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IMMUNOLOGICAL SIGNATURE IN NAÏVE AND SUNITINIB-TREATED SOFT TISSUE SARCOMA PATIENTS: ROLE OF MYELOID CELLS

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I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician: he is also a
child placed before natural phenomena which impress him like a fairy tale.

—Marie Curie

Abbreviations

ASPS	alveolar soft part sarcoma
ATP	adenosine triphosphate
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CCS	clear cell sarcoma
CD	cluster of differentiation
CML	chronic myeloid leukemia
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T-Lymphocyte Antigen 4
CRT	calreticulin
DAMP	damage-associated molecular pattern
DC	dendritic cell
FASL	Fas ligand
FISH	fluorescence in situ hybridization
FLT3	fms-related tyrosine kinase 3
FOXP3	forkhead box P3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GZMB	granzyme B
HLA	human leukocyte antigen
HMGB	high-mobility group box
IDO	indoleamine 2,3-dioxygenase
IHC	immunohistochemistry
IL	interleukin
IMCs	immature myeloid cells
LPS	lipopolysaccharides
M-CSF	macrophage colony-stimulating factor
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PD-1	programmed cell death-1
PD-1L	programmed death-ligand 1
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PGE2	prostaglandin E2
RCC	renal cell carcinoma
SFT	solitary fibrous tumors
STAT	saignal transducer and activator of transcription
STS	soft tissue sarcoma
TAA	tumor-associated antigen
TAM	tumor associated macrophage
T-bet	T-box transcription factor
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TSA	tumor-specific antigen
Treg	regulatory T cell
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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LIST OF PUBLICATIONS *not included in this thesis*

Camisaschi C, **Tazzari M**, Rivoltini L and Castelli C. Monitoring the frequency and function of regulatory T cells and summary of the currently used approaches to limit regulatory T cells expansion. *Methods Mol Biol.* 2014 1139:201-21. doi: 10.1007/978-1-4939-0345-0_18. *(Book Chapter)*

Castelli C, Rivoltini L, Rodolfo M, **Tazzari M**, Belgiovine C, Allavena P. Off-target effects of cancer therapies: focus on immunity. *Cancer Immunology Immunotherapy. (Review)* [under revision]

Camisaschi C, Filipazzi P, **Tazzari M**, Casati C, Beretta V, Pilla L, Patuzzo R,Maurichi A, Cova A, Maio M, Chiarion-Sileni V, Tragni G, Santinami M, Vergani B, Villa A, Berti E, Umansky L, Beckhove P, Umansky V, Parmiani G, Rivoltini L, Castelli C. Effects of cyclophosphamide and IL-2 on regulatory CD4+ T cell frequency and function in melanoma patients vaccinated with HLA-class I peptides: impact on the antigen-specific T cell response. *Cancer Immunol Immunother.* 2013 62(5):897-908.

Activity of sunitinib in extraskeletal myxoid chondrosarcoma. Stacchiotti S, Pantaleo MA, Astolfi A, Dagrada GP, Negri T, Dei Tos AP, Indio V, Morosi C, Gronchi A, Colombo C, Conca E, Toffolatti L, **Tazzari M**, Crippa F, Maestro R, Pilotti S, Casali PG. *Eur J Cancer.* 2014 Apr 2. doi: 10.1016/j.ejca.2014.03.013.

PART I

1. ABSTRACT

Although designed to directly target cancer cells and tumor associated-vasculature, anti-angiogenic drugs (e.g. sunitinib), have been described to influence tumor-host interactions. Sunitinib is currently in use at our Institute for the treatment of progressive, advanced soft tissue sarcomas (STS) of different histology. However, the systemic and local immune responses and their modulation by anti-angiogenic therapies are unknown in these neoplasms, namely solitary fibrous tumors (SFTs), clear cell sarcoma (CCS) and alveolar soft part sarcoma (ASPS). This thesis aims to shed light on the immunological status of these STS patients and to address the question to which extent sunitinib induces immune modulation in these patients. Thus, my research focused on the characterization of both tumor-infiltrating and circulating immune cells of STS patients. Fine analysis of the immune contexture at the tumor site in naïve and in sunitinib-treated tumors revealed that myeloid cells, namely tumor-associated macrophages, represent a key component of the tumor microenvironment and that their reprogramming is part of the response to sunitinib treatment. Immune monitoring of circulating cells in these STS patients indicated that circulating myeloid suppressor cells were associated to disease progression and were the major player in mediating the immune-suppressive status in naïve and in sunitinib-treated SFT patients. Moreover, evidence have been provided that, in sunitinib-treated SFT patients, myeloid suppressor cells may be part of acquired resistance, thus supporting the notion that myeloid cells are the most relevant hurdle in the efficacy of anti-angiogenic treatments. Collectively the results of this thesis shed light on an unappreciated phenomenon of immune dysfunction in STS patients and indicate that in SFTs sunitinib transiently relieves systemic immunosuppression and reprograms the immune microenvironment. Moreover, for the first time, an antigen-specific T cell response has been evidenced in CCS, and, this tumor-specific response has occurred in association to sunitinib-induced immune modulation. Overall, this thesis poses the rationale for the development of immune-based clinical approaches aimed at achieving a more durable disease control in these cancer patients, for which effective medical therapies are still needed.

2. STATE OF THE ART

2.1 Cancer immunology

The concept that immune system can control cancer has its roots in the early 1800s when Rudolf Virchow was the first to observe the infiltration of tumors by leukocytes, indicating a possible cross-talk between the immune system and the malignant tissue. Later, in the 1890s, William B. Cooley observed that some cancer patients (interestingly, they were patients suffering of a rare sarcoma) experienced spontaneous remission when they contracted acute infection [1]. However it was only 50 years later that the *cancer immunosurveillance* hypothesis was formulated by Lewis Thomas and MacFarlane Burnet [2,3]. The core of the cancer immunusurveillance hypothesis is based on the assumption that in each individual the immune system is responsible for eliminating precancerous or cancerous cells before these cells could indeed become a clinically apparent tumors. This idea was based on the observation that some cancer patients do not progress for prolonged period of time, and some even exhibit spontaneous regression. While this theory was mainly challenged in its infancy, in the 1990s it has regained favor thanks to some crucial murine experiments demonstrating that mice lacking either IFN-γ responsiveness or adaptive immunity were more susceptible to carcinogen-induced and spontaneous primary tumor formation [4,5]. Later on, many laboratories added similar findings, thus documenting that the immune system can function as an extrinsic tumor suppressor [reviewed in 6].

2.1.1 Cancer immunoediting

In 2001 it was described that the immune system, besides controlling tumor formation, can also shape the tumor to become less immunogenic [7,8]. This prompted a major revision of the *cancer immunosurveillance* hypothesis and posed the basis for *the cancer immunoediting* theory, which stresses the dual host-protective and tumor-promoting actions of immunity on developing tumors. This model proposes three distinct sequential phases: elimination, equilibrium and escape. The elimination phase looks like an updated version of cancer immunosurveillance. Various factors alert the immune system to the presence of the tumor. Among them are the so called “danger signals”, such as damage-associated molecular pattern (DAMP) molecules derived from dying cells [9]. They lead to the activation of innate

immune cells, which in turn coordinate the activation of the adaptive immune arm in order to destroy the tumor. The existence of this phase is supported by the fact that mice and human with deficient or suppressed adaptive immune response (RAG-2 KO mice, IFN-γ deficient mice, or immunosuppressed patients) display a greater incidence of tumor development [10 and reviewed in 6]. During "equilibrium" tumor cells and immune cells interact to induce reciprocal modifications. At this phase the immune system sculpts the immunogenicity of the tumor cells maintaining them in a functional state of dormancy [11] in the case of a protective immunity. However, at this stage the immune pressure may also lead to the outgrowth of tumors with more aggressive features and less sensitive to immune recognition [12]. Finally, the tumor itself may actively acquire the ability to circumvent immune recognition to an extent that it can escape from, and even suppress, the immune system. Indeed, immune evasion has been recently recognized as an emerging hallmark of cancer (**Figure 1**).

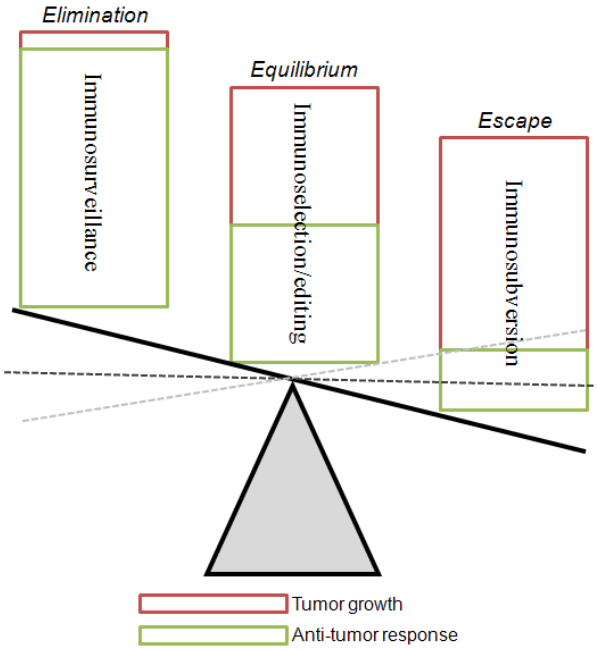

Figure 1. The balance of tumor elimination, tumor immunoediting and tumor immune escape

Immune evasion relies on the setting of both passive and active tolerizing condition [13]. At the tumor-cell-level (*passive tolerization*), mechanisms of escape might include: *i)* reduced immune recognition due to the loss of antigens or to defects in antigen processing and presentation [14,15]; *ii)* lack of susceptibility due to increased tumor-intrinsic-resistance, for example through the over-expression of anti-apoptotic molecules [16] or the up-regulation of immunosuppressive receptors that can directly kill T cells (FasL, TRAIL) [17] or preclude

their destruction by T cells [18]; *iii)* over-expression of “don’t eat me” signals (i.e. CD47) which inhibit the phagocytosis by macrophages.

In addition, tumor immune escape occurs also because severals mechanisms are actively operated by tumor cells to establish an immunosuppressive state both at the tumor site and systemically (*active tolerization*). In particular, this will be discussed in details in the next chapter.

2.1.2 Tumor-induced immunesuppression

Tumor immune escape is a complex process that relies on the establishment of an immunosuppressive state. Tumor cells can promote the development of such a state by secreting a myriad of immunosuppressive molecules (e.g. vascular endothelial growth factor (VEGF), transforming growth factor (TGF)β, Interleukin (IL)-10, prostaglandin E2 (PGE2) [19, 20, 17]. These factors are toxic for optimal cytotoxic effector T cell (CTL) functions as well as are crucial for the accumulation (through *de novo* induction and recruitment) of suppressive, tolerogenic and regulatory innate and adaptive immune cells that function both to suppress the anti-tumor function of CTL and to promote tumor dependent angiogenesis as well as tumor invasion and metastasis. Many of these secreted factors are expressed by many types of cancer and correlate with advanced disease stage. Moreover, we should also take into account that the expression of many tumor-promoting factors is not confined to tumor cells but they are also produced by by-stander immune cells recruited and activated by the tumor itself [21].

