colture with bone marrow-derived macrophages, Ap2α expression decreases in B16-F1 cells correlating with the reduction of A-SMase levels. Other experiments in which Ap2α is silenced are required to confirm unambiguously the dependence of A-SMase expression on this transcription factor.

All these data further support the existence of a tight cross-talk between various cell types at the tumour site which accounts for A-SMase downregulation in melanoma cells thus leading to more aggressive tumours, as shown in our previous work (Bizzozero, Cazzato et al. 2013). As tumour microenvironment can be considered the product of a developing cross-talk between different cells types, we hypothesized that the molecular changes we

observed in tumour cells could, in turn, give pro-tumoural and immunosuppressive features to the surrounding microenvironment. In this scenario, our data clearly revealed a close correlation between the extent and type of tumour infiltrate and A-SMase levels in-melanoma, in particular, A-SMase decrease accounts for the establishment of an high immunosuppressive and pro-tumoural microenvironment. This is indicated by our observation that tumours with low A-SMase levels, B16-F1 at late stage of development and B16-F1_psil10 at early stage, displayed high MDSCs infiltration, already known to be responsible for the establishment of immunosuppression (Umansky and Sevko 2012). Our data are in line with previous findings describing an high immunosuppressive microenvironment as typical of melanoma lesions (Meyer, Sevko et al. 2011; Umansky and Sevko 2012).

MDSCs exert their function through the secretion of immunosuppressive cytokines leading to the impairment of DCs and cytotoxic lymphocytes (Rodriguez, Quiceno et al. 2007; Ostrand-Rosenberg, Sinha et al. 2012; Schiavoni, Gabriele et al. 2013), and to an increased recruitment of regulatory $CD4^+/CD25^+/Foxp3^+$ T lymphocytes (T_{REGS}) (Schlecker, Stojanovic et al. 2012). Accordingly, in tumours with downregulated A-SMase, DCs were not only diminished, but also immature and anergic. Moreover the same tumours displayed increased T_{REGS} infiltration.

We clearly showed that the ability to create this immunosuppression together with the acquisition of a more aggressive phenotype (Bizzozero, Cazzato et al. 2013), both depend on the naturally occurring A-SMase decrease in melanoma cells during tumour progression.

The broad role of A-SMase in tumour pathogenesis we identified, indicates also that the enzyme is at the crossroad of key pathways in tumourigenesis. This aspects has clear potential in therapeutic perspective in which A-SMase overexpression or administration might be consider as an useful adjuvant for cancer therapy.

To date, studies based on the use of A-SMase for tumour treatment, have concentrated mainly on its effect in sensitizing tumour cells to chemotherapy (Smith and Schuchman 2008; Savic, He et al. 2013), but the consequences of restoring A-SMase levels on tumour growth and on tumour microenvironment haven't been studied yet. Here we demonstrated for the first time that restoring A-SMase expression in melanoma cells not only reduces tumour growth and immunosuppression, but moreover accounts for a high, unexpected recruitment of immune cells with an anti-tumoural function in the tumour microenvironment. This is indicated by our observation that tumours that overexpress A-

SMase during all their progression display poor homing of MDSCs and T_{REGS}. Moreover A-SMase expression accounts for the creation of an inflammatory microenvironment, as shown by the decrease in the percentage of TAMs, which role is mainly pro-tumoural (Chen, Huang et al. 2011; Lin, Zheng et al. 2013), together with strong recruitment of DCs, $CD8^+$ and $CD4^+$ T lymphocytes.

In conclusion our results demonstrated for the first time the central role of A-SMase expressed by melanoma cells in orchestrating the cross-talk with the surrounding microenvironment. These interactions are crucial for tumour fate, lying on its rejection or progression. Our observation that A-SMase overexpression "educate" tumour microenvironment against cancer cells, further encourage the use of this enzyme as an adjuvant for cancer therapy. Indeed, the discovery of this new function that A-SMase exerts on the tumour immune system, adds to the previously observed one exerted on cancer cell death, thus suggesting that the antineoplastic effect of this enzyme occurs through more than one mechanisms.

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