The major types of immunosuppressive leukocyte populations relevant to the topic of this thesis are regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). In the setting of human tumors, their main phenotypic features and functional activities are here summarized and discussed (**Figure 2).**

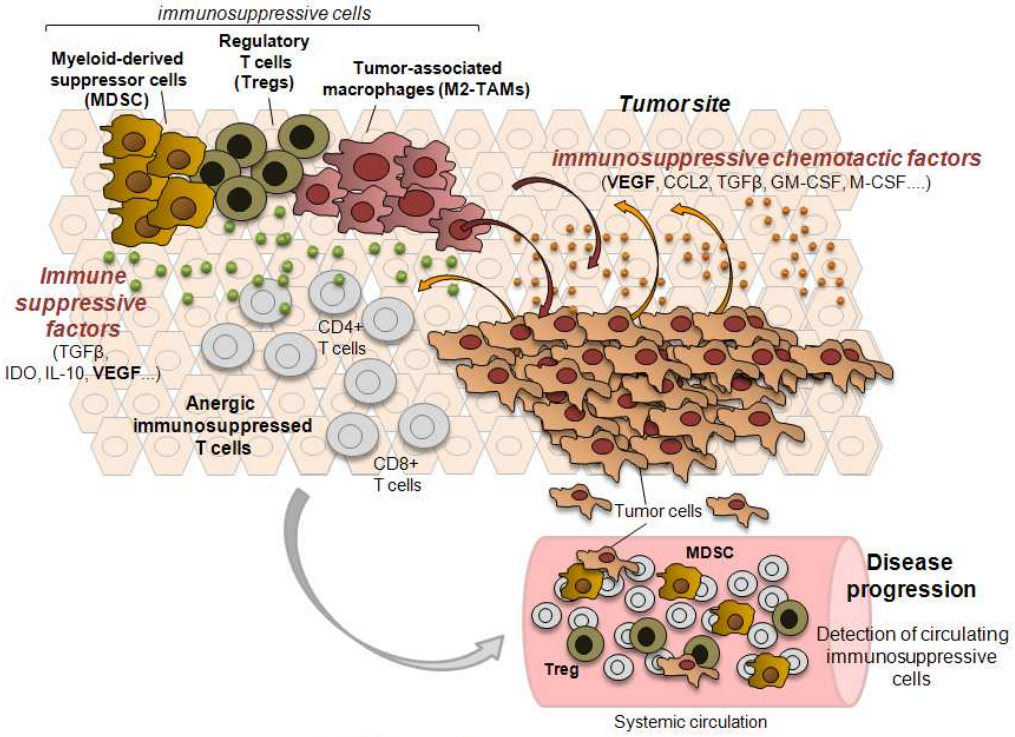

Figure 2. Local and systemic immune suppression

2.1.2.1 T cell dysfunction

Successful T cell activation requires several signals: interaction of the peptide-HLA (*human leukocyte antigen*, also known as major histocompatibility complex (MHC)) complex with the TCR of the right specificity (signal 1), co-stimulatory signals (signal 2), and optimally also the presence of immunogenic signals (signal 3), such as proinflammatory cytokines or "danger signals" (**see section 2.1.1**). Stimulation in the absence of co-stimulation will induce tolerance and anergy in the antigen-specific T cells, a mechanism to prevent auto-reactivity. Tumor-induced T cell anergy has been shown to affect both $CD8^+$ and $CD4^+$ T cells and occur in T cells that infiltrate tumors [22]. The presentation of antigen to a cognate T cell receptor (TCR) is the crucial point in the initiation of an immune response. It results in T cell activation and clonal expansion. It has been shown that in the context of tumor a T helper type 1 (Th1) response, characterized by T-bet (transcription factor that directs Th1 lineage commitment) and by the release of IFN-γ and IL-2 rather than a Th2 response, is necessary for immune-mediated tumor rejection. Yet, most of the clinically apparent tumors subvert the immune response such that T cells are dysfunctional, and selectively deficient in several of the activities necessary to generate a Th1 response, such as the production of IFN-γ [23-25]. Moreover, besides co-stimulatory signals (e.g. CD28) T cells express also co-inhibitory

receptors, such as programmed-death 1 (PD-1) and cytotoxic T lymphocyte activation marker 4 (CTLA-4). These molecules are up-regulated after T cell stimulation and they represent a physiological "immunologic brake" crucial for the control of an on-going immune response also in other physiological setting, such as anti-viral responses. CTLA-4 binds to CD80 and CD86 like CD28, but with higher affinity, and conveys negative signals that lead to reduce proliferation and cytokine production. PD-1 interacts with PD-L1 (B7-H1) and PD-L2 (B7-DC). The fact that deficiency in CTLA-4 as well as PD-1 is associated with severe autoimmune diseases illustrates the importance of this negative regulation of T cell function [26]. Of note, the tumor can make use of these inhibitory pathways to control anti-tumor immunity. Many type of tumors have been reported to express PD-L1 [18,27] and are therefore able to inhibit T cells [28]. Consequently, high expression of PD-1 on tumor infiltrating T cells or PD-L1 on tumor cells has been found to correlate with poor survival in cancer patients [29,30]. Importantly, many of the above cited mechanisms of T cell dysfunction occur at the tumor site where effective anti-tumor response takes place. Thus in order to exert their anti-tumor activities T cells should be able to successfully transmigrate through the tumor endothelial barrier. However, several tumor types have developed a number of unique ways to prevent homing of effector T cells to the tumor site. Among those mechanisms, deregulation of chemokine loops (reduction of T-cell attracting chemokines, such as CCL2, CCL5, CXCL10, CXCL11) [31] and the prohibitive/suppressive nature of the tumor endothelium [32,33] have been described. Thus, lack of homing of T cells represents itself a T cell dysfunction phenomenon.

2.1.2.2 Regulatory T cells (Tregs)

Tregs are a subset of T lymphocytes that in humans represent less than the 10% of circulating CD4+ T cells. The high expression of the surface marker CD25 and the positivity for the intracellular transcription factor forkhead box P3 (FOXP3) are cardinal phenotypic features of these cells. CD25 is also known as IL-2 receptor subunit α (IL-2Rα), and FOXP3, is considered the lineage specific factor for this T cell subset. The crucial role played by FOXP3 in Treg fate determination and immune homeostasis, is strikingly evident in patients with FOXP3 mutations. In fact, these patients develop severe autoimmune disease [34]. Another characteristic of Tregs is their potent suppressive capacity [35]. Multi-parametric flow cytometry analysis is required to identify Treg *ex vivo*. In this context, we also contributed in

suggesting guidelines for an accurate *ex vivo* identification of human Tregs [36].
A great number of publications have shown that Tregs are increased in many different human cancers and often correlate with poor prognosis [reviewed in 37]. Moreover, Tregs express receptors for chemokines such as CCR4, CXCR4 and CCR10 that could induce their migration towards the tumor [38, 39]. Indeed, in cancer patients, an increased Treg/Tconv (conventional T cells) and Treg/CD8 T cell ratios is often observed in the blood [38, 40], in tumor draining lymph nodes [38], and in the tumor [38, 41]. Besides Tregs infiltration into the tumor, the accumulation of Tregs at tumor sites may be due to the conversion of $CD4^+$ TILs into Tregs, to the selective expansion of Tregs displaying a survival advantage in the hypoxic tumor microenvironment (TME), and finally the proliferation of pre-existing Tregs. Several lines of evidence clearly documented the role of Tregs in restraining anti-tumor immune responses. Suppression exerted by Tregs might mask antigen-specific responses that become detectable only after Treg depletion [42].
Many strategies have been used to manipulate Tregs, including: Treg depletion, inhibition of Treg function or blockade of Treg trafficking into lymph nodes or tumors [36].

2.1.2.3 Myeloid-derived suppressor cells (MDSCs)

A large number of studies attest to the remarkable plasticity of the myeloid lineage [43]; tumors take advantage of this plasticity to re-direct myeloid differentiation toward the acquisition of immune suppressive subsets that effectively interfere with the anti-tumor immunity. This is the case of MDSCs and TAMs.

MDSC are a heterogeneous population of variably immature myeloid cells (IMCs) with suppressive activity, containing myeloid progenitor cells and precursors of granulocytes, macrophages and DC. Elevated levels of MDSC have been reported in the blood of cancer patients bearing several types of tumors and they seem to represent a major contributor to cancer-related immune suppression [21]. In cancer patients, increased MDSCs are translated in inhibition of autologous T cell proliferation and IFN-γ production. Definitively, their peripheral blood accumulation has been correlated with tumor progression (tumor stage and burden) and poor prognosis [44-46]. The heterogeneity of MDSC in human malignancies is striking, thus for their characterization, as for Tregs, the application of multi-parametric flow cytometry approach is mandatory. Indeed, a great number of MDSC phenotypes has been

described in many different human cancers. Some of these phenotypes overlap, at least partially, while others are mutually exclusive. Agreement in the scientific community indicates three main subsets of MDSC [reviewed in 47,48]. **Table 1** summarized MDSC phenotype described in patients with different tumor histologies. The majority of human MDSC subtypes expresses common myeloid markers, such as CD11b and CD33, but have low or absent expression of the MHC class II molecule HLA-DR. Many reports described MDSC as $CD33^{+}HLADR^{-/low}$ and Lineage (Lin) negative, meaning that they do not express CD3, CD19, CD56 and CD14, markers characteristic of T, B or NK cells and monocytes, respectively.

These myeloid precursors, although might appear to be granulocytic-like, they are defined as *lineage-negative MDSC* as long as they do not express the CD15 granulocytic marker ($CD33^{+}Lin^{-}HLA\text{-}DR^{-}CD15^{-}$) [44, 65, 70]. Furthermore, a number of studies have detected the expression of the granulocytic markers CD15 or CD66b in $Lin^{-}CD33^{+}HLADR^{-/low}$ cells, indicating that these populations partially overlap [23, 61, 71-73].

Disease type	Phenotype	Ref.
Monocytic MDSC (mMDSC)		
Melanoma	$CD14^{+}HLADR^{neg/low}$	[49]
		[50]
	$CD14^{+}IL4R\alpha^{+}$	[51]
RCC	$CD14^{+}HLADR^{neg/low}$	[52]
Colon carcinoma	$CD14^{+}IL4R\alpha^{+}$	[51]
HNSCC	$CD14^{+}$	[53]
Multiple myeloma	$CD14^{+}HLADR^{neg/low}$	[54]
	$CD14^{+}$	[53]
HCC	$CD14^{+}HLADR^{neg/low}$	[55]
Prostate Cancer	$CD14^{+}HLADR^{neg/low}$	[56]
T Cell NHL	$CD14^{+}HLADR^{-}B7\text{-}H1^{+}$	[57]
Granulocytic MDSC (gMDSC) and *Lineage-negative MDSC*		
RCC	$Lin^{-}HLADR^{-}CD33^{+}$	[58]
		[59]
	$CD33^{+}HLADR^{-}$	[23]
	$CD11b^{+}CD14^{-}CD15^{+}$	[60]
		[61]
	CD15+CD14-	[23]
NSCLC	$CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$	[62]
Melanoma	$Lin^{-}HLADR^{-}CD33^{+}$	[63]
	$CD15^{+}IL4R\alpha^{+}$	[51]
Colon carcinoma	$CD15^{+}IL4R\alpha^{+}$	[51]
	$CD15^{+}$granulocytes	[64]
HNSCC	$Lin^{-}HLADR^{-}$	[65]
	$CD11b^{+}CD14^{-}CD33^{+}$	[66]
		[67]
	$SSChiCD66b^{+}$	[68]
Breast carcinoma	$Lin^{-/low}HLADR^{-}CD33^{+}CD11b^{+}$	[44]
	$CD15^{+}$granulocytes	[64]
Pancreatic carcinoma	$CD15^{+}$granulocytes	[64]
Lung carcinoma	$CD11b^{+}CD33^{+}$	[69]

Table 1. Adapted from Filipazzi et al, 2012

These polymorphonuclear (PMN)/granulocytic-MDSC (hereafter called *gMDSC*) have been suggested to be activated (i.e. "suppressive") neutrophils distinct from their normal counterparts. While mature PMN are short-live, and mostly excluded when peripheral blood mononuclear cells (PMBC) are isolated by gradient centrifugation, gMDSC have a low density phenotype allowing to co-purify with PBMC [61, 64, 68], and have increase resistance to apoptosis [61, 68]. Moreover, according to what described by our group in advanced melanoma patients [49], and then by others in several cancer hystologies [45, 46, 50, 55, 74, 75] a third MDSC can be defined: $CD11b^{+}CD14^{+}HLADR^{-/low}$ monocytic-MDSC (*mMDSC*). They resemble monocytes in size and light scatter characteristics as well as express the CD14 monocytic marker. Interestingly, their presence across different human cancers, strongly suggest that the ability to affect myeloid differentiation towards immature and defective monocytes might be a common feature of human tumors. Interestingly, in human cancer these mMDSC have a prognostic significance. In cancer of different hystologies, the frequency of this MDSC subset in the blood correlates with tumor burden [46,50] and is inversely associated with the response to cancer vaccines [49,74], reinforcing their detrimental effect in tumor immunity.

Moreover, Weide and colleagues recently reported in melanoma patients a "triple-correlation" between high levels of mMDSC, the absence of antigen-specific T cells and poor clinical prognosis, thus suggesting a causal relationship where mMDSC counteract the development of tumor-reactive T cells [45]. Regarding the overall MDSC phenotype, additional and more specific molecules have been reported as defining human MDSC. Similar to murine studies [76] the IL4Rα (CD124, the receptor for Interleukin-4) has been suggested as a specific marker for tumor-derived MDSC with suppressor function [51]. As far from our experience with melanoma MDSC, we could not detect $IL4R\alpha^{+}$ cells either in the monocytic or in the granulocytic subpopulations (*Tazzari M, unpublished observation*). VEGFR1 is another marker, which has recently been described in renal cancer patients to be expressed in gMDSC [61]. Surprisingly, a recent paper showed that mMDSC from melanoma patients could express even markers of more mature myeloid cells, namely CD80 and CD83 [50]. This large amount of novel candidate markers further emphasizes the complexity in defining these cells. In cancer, one common denominator of these cells is their reliance on tumor-derived factors. In healthy individuals, bone marrow-derived IMCs will distribute throughout the body and differentiate into macrophage, granulocytes or DC. Instead, the presence of tumor-derived soluble mediators arrests IMCs in their immature state, leading to

MDSC expansion and activation. Evidence supporting this conclusion derives from studies revealing a decline of circulating MDSC after surgical resection of tumors [46], and by *in vitro* experiments which showed that culturing normal neutrophils and/or monocytes with conditioned medium from melanoma, renal cell carcinoma (RCC) cells and other solid cancers prevented their differentiation, thus leading to MDSC generation [77, 78]. Indeed, these studies, although limited to the *in vitro* setting, underline the crucial role played by cancer cells in MDSC recruitment/activation, again pointing to the TME as a crucial site where the most immune dysfunctions involving the myeloid compartment stem from. However, compare to mouse studies, gaining such *in situ* data in cancer patients is obviously challenging. The number of potential MDSC-inducing and MDSC-activating factors is large, including VEGF, IL-6, PGE_2, IL-1β, stem cell factor (SCF), macrophage- and granulocyte-macrophage-colony stimulating factors (M-CSF and GM-CSF) [49] IL-4, IL-13 and TGFβ. Clearly, the secretory profile of each tumor is diverse, which can help to explain the heterogeneous appearance of MDSC induced by cancers of different hystologies. Thus, the prevalence of one MDSC subsets to another in a given tumor histology, might be the result of the pressure exerted by the tumor cells able to skew/shape the MDSC composition and function. Noticeably, in melanoma and in soft tissue sarcoma lesions, sharing the same mesenchymal origin, cells expressing monocyte/macrophage markers are quantitatively predominant, while granulocytes are rarely detected (see **manuscript I**). In contrast, gMDSC represent the predominant population in renal cancer [23, 60, 61], in glioblastoma [73], bladder cancer [72], and advanced-stage non-small cell lung cancer [62]. Moreover, it is also likely that multiple populations are present in the PBMC of patients with a single type of tumor. Of note, the majority of the tumor-derived factors implicated in MDSC expansion ultimately converges in a common signalling pathway, the Janus tyrosine kinase (JAK) protein family members and signal transducer and activator of transcription 3 (STAT3), involved in cell survival, proliferation, differentiation and apoptosis [79]. STAT3 is a member of the STAT family of transcription factors [80]. STAT3 is constitutively activated in many tumor cells, and thereby it contributes to tumor cell survival and proliferation. In cancer cells STAT3 activation promotes the production of immunosuppressive molecules which down-regulate the immune response. Moreover, increased levels of phosphorylated STAT3 has been observed in MDSCs from tumor-bearing mice [81] and more recently it has been confirmed in mMDSC from melanoma patients as well [50]. It appears that persistent activation of STAT3 in myeloid progenitors prevents their differentiation and increase their

proliferation and survival, possibly through up-regulation of STAT3-targeted genes like Bcl-xL, cyclin D1, c-myc, survivin [79]. Moreover, over-expression of a constitutively active form of STAT3 has been proven to increase the MDSC-mediated suppression of T cell activation, while its *in vitro* pharmacological inhibition facilitates the differentiation of MDSC in myeloid cells without suppressive activity [50, 82]. MDSC exert their suppressive activities by a variety of different mechanisms. Factors implicated in the suppression of T cell functions [21] include reactive oxygen species (ROS) production, L-arginine depletion by arginase I [60, 61], TGFβ secretion [49], depletion of cysteine, upregulation of cyclo-oxigenase 2 (COX2) and PGE2, induction of Tregs [55]. Each of these functions has been primarily linked to a certain MDSC subtype, although they might use more than a single mechanism at once. gMDSCs, have been reported by many to inhibit T cell functions predominately via L-arginine depletion, consequently associated with an increased levels of plasma arginase activity [60, 61]. In addition to impair autologous T cell proliferation and IFN-γ production in response to TCR triggering, MDSC can block T cell activation by inhibiting T cell trafficking to antigen-containing sites (i.e. draining lymph nodes and tumor tissues) [83]. There is also evidence that MDSC are involved in a whole array of non-immunological functions, such as promotion of angiogenesis, tumor local invasion and metastases [21].

2.1.2.4 Tumor-associated macrophages (TAMs)

Macrophages play an essential role in innate immunity and are involved in a variety of immune functions, including host defence and wound healing. They are mature-tissue resident myeloid cells derived from circulating monocytes.

During the course of an immune response macrophages become activated and, depending on the cytokine network they encounter, become either highly effective in destroying potential pathogens and activating the adaptive immune system, or become attenuators of the inflammatory response. These two functional states are classified as M1 or M2 polarization, a nomenclature reflecting the Th1/Th2 dichotomy [84]. M1 or “classically” activated macrophages are induced by inflammatory stimuli and danger signals, such as lipopolysaccharide (LPS) and IFN-γ. When activated, they express HLA-DR molecules at high level and release proinflammatory cytokines (e.g. IL-1β, IL-12 and tumor necrosis factor (TNF)-α). They have an enhanced ability to present antigens and promote the

differentiation of naïve $CD4^+$ T cells into Th1 effector cells. By contrast, M2 or "alternatively" activated macrophages, which are activated by IL-4, IL-10 and IL-13 stimulate $CD4^+$ Th2 cell and Treg differentiation and are characterized by higher production of the anti-inflammatory cytokine, IL-10 [85]. However, it stands clear that the M1/M2 paradigm might be too semplicistic and that a broad range of phenotypes that are in between the two extremes M1 and M2 are more likely to occur *in vivo*. In fact, TAMs have been shown to display a high degree of heterogeneity and functional plasticity and their activation state is primarily dictated by the soluble factors present within the local microenvironment produced either directly by tumor or [24], by intratumoral T helper-cells [86].

In cancer, TAMs are considered to be a tumor-induced type of M2-polarized macrophages. TAMs are not only ineffective as antigen-presenting cells but they also exert pro-tumor functions by the direct release of various immunosuppressive factors (while producing low levels of M1 mediators). M2-derived factors support tumor cell resistance to apoptotic stimuli (e.g. protection of tumor cells from chemotherapy-induced apoptosis [87]) and stimulate the proliferation and invasion of malignant cells. Increased TAMs density is usually associated with advanced progression and poor prognosis in multiple human epithelial malignancies, including breast, prostate, endometrial, kidney, bladder and anaplastic thyroid carcinomas [88, 89 and reviewed in 90]. In contrast, accumulation of TAMs remains less well characterized in mesenchymal tumors, such as sarcomas [91].

At the tumor site TAMs certainly derive from the local differentiation of monocytes actively recruited by tumor-derived cytokines/growth factors such as VEGF, M-CSF and CCL2. Moreover, a relationship between MDSC and TAMs has been also suggested, at least in a mouse model [67]. In addition to monocytes, in a murine study, circulating MDSCs have been shown to be a plausible precursor of TAMs.

In situ characterization of macrophages is an important issue and a precise characterization of tumor-infiltrating myeloid cells requires the use of immunohistochemical technique (IHC). CD68 and CD163 in combination with the specific monocyte/macrophage marker CD14 are used to identify and quantify macrophages in tissue sections. These two markers are not equivalent in the identification of macrophages [88, 89, 92,93]. Indeed, CD163 is a hemaglobin scavanger receptor expressed on most subpopulations of mature tissue macrophages [94]. It is believed to be mainly associated with M2 macrophages, owing to its upregulation by anti-inflammatory cytokines (IL-4, IL-10) important for M2 polarization *in vitro*. Thus, $CD163^+$ cells have been recognized by many to identify *in situ* TAMs [93]. In contrast, CD68, in association with the HLA-DR maturation marker, has been used to

identify a tumoricidal macrophage subsets, more associated with immunostimolatory properties [88,89]. Furthemore, although CD163 in IHC studies has been pinpointed as a specific macrophage marker, a minor subset of $CD34^+$ stem/progenitor cells [95] and MDSC [96] are found to express CD163 in flow cytometry analysis. Thus, it cannot be fully excluded that a minor subsets of the $CD163^+$ cells are IMCs.

Beside their phenotypic diversity, macrophages adopt different geometries in vivo. Within a tumor, it has been recently noted that TAMs might adopt a *ramified morphology* forming a network in close contact with cancer cells and blood vessels, in contrast to the absence of ramification and the *ameboid shape* of M1 macrophages [88]. Moreover, a recent in vitro study confirms this point showing that elongation itself enhances macrophage polarization towards the M2 phenotype [97].

Their abundant presence and contribution to tumor progression as well as their plasticity has prompted researchers to develop therapeutic agents that specifically target or "re-educate" these cells. These efforts might be reassumed in three main goals: *i*) inhibition of monocytes recruitment into tumors; *ii*) depletion of TAMs; *iii*) neutralization of TAM-derived molecules. Moreover a more recent approach consists in repolarizing TAMs into M1 macrophages, able to exert anti-tumor responses protective for the host.

2.1.3 The immune contexture in human tumors

It is now accepted that tumors cannot be simply considered as formed only by neoplastic cells. Tumors are 'aberrant organs', made up by different cell types and components; these include epithelial cells, vascular and lymphatic vessels, and immune cells. All these cell subsets are connected to each other by reciprocal cross-talk and altogether, they compose the TME. Histopathological analyses of human tumors have provided evidence that variable numbers of infiltrating immune cells are found in different tumors. Moreover, this large collection of *in situ* data has allowed the identification of components of the TME that are beneficial, as well as those that are deleterious, to patients' prognosis. Thus is clearly emerging the concept that anti-cancer immunity is indeed a dymamic equilibrium in which each subset of the immune sytem, exerting pro or anti-tumor activity, can be contemporarily present at the tumor site. For example, chronic inflammation and the presence of MDSC or M2 type macrophages favor tumor growth and spreading in most cancer types [98]. While, in contrast infiltrating lymphocytes with a Th1 polarization are strongly associated with good outcome. Indeed, tumor-infiltrating lymphocytes (TILs), their density and localization inside

the tumor nest as well as their functional polarization is a strong independent prognostic factors in different type of cancers [reviewed in 99,100]. Efforts in the scientific community are currently on-going to construct precise algoritsm for defining an 'immunescore' to be used then as prognostic value [101].

2.1.4 Immunotherapy in cancer

In advance stages, when tumors have escaped immune control, immunotherapy approaches are designed to enhance anti-tumor T cell reactivity and thus reverte the relation between immune system and tumor cell growth to a phase of *equilibrium* (**Figure 3**).

This goal can be achieved by actively boosting the positive anti-tumor response and by counteracting the immune suppression. Cancer cells express a variety of antigens that are able to trigger the host immune response. Among these are the so-called Tumor-Associated (self) Antigens (TAAs), such as melanocyte differentiation antigens (gp100/HMB45, Melan-A/MART-1).

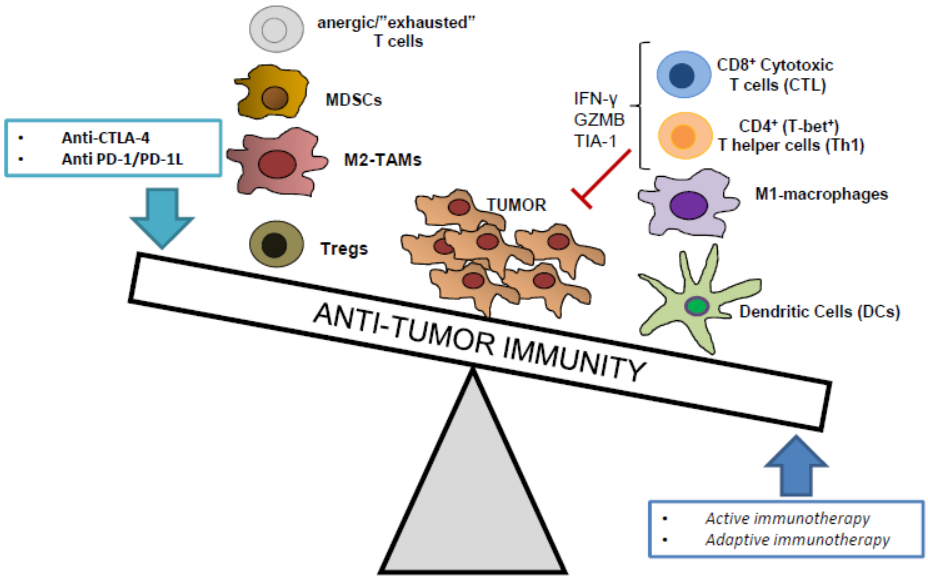

Figure 3. Restoration of tumor equilibrium by immunotherapy

Moreover, tumors are characterized by the accumulation of genetic alterations that lead to the generation of neoantigen-containing epitopes (Tumor-Specific (non-self) Antigens (TSAs)) that can be recognized by T cells [102]. Many different strategies of immunotherapy have been developed over the past that include: *i)* vaccine-based strategies [103]; *ii)* cytokine therapies [104], both belonging to *active immunotherapy*; *iii)* approaches involving adoptive transfer of in vitro expanded, naturally arising, or genetically engineered tumor-specific

lymphocytes [105] that belong to *adaptive immunotherapy*. One of the mechanism by which tumors disable immune response relies on antigen persistence and chronic antigenic stimulation. This situation leads to a overstimulation of T cells that thus became anergic and overexpress the inhibitory receptors CTLA-4 and PD-1. Monoclonal antibodies (mAbs) directed to these molecules (anti-CTLA-4/Ipilimumab or anti-PD-1/Nivolumab) release immune effector T cells from their natural restraints and appeared to be a very fruitful intervention for boosting anti-tumor immunity in the clinical setting [106,107]. In addition, the development of successful immunotherapy is likely to depend on identifying *dominant immune suppressive mechanism* in a given tumor type, allowing to design rational combinatorial approaches. Ideal candidates for "immunosensitizing drugs" would be for example those able to reduce frequency and function of immunoregulatory cells (e.g. MDSC, Tregs) or those that will enhance T cell trafficking and infiltration into the tumor bed.

2.2 Targeted-therapy molecules

Malignant transformation is characterized by alteration in the intracellular signalling pathways that regulate cell proliferation, survival, differentiation and metabolism. Key components in the activation of such pathway are protein kinases that upon the phosphorylation of target molecules, induces signalling cascades that culminate in the activation of gene transcription and modulation of protein expression or function. In the past decade, improvement in the knowledge of the transformation process have allowed the design of "molecular targeting" therapeutic approaches that from a clinical standpoint have represented a new weapon beyond aspecific cytotoxic agents (radiation/chemotherapy). While chemotherapeutic agents interfere with DNA synthesis, or produce chemical damage to DNA, targeted-therapy molecules activity relies on the inhibition of those molecular events responsible for the maintenance of the malignant phenotype. Based on this, specific tyrosine kinase inhibitors (TKIs) have been developed and demonstrated to have significant antitumor efficacy. Functionally, TKs can be classified into receptor kinases (i.e. receptors of growth factor that regulate cell behaviour in response to extracellular stimuli) and non-receptor kinases (i.e. those involved in intracellular signalling that are frequently downstream of RTKs). The modifications that promote TK-mediated malignant transformation are diverse (e.g. mutations in the catalytic domain, chromosomal recombination that modulate the catalytic activity as well as RTK abnormal activation due to tumor-derived overexpression of the ligand). The first TKI approved by the US Food and Drug Administration (FDA) was the

BCR-ABL inhibitor imatinib mesylate (ST1571; Glivec, Novartis; hereafter indicate as imatinib) for the treatment of Philadelphia chromosome positive chronic myeloid leukemia (CML) [108]. Of note, this compound also efficiently inhibits TKs other than BCR-ABL, including c-KIT, PDGFR, M-CSF receptor and the fms-like tyrosine kinase-protein kinase 3 (FLT3). This has led to its approval also for c-KIT$^+$ unresectable metastatic gastrointestinal stromal tumors (GISTs) [109] and dermatofibrosarcoma protuberans (DFSP) patients [110]. Owing to the clinical success of imatinib in CML patients several others targeted-drugs have been developed. It is now widely accepted that tumor neo-angiogenesis is a crucial step in tumor development and progression [111]. Indeed, among the class of targeted-therapy molecules a special place is deserved to anti-angiogenic drugs [112]. Among them, Bevacizumab (Avastin, Genentech/Roche), a VEGF-trapping monoclonal antibody [113]; and many RTKIs, such as sorafenib (BAY43-9006, Nexavar, Bayer) [114], sunitinib malate (SU11248; Sutent, Pfizer, hereafter indicate as sunitinib) [115] pazopanib (GW-786034, Votrient; GlaxoSmithKline) [116] and cediranib (AZD2171, Recentin; AstraZeneca) [117] have been approved by FDA for clinical use. Currently, there are numerous clinical trials as well as off-label medical applications testing the use of these drugs in different tumors, and many others antiangiogenic TKIs are being clinically evaluated (e.g. axitinib). Often patients develop resistance to TKIs, which might be caused by a tumor cell intrinsic-adaptation, such as the acquisition of secondary genetic alterations or the activation of alternative signalling pathways [118]. Understanding the molecular mechanisms by which cancer patients develop resistance to TKIs is crucial and is a challenge for achieving long-term disease control.

2.2.1 Immunostimulation by anticancer drugs

Considering the intense interplay of the different cells composing the TME **(see previous paragraphs**), it is not surprising that effects delivered to tumor cells may impact normal host cells, especially those of the immune system. Data obtained both in some human clinical settings and in animal models, strongly demonstrated that conventional chemo and radiotherapy treatment, as well as TKIs possess immuno-modulating activities [119]. These by-stander immune related effects stem from their capacity to affect *directly* the signalling pathways regulating the functional activities or the maturation/differentiation programs of immune cells and/or from their ability to modulate the immune-related features of cancer cells. Imatinib represents the paradigm of this double activity. It directly induces the host DCs to promote NK activation, and this immunological effect was associated with prolonged

disease free survival in imatinib-treated GIST patients [120]; on the other hand, it reduces the release of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) by tumor cells [121]. Some cytotoxic drugs (e.g. oxaliplatin, doxorubicin, cyclophosphamide) have been shown to affect the immune system by inducing the immunogenic cell death of tumor cells [122, 123]. Thus, dying tumor cells release/exposure specific signals (i.e. calreticulin (CRT), high mobility group box 1 (HMGB1), ATP) which trigger phagocytosis and promote the maturation of dendritic cells, initial events for the induction of a protective immunity. Anticancer agents can also favor anti-tumor immunity by increasing the expression or presentation of TAAs by cancer cells, as demonstrated for vemurafenib, a specific BRAF inhibitor approved for the treatment of melanoma [124]. On the other hand, anticancer drugs subvert tumor-induced immunosuppression or exert a stimulatory effect on immune effector cells. Both MDSC and TAMs represent interesting therapeutic targets and some of the currently anti-cancer therapies actively modulate myeloid cell functions and frequency. For instance, gemcitabine reduces the levels of MDSC in patients with advanced pancreatic cancer [125]. Moreover, some cytotoxic drugs may also actively stimulate the effector arms of the immune systems. Indeed, immune monitoring of patients with advanced non-small cell lung cancer revealed that treatment with paclitaxel [126] restored Th1 cellular immunity (i.e. IFN-γ and IL-2 secreting $CD4^+$ T cells).

2.2.1.1 *A focus on anti-angiogenic drugs*

At the tumor site, neo-angiogenesis is promoted by pro-angiogenic tumor-derived factors (in particular VEGF) and results in the formation of new, highly abnormal blood vessels displaying a heterogeneous distribution, irregular blood flow and increased permeability [111]. Sunitinib is an orally bioavailable multi-target TKI that inhibits a broad array of RTKs. Indeed, beyond VEGFR-1 and -2, it also targets c-KIT, PDGFRβ, FLT3 and RET [127].
Anti-angiogenic therapies were developed to inhibit new blood vessel growth and thus starve tumors. However, besides to its well-characterized role in angiogenesis, VEGF may: *i)* promote Treg proliferation [128], *ii)* inhibit the maturation and function of DCs [129], *iii)* stimulate MDSCs accumulation [130]. Indeed, drugs inhibiting VEGF-mediated signalling, such as sorafenib and sunitinib, have been shown to affect the balance of these cell subsets and impact the anti-tumor immune response. In mouse models and in RCC patients sunitinib reduces the frequency of circulating Tregs and different subsets of MDSCs [25, 131]. Indeed,

mMDSC as well as MDSC defined as, $CD33^{+}HLA\text{-}DR^{-}$ and $CD15^{+}CD14^{-}$ are downmodulated in the blood of RCC receiving sunitinib treatment [23]. Furthermore, van Crujsen and colleagues showed, in a subset of RCC patients experiencing tumor regression, that sunitinib induced the reacquisition of a normal frequency of $CD1c^{+}$ myeloid DC [52]. Due to the singularity of each immunosuppressive network put in play by each cancer histology (see **section 2.1.2**) the raising question is: "Are these effects confined to RCC patients or they represent a general phenomenon?" Moreover, pro-angiogenic factors and abnormal tumor vasculature strongly limits the leukocyte-endothelial interaction and the subsequent extravasation of effector cells into the tumor site [132]. Conversely, anti-angiogenic treatments, while normalizing blood vessels, enhance immune infiltration, as was recently shown in different animal models [133,134].

Altogether, the evidence summarized in these two last paragraphs, strongly indicate that part of the clinical efficacy of many anticancer agents relies on restoring an active anti-tumor immunity. Of interest, in a murine breast cancer model Huang Y. and colleagues recently showed that the efficacy of a cancer vaccine therapy was greatly increased by antiangiogenic treatment that, at the tumor site, re-directed TAMs to an immuno-supportive M1-like phenotype [135]. Studies that analysed the modulation of the immune contexture at the tumor site induced by TKIs are still few, especially considering the wide application of these targeted-drugs in tumors of different histology. In this respect, the only documented example is reported in melanoma patients in which tumors surgically removed after short-term treatment with vemurafenib, clearly displayed enhanced infiltration with activated $CD4^{+}$ and $CD8^{+}$ T lymphocytes [136]. Thus, in the human setting, the ability to shape the immune cell repertoire at tumor site needs to be further address and hold interesting promise for the development of strategies that combine TKIs with immunotherapeutic approaches.

2.3 Soft Tissue Sarcomas and the immune system

Soft tissue sarcomas (STS) are a sundry group of solid tumors that till recently were traditionally categorized together based on their mesenchymal origin. However, inside STS, current studies are now considering each single histology as a separate entity with unique biological and clinical features [137]. The Fondazione IRCCS Istituto Nazionale Tumori, Milan has a long-standing interest in different STS subtypes and it is among the major referral centre in Italy for these rare diseases. Immunohistochemical and molecular biology studies are necessary for their adequate characterization; however, biological behavior,

staging and grading are essential for an accurate prognosis and for planning the most adequate therapy. The mainstay of treatment for localized STS is surgery, also applied after radio or chemotherapy treatment (adjuvant setting). Of note, some STS patients, can be made virtually disease free (i.e. by surgery), but are known to be a high risk for relapse. Metastasis can also occur, with lung being the most frequent site of dissemination. STS are mainly insensitive to the most applied chemotherapy regimens (anthracyclines and isosfamide). In the last years, great advances have been made in the understanding of sarcomas' molecular biology [138] leading to the testing of new targeted-compounds in order to improve efficacy and outcome achieved with classical drugs. A number of individual sarcoma subtypes responds to TKIs that inhibit both VEGFR and PDGFR. While all these drugs have achieved relative success in aggressive metastatic STS, they have failed to cure patients, and the clinical responses can be short-lived, due to the occurrence of resistance. Further options for the treatment of sarcoma are needed, not only to improve the rate of response to treatment, but also to improve the duration of elicited responses and disease stabilization. In an attempt to improve response rates, one of the strategies that are currently ongoing is the combination treatment with TKIs and conventional cytotoxic drugs **[**139], however these approaches are associated with an increased risk of toxicity (e.g. cardiac toxicity). Thus, the discovery of new safer synergistic combination is essential.

As opposite for other human tumors, including carcinomas and tumors of mesenchymal origin such as melanoma, for STS very little is still known on the role of the immune system in disease progression and in the response to treatment as well. So far, few studies have examined the systemic and local immune status in selected STS subtypes. However, the immunological behaviour of STS is now gaining interest in the scientific community and a very recent study described the impact of radiotherapy on the quality of the tumor-associated immune infiltrate in a cohort of heterogeneous sarcoma patients. Interestingly, radiotherapy induced the *in situ* accumulation of cells and molecules characteristic of a protective immunity [140]. Studies at the tumor site in STS are of course hampered by the paucity of cases. Indeed, the TME's role as a non-neoplastic component of tumor has been studies extensively in carcinoma but remains very poorly characterized in sarcomas. Lee CH and colleagues, showed in leiomyosarcoma (LMS) that a high density of TAMs ($CD163^{+}$), likely attracted to the primary tumor site by secretion of M-CSF by tumor cells [141], predict poor patient outcome [91]. Moreover LMS tumor cells have been shown to express the anti-phagocytic molecule CD47 and the potentially anti-tumor efficacy of targeting this pathway have been demonstrated [142]. In these last years, few studies have discussed the utility of

immunotherapy approaches in STS [143]. Of note, many STS are characterized by tumor-specific chromosomal translocations, which produce neo-antigens that might be seen as foreign by the immune system [144]. Moreover, other STS display cancer-testis antigens aberrantly [145] and other express differentiated antigens in common with melanoma [146]. Overall, these antigens could represent an attractive target for immunotherapy. However a better understanding of the meachanisms of tumor-induced immunesubversion in individual STS is needed to design combination treatment that includes targeted drugs and immunetherapy. Hereafter I will introduce the three STS subtypes focus of the present thesis.

2.3.1 SOLITARY FIBROUS TUMOR (SFT)

Solitary fibrous tumor (SFT) is a rare STS, characterized by CD34 positive fibroblastic appearing tumor cells. It can occur in several anatomical sites: meninges, pleura, peritoneum, extremities and viscera; most frequently in middle-aged patients. Only very recently, Robinson and colleagues found recurrent fusion transcripts in SFTs caused by inversion at chromosome 12q13 involving NAB2 and STAT6, adjacent genes normally transcribed in opposite directions [147,148]. Normally, NAB2 is an endogenous inhibitor of EGR1, but in the fusion gene NAB2 loses its repressor domain and gains the transcriptional activation of STAT6. Thus, the NAB2-STAT6 fusion protein acts to induce expression of EGR1 targeted genes. While most SFTs have an indolent course and can be cured by surgery, 15-20% of SFTs progress with either local recurrence or distant metastases [149,150]. In addition to the classical SFT (CSFT), two more aggressive clinical-pathological variants of SFTs are currently recognized: malignant (MSFT) and dedifferentiated (DSFT), the latter showing a higher metastatic rate [151,152]. While the standard treatment for CSFT and localized disease is surgery, medical therapy is needed in case of locally advanced or metastatic M/DSFT. New agents are currently under evaluation, along with radiotherapy and cytotoxic chemotherapy. Chemotherapy has been mainly described to be ineffective, but of note dacarbazine have recently reported to be active in patients with progressive pre-treated advanced SFTs [153]. Involvement of the platelet derived growth factor receptor (PDGFR) β and vascular growth factor receptor 2 (VEGFR-2) pathways were reported in SFTs [154,155]. In line with this, sunitinib has been recently reported to exert anti-tumor activity in unresectable, progressive M/DSFT patients [154,155]. Moreover, in the clinical setting, preliminary data point to the potential antitumor activity of other antiangiogenic agents like sorafenib, pazopanib, and

bevacizumab combined with temozolomide [156]. Interestingly, in the larger retrospective series of M/DSFT patients receiving sunitinib [155], the levels of activation of the direct targets of this agent, PDGFRβ and/or VEGFR-2, as evaluated by IHC in tumor lesions, did not fully account for the response to treatment. Moreover, very recently, in a xenograft model of DSFT, sunitinib monotherapy was found to have low efficacy [153]. These observations strongly supported the hypothesis that additional off-target mechanisms may sustain the activity of sunitinib in this clinical setting.

2.3.2 CLEAR CELL SARCOMA (CCS)

Clear Cell Sarcoma (CCS) is a very rare and aggressive soft tissue sarcoma (STS), usually arising from deep soft tissue or viscera [157], and marked by a very high metastatic risk resulting in a 5-year overall survival of about 50% [158, 159]. In contrast with other STS, and similarly to melanoma, its metastatic sites include lymph nodes. CCS, initially named, malignant melanoma of soft parts, are molecularly characterized in most cases by a specific translocation t(12;22)(q13;q12), which results in fusion of the Ewing's sarcoma gene, EWS, with the cyclic AMP (cAMP) regulated transcription factor, ATF1, a member of the cAMP-responsive element binding protein (CREB) family [160]. The EWS-ATF1 chimeric fusion protein interacts with the MITF (melanocyte master transcription factor) promoter, thus it directly and aberrantly activates MITF expression. Consequently, CCS is characterized by the expression of the melanocytic differentiation markers HMB45/gp100 and Melan-A/MART-1 [146]. Overall, several immunophenotypic and molecular features are shared between CCS and malignant melanoma. Thus, clinical presentation together with FISH or RT-PCR analysis for the specific translocation is crucial to distinguish the two entities. Receptor tyrosine kinase expression/activation [161] and gene expression analysis [162], indicate that MITF drives the same down-stream pathways in CSC and in melanoma and that PDGFRβ and the hepatocyte growth factor receptor (c-Met), are expressed by CCS [163]. CCS is poorly sensitive to chemotherapy and anecdotal responses to regimens containing dacarbazine, vincristine, anthracycline, and cyclophosphamide and to interferon-alpha-2b [164] have been reported. Based on the molecular features described above, multi-kinase inhibitors have been used as therapeutic agents in this STS and objective responses to sunitinib, and sorafenib treatments have been recently reported [165, 166].

2.3.2 ALVEOLAR SOFT PART SARCOMA (ASPS)

Alveolar soft part sarcoma (ASPS) is a rare malignancy that tends to strike young adults and adolescents. Patients with ASPS often present with extensive metastatic disease, frequently involving the lungs and sometimes the brain. The clinical management of patients with unresectable, metastatic disease is still challenging. ASPS expresses an array of potentially therapeutically targetable, angiogenesis-related molecules and, importantly, it has a distinctive angiogenic phenotype marked by a peculiar tumor-associated vasculature [167]. Base on this, alveolar soft part sarcoma have been shown to respond to both sunitinib [168] and cediranib [117]. ASPS carries an unbalanced recurrent t(X;17)(p11;q25) translocation, leading to the chimeric transcription factor ASPL-TFE3 [169]. The product of this peculiar translocation induces c-Met transcriptional up-regulation and activation [170]. Thus, MET may represent a potential therapeutic target in ASPS patients and studies on selective MET inhibitors, have been proposed [171].

3. AIM OF THE PROJECT

It is now clear that the tumor-host interplay represents a key component in the response to treatment. Thus, patient's immune status, as well as the dynamic changes in the tumor microenvironment, needs to be deeply investigated during anticancer treatment. So far, no information is available about the *systemic immunological status* and no accurate histological description of the *immune contexture at the tumor site* exists for the STS subtypes topic of my thesis. Consequently, the impact of anti-angiogenic therapies on the immunity of these patients remains unexplored. My study aimed to fill these gaps:

- The first objective was to investigate the *in vivo* presence and quality of circulating immune cells and tumor immune infiltrates in patients with STS.

- The second goal was to explore whether anti-angiogenic treatment (sunitinib) were actively interfering with the immunological status of these patients locally, at tumor site, and systemically.

4. MAIN RESULTS

Submitted Manuscript I - *Adaptive immune contexture at the tumor site and downmodulation of circulating myeloid-derived suppressor cells in the response of solitary fibrous tumor patients to anti-angiogenic therapy.*

The first interesting observation of our *in situ* analysis on anti-antiogenic naïve SFT specimens was the presence of a very dense infiltrate of ramified $CD163^+$ myeloid cells diffusely interdispersed among the cancer cells (**Figure 1, panel a**). These cells, mostly CD68 negative (**b**), included, as shown by confocal analysis, $CD163^+CD14^+$ M2-type TAMs (arrows) and $CD163^+CD14^-$ cells (circle), likely representing IMCs (**c**). Conversely, $CD3^+$ lymphocytes were mainly absent (**d**), and when present, they were enriched in suppressive $Foxp3^+$ Tregs (**e and f**). Collectively, these IHC and confocal analyses indicated an immunological status skewed toward immune suppression.

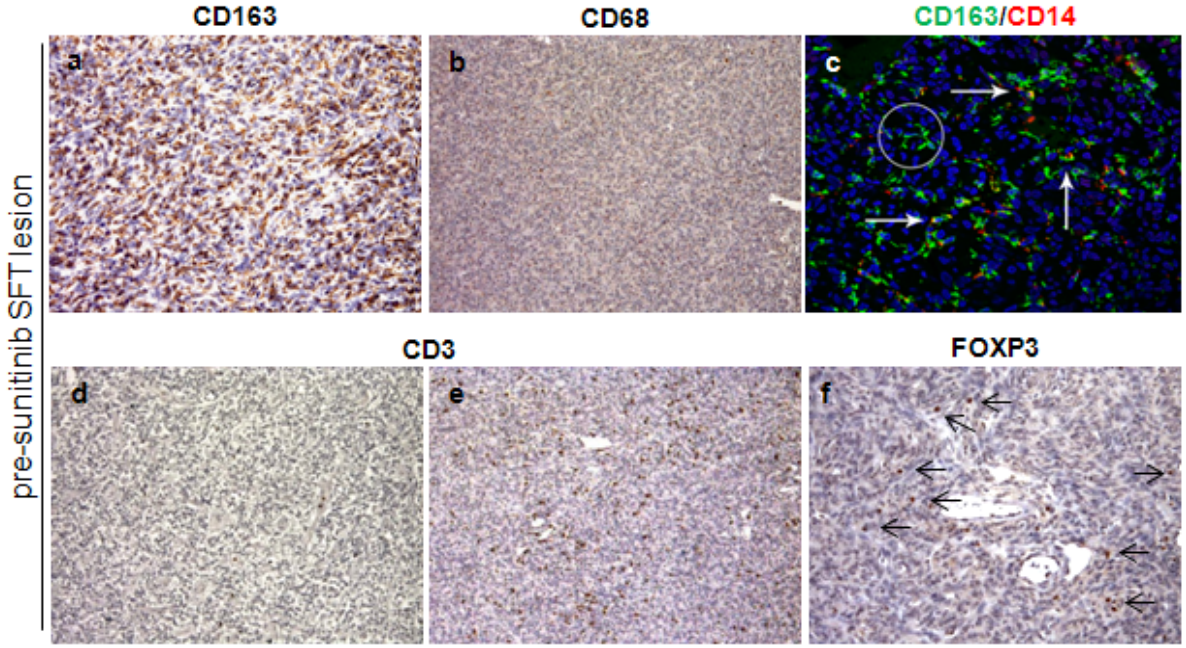

Figure 1

Then, we wondered whether this local immunosuppressive signature could be reversed by anti-angiogenic treatments. We observed that, as opposed to sunitinib-naïve tumors, M/DSFT lesions surgically removed from patients who received sunitinib in neo-adjuvant setting were all characterized by a high density of activated ($HLADR^+$) $CD3^+$ tumor-infiltrating T

lymphocytes (TILs), which included both Th1-polarized (T-bet$^+$) CD4$^+$ T cells and cytotoxic competent CD8$^+$ T cells (GZMB$^+$ and TIA-1$^+$) (**Figure 2A**).

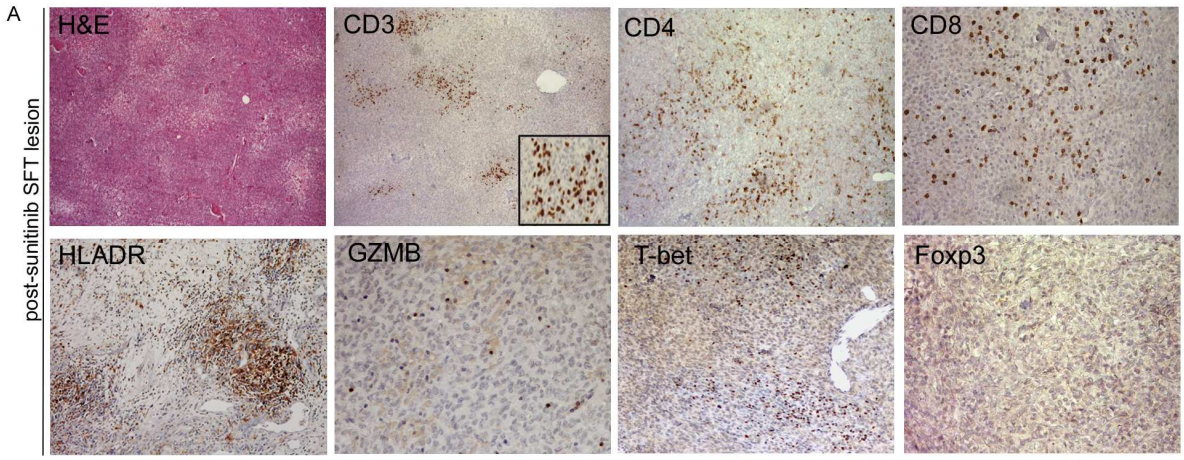

Figure 2

These data were further confirmed by *ex vivo* analysis on TILs purified from a sunitinib-treated MSFT lesion (**Figure 2B**).

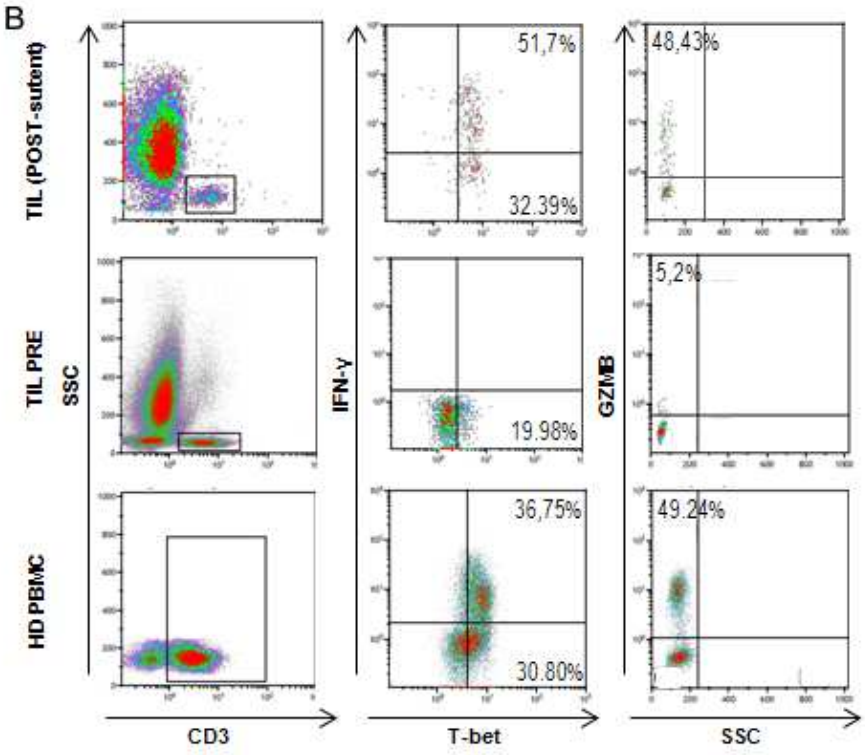

Figure 2

Interestingly we found that in post-therapy lesions, activated T cells correlated with the concomitant presence of a newly acquired population of $CD68^{+}CD14^{+}$ macrophages rarely found in untreated tumors (**Figure 3A and 3B panels a and b**).

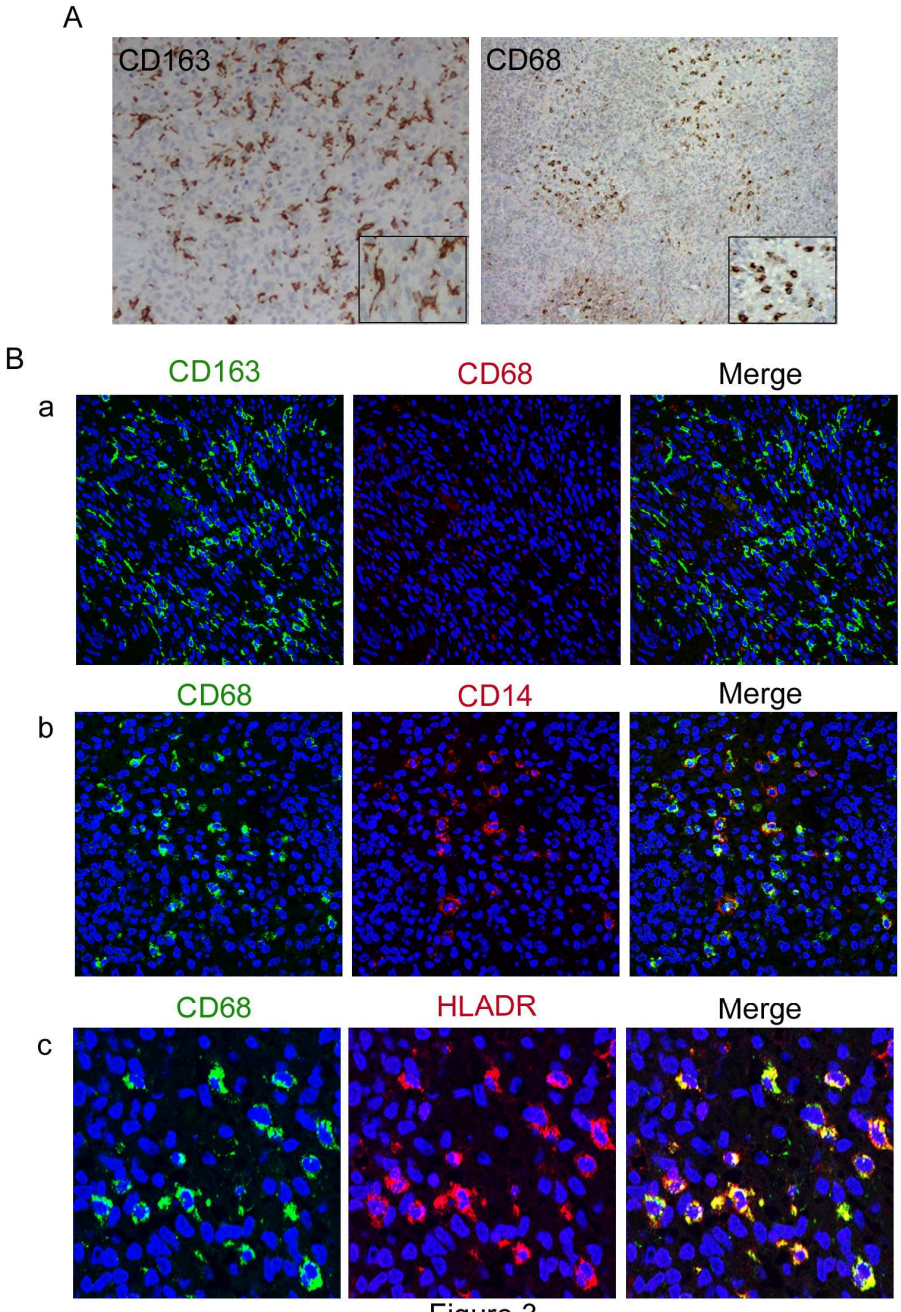

Figure 3

Indeed, we observed that, while $CD163^{+}$ cells in the pre–treated lesions showed an elongated and often ramified morphology, these $CD68^{+}$ macrophages displayed the round-shape morphology typical of the M1 polarization (**Figure 3A**) [97], expressed high level of HLA-DR (**Figure 3B panel c**), and were mainly organized in clusters around areas of tumor regression. Thus, proximity to T cells, morphology and phenotype features suggest their

immunostimulatory and anti-tumor activity. Altogether, the IHC analysis results were consistent with an ongoing adaptive immunity in post-sunitinib M/DSFT lesions.

We then evaluate the systemic immunological status of SFT patients. Multiparametric flow cytometry approach was applied to identify the different subsets of immune suppressive cells and to quantify their frequency. We found that:

a) $CD3^{+}CD4^{+}CD25^{hi}Foxp3^{hi}$ Tregs, were significantly expanded in M/DSFT patients compared with age-matched healthy donors (HDs) (**Figure 4A**).
b) No differences in the percentages of *Lineage-negative MDSCs* ($Lin^{-}HLADR^{-}CD33^{+}$), were detected between patients and HDs.
c) The percentage of $CD11b^{+}CD14^{+}HLADR^{-/low}$ *mMDSCs*, was significantly higher both in subjects with CSFTs and M/DSFTs than in HDs **(Figure 4B**).
d) *gMDSCs*, identified both as percentage of $Lin^{-}HLADR^{-}CD66b^{+}$ and $CD66b^{+}CD15^{+}$ cells in live PBMCs as well as considered as neutrophil count in parallel with the arginase activity detected in plasma, were found to be increased in untreated SFT patients compare to HDs. Concomitantly, by intracellular staining, circulating $CD3^{+}$ T cells displayed a decreased functionality and they showed a reduced production of IFN-γ and IL-2 (**Figures 4C and 4D**).

Altogether, these phenotypic and functional assays testify a status of systemic immunosuppression in SFT patients.

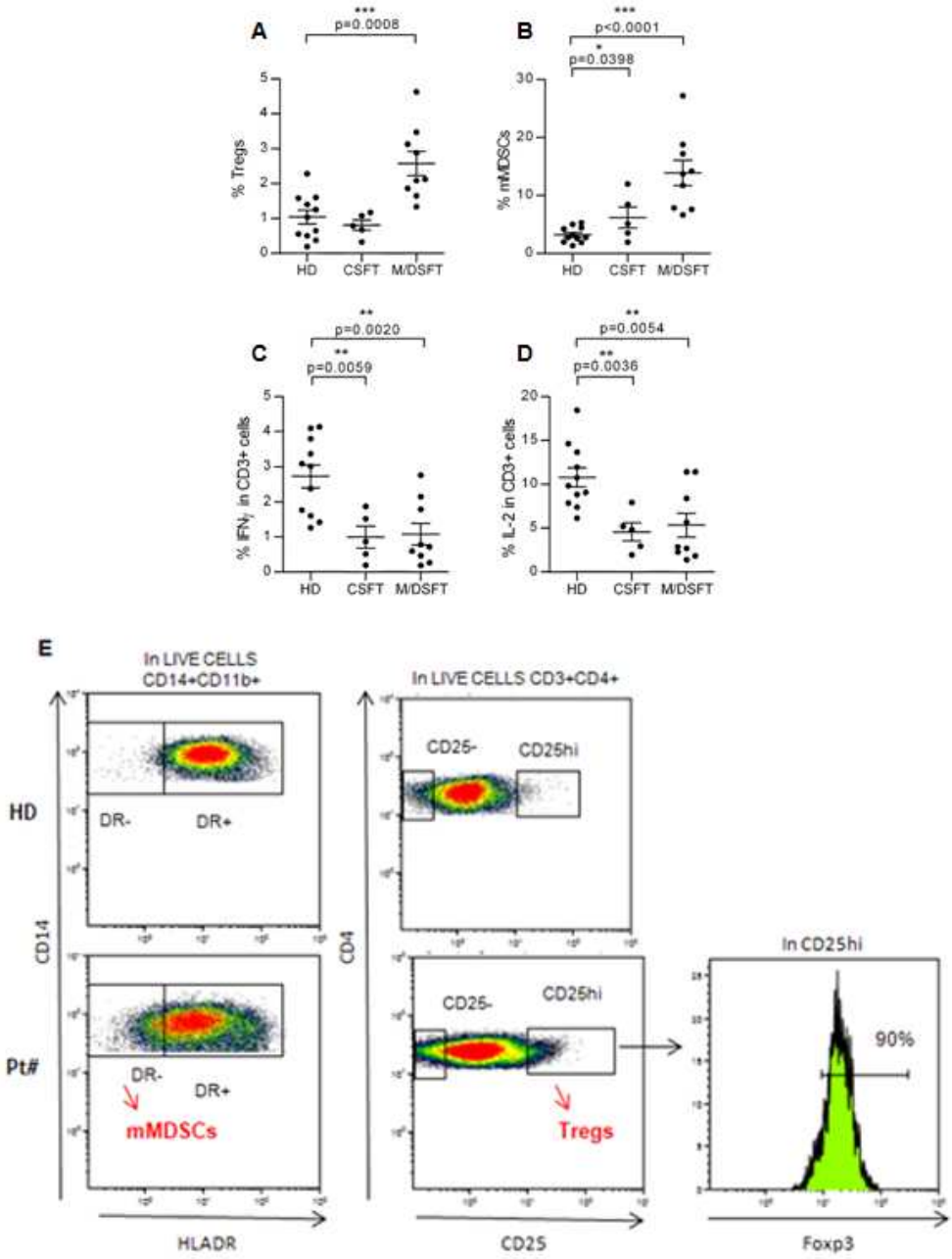

Figure 4

We monitored the frequency of circulating Tregs and the two MDSC subsets (mMDSC and gMDSC) in patients with M/DSFTs collected at different time points during sunitinib therapy. We found that:

a) The frequency of both Tregs and gMDSC decreased during treatment and remained low at time of disease progression. Interestingly, the frequency of circulating mMDSCs was significantly reduced at day 15 (T15) but, although patients were still under drug treatment, a rebound in the number of mMDSCs was observed at disease progression (**Figure 5A**).

b) The decrease functionality of the circulating $CD3^+$ T cells, assessed by intracellular staining as reduced IFN-γ and IL-2 production, was quickly relieved at T15 but then re-established concomitantly with the increase in mMDSCs at progression (**Figures 5B and 5C**).

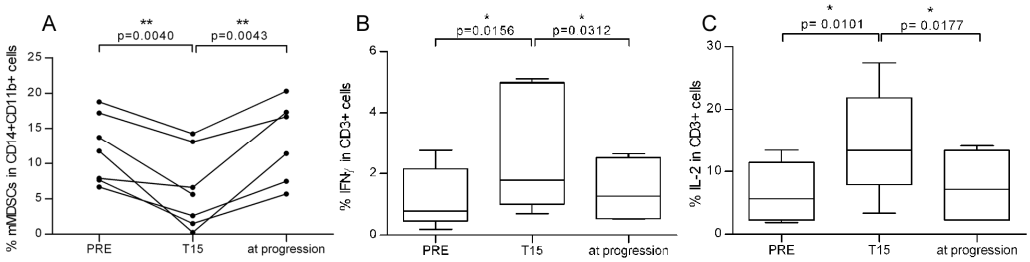

Figure 5

c) Three patients displaying a long lasting response to sunitinib treatment consistently displayed percentages of mMDSCs comparable to HDs (**Figure 6A Panel a**) and no evidence of $CD3^+$ T cell dysfunction in the peripheral blood could be detected (**Figure 6A Panel b and c**).

d) mMDSCs from patients at time of disease progression were assessed for the activation of STAT-1, 3, 5 and 6 in response to the *ex vivo* cytokine stimulation. Interestingly, *ex vivo* analysis of $CD14^+CD11b^+HLADR^{-/low}$ mMDSCs from peripheral blood of patients progressing during sunitinib treatment evidenced an IFNα-mediated STAT3 phosphorylation that did not occur in mMDSCs of sunitinib-responding patients (**Figures 6B**).

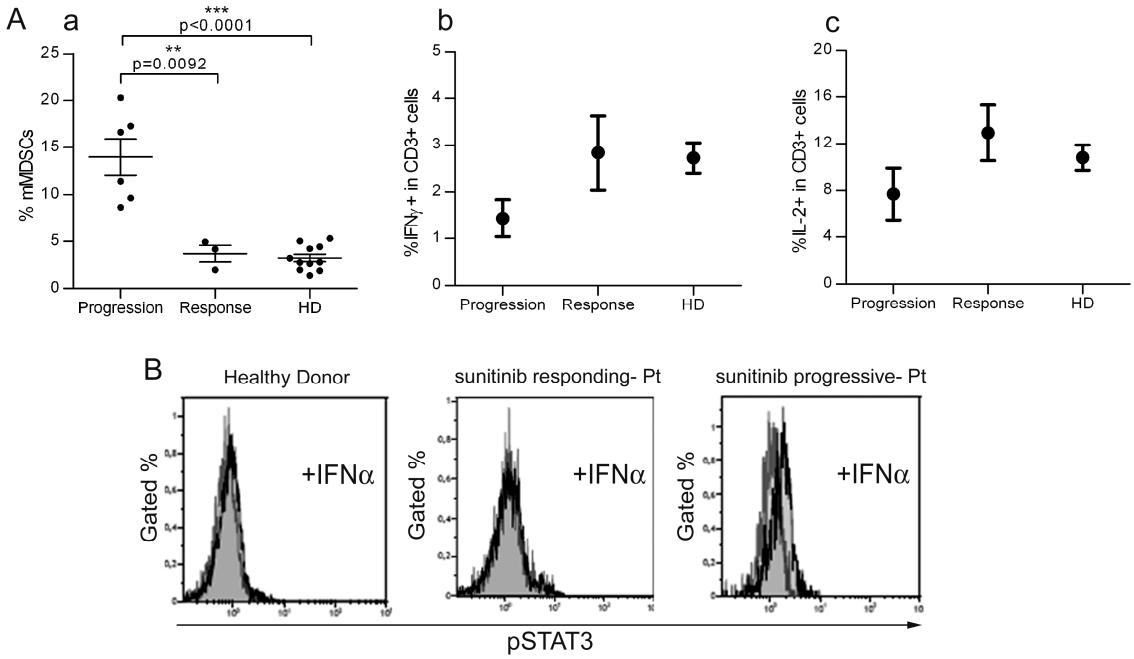

Figure 6

Conclusions: Collectively, our results provided phenotypic and functional evidence of a local and systemic immunosuppressive status that could be relieved by anti-angiogenic therapy. Moreover, high levels of mMDSCs and impaired T cell functions were found to be associated with tumor progression, while long-lasting low mMDSC frequency in sunitinib-treated patients paralleled the disease control. Initial functional characterization of mMDCS at progression suggests that sunitinib-treatment might induce/select a qualitatively different mMDSCs population, possibly representing an immune-mediated mechanism of acquired resistance.

Manuscript II [in preparation]- *Melan-A/MART-1 immunity in a clear cell sarcoma patient treated with sunitinib: a case report.*

Unlike other tumors, such as malignant melanoma, there are not currently bona fide TAAs or TSAs in SFT. Of note, as melanoma, CCS express a MITF-regulated expression of melanocyte differentiation antigens (HMB-45/gp100 and Melan-A/MART-1). Thus, I had the possibility to directly study the antigen-specific T cell response in a CCS (HLA-A*0201) patient with advanced disease that displayed a long-lasting response to treatment with the anti-angiogenic drug sunitinib.

In situ analysis revealed that, tumor specimen removed after treatment with sunitinib displayed signs of pathological regression associated with $CD3^+/CD8^+$T cell infiltration and a selective loss of MART-1 expression at the tumor level, while retaining the positivity for HMB-45 and S-100 (**Figure 1A and 1B**).

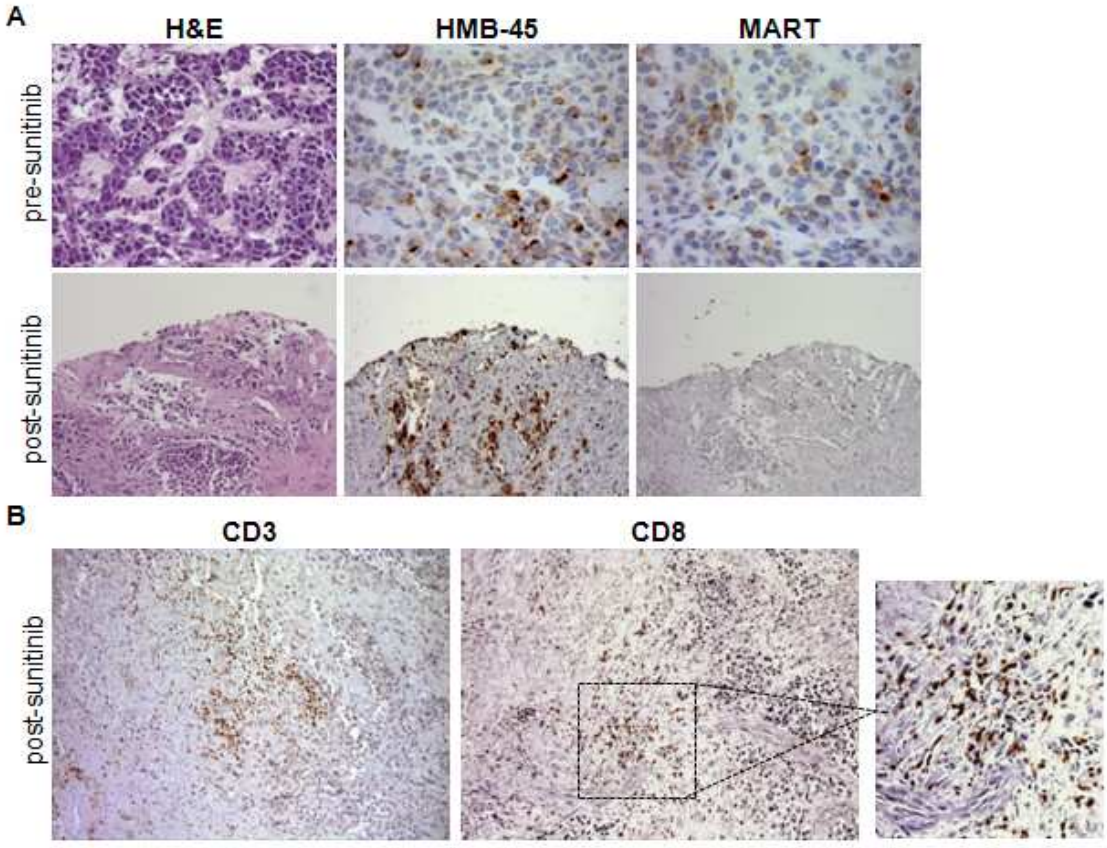

Figure 1

The *in vivo* generation of the MART-1 loss antigen variant was associated with the presence of an anti-MART-1 systemic immunity. In fact, after *in vitro* sensitization with the immunogenic HLA-A*0201 restricted peptide Melan-A/MART-1$_{[27L]}$, functionally active MART-1 specific CD8^{+}T lymphocytes were detected by pentamer staining (**Figure 2A**) and by ELISpot assay (**Figure 2B**).

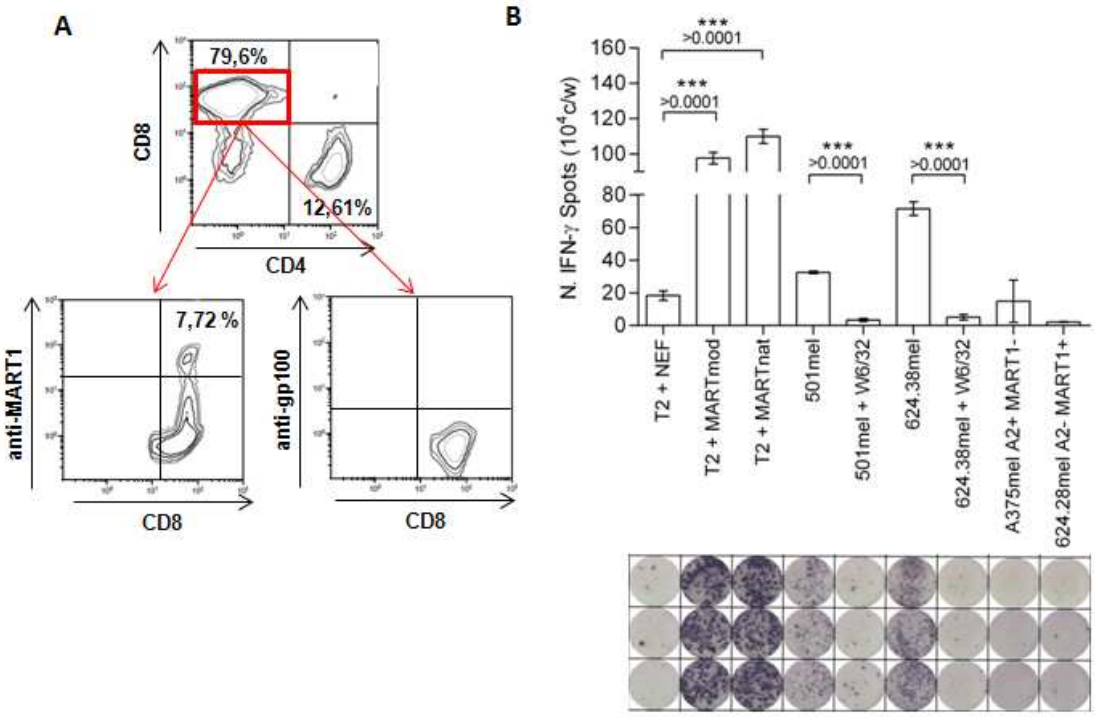

Figure 2

As for SFT, sunitinib treatment induced a sustained down-modulation of the frequency of immune suppressive cells, Tregs and mMDSCs, in this patient. Modulation of these cell subsets paralleled a T cell re-activation (**Figure 3)** that likely included also the anti-MART-1 specific T cells. Indeed, in the peripheral blood the percentage of IFN-γ (red line) and IL-2-producing (blue line) T cells were inversely correlated with that of immunosuppressive cells.

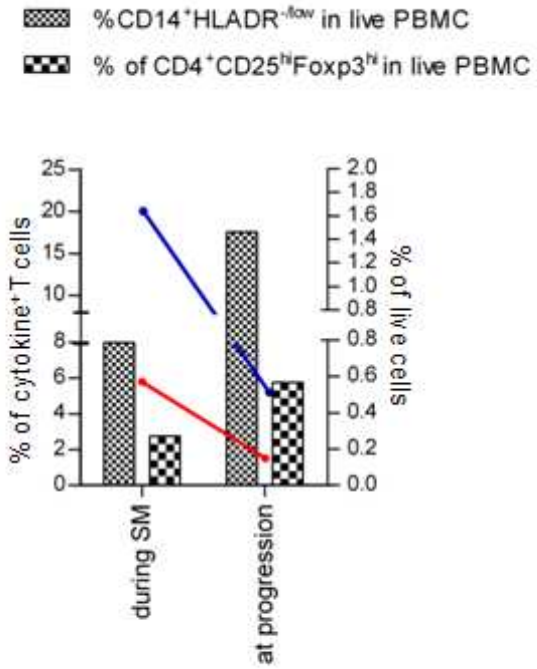

Figure 3

Conclusions: These data demonstrate a previously undescribed immune response directed toward melanoma antigen in a CCS patients. Altogether the provided evidence support the hypothesis that the post-sunitinib MART-1 negative tumor variant was the *in vivo* outcome of T cell-mediated, immune selection occurring in CCS patient likely operated by the anti-MART-1 T cells. This anti-MART-1 specific immunity may have been reactivated/unleashed following the release in the immune suppression induced by the sunitinib treatment.

Published Paper III - *Structured myeloid cells and anti-angiogenic therapy in alveolar soft part sarcoma.* [Commentary]

In line with the characterization of myeloid/macrophage cells previously described in SFT patients, we explored the presence and the localization of cells expressing myeloid markers in the inflammatory infiltrate of metastatic alveolar soft part sarcoma (ASPS). As stated in the introduction this tumor is characterized by a peculiar tumor-associated vasculature [167]. By IHC and confocal analysis, we found that myeloid cells expressing CD14 and CD163 markers constitute the prominent cells in the inflammatory infiltrate of naïve ASPS (**Figure 1, panels A and D**). Within the TME, these M2-like $CD14^{+}CD163^{+}$ macrophages were structurally organized in two distinct localizations. $CD14^{+}CD163^{+}$ cells formed a network surrounding $VEGFR2^{+}$ $CD31^{+}$ endothelial cells (arrows) or, as single cells, they were interspersed in tumor nests (circle), keeping deep contact with tumor cells (**panels B, C and E**).

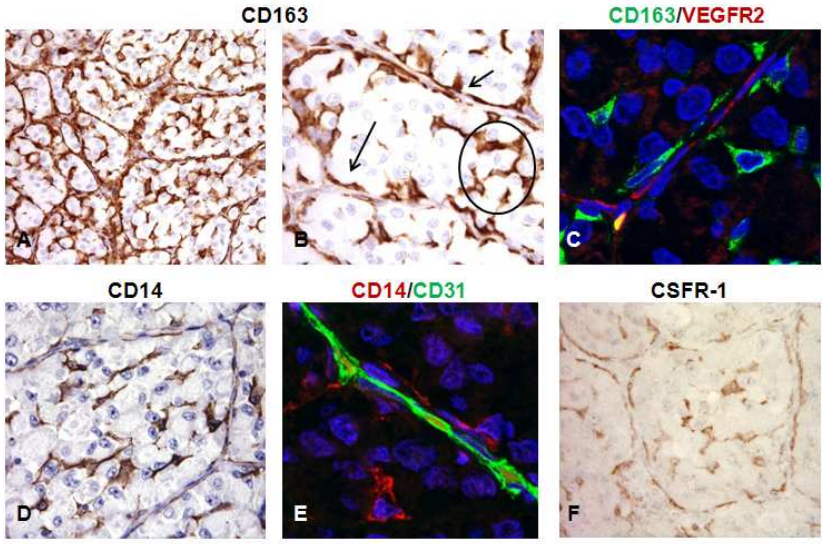

Figure 1

These myeloid cells might function as active inflammatory components promoting VEGF-mediated vasculogenesis and, although not physically part of the vasculature, they are thought to provide trophic support to the characteristic ASPS vascular network. We discuss these findings in relation to a published paper by Kummar and colleagues on metastatic alveolar soft part sarcoma (ASPS) treated with the anti-angiogenic drug cediranib [117]. Interestingly, molecular analysis of ASPS after treatment showed a strong modulation of transcripts related not only to angiogenesis/vasculogenesis, but also to inflammatory myeloid cells.

Conclusions: We showed for the first time that ASPS are heavily infiltrated by M2-like $CD14^{+}CD163^{+}$ macrophages structurally organized to support vasculature or likely exerting tumor trophic functions. Our morphological observation provide the rationale for considering the tumor infiltrating myeloid cells as potential targets of antiangiogenic therapies such as cediranib and suggest that their numeric or functional modulation can be part of the response to treatment.

5. CONCLUSIONS AND FUTURE PROSPECTS

The results collected in this thesis provided evidence of a local and systemic immunological status skewed toward immunosuppression in these STS patients, status that was previously ignored and that have crucial importance for directing appropriate clinical intervention in these types of neoplasms. The described immunomodulatory activities of sunitinib, together with the preliminary evidence that STS might be immunogenic *in vivo* (as we showed in the case report about the CCS subtype) strongly suggest that a re-activated tumor immunity could be part of the response to treatment. Data collected in this thesis provide the rationale for considering the manipulation of the immune system as a therapeutic approach at least in advanced SFT and CCS patients. Indeed, the observed phenomenon that sunitinib transiently normalizes disturbed myeloid differentiation status, while sparing lymphocytes and even enhancing their function, suggests that this treatment might provide a window, in which these patients may benefit from active immunotherapeutic approaches. Thus antibodies directed to immunological checkpoints, such as ipilimumab (anti-CTLA-4) or nivolumab (anti-PD-1) [106, 107], now in use for melanoma patients, may offer, alone or in association with targeted-therapies, a new therapeutic option for achieving more durable disease control in this category of STS patients. Of note, both SFT and CCS bear a causative chromosomal translocation that encodes for a new fusion protein. This protein, exclusively expressed by tumors cells, present in tumor at the early stages and maintained in the advanced metastatic disease, can be a potential and ideal source of tumor-specific, unique, non-self antigens toward which direct/re-direct the immune response by active vaccination or adoptive therapies.

The involvement of myeloid cells in maintaining an immune suppressive state in the peripheral blood, together with the heavy infiltration of TAMs observed across different naïve STS subtypes, clearly demonstrated by the results provided in this thesis, pose the rationale for combination therapies that include drugs limiting the function, differentiation or recruitment of myeloid lineage cells in association with other targeted-therapies. Some of these drugs are already used in the clinical settings [172-174] with promising results. The rebound of the myeloid suppressive cell population documented in the peripheral blood of SFT patients at disease progression, although patients were still under sunitinib treatment, poses the crucial question whether, in addition to participate to a clinical response, the immune system may also take part in the phenomenon of the acquired resistance to treatment.

The initial characterization performed in this thesis on the functional features of the mMDSC present/selected in the presence of sunitinib at disease progression seem to indicate that this is a realistic hypothesis that deserves accurate and further investigation.

In conclusion, the results of this thesis pose the rationale for immune based intervention in the subtypes of STS here analyzed. Futures studies should be designed to:

1. shed light on the antigen specificity of the autologous anti-tumor response in SFT and CSC patients and defining immunogenic unique, non self-epitopes for vaccine-based, or specific adoptive therapies;
2. dissect the contribution of the immune system in the ‘acquired resistance‘ to targeted-therapies.

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PART II

Submitted Manuscript I

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Title: Adaptive immune contexture at the tumour site and downmodulation of circulating myeloid-derived suppressor cells in the response of solitary fibrous tumour patients to anti-angiogenic therapy.

Running Title: Host immunity in solitary fibrous tumours

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Abstract

Background: Host immunity is emerging as a key player in the prognosis and response to treatment of cancer patients. However, the impact of the immune system and its modulation by therapies are unknown in rare soft tissue sarcomas such as solitary fibrous tumours (SFTs), whose management in the advanced forms includes anti-angiogenic therapy. Here we studied the *in situ* and systemic immune status of advanced SFT patients and the effects of sunitinib malate (SM) in association with the clinical efficacy. **Methods:** Immune contexture of SFT was assessed by immunohistochemistry in lesions from untreated or SM-treated patients. Frequency of circulating myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs) and T cell functions were assessed *ex vivo* in SFT patients prior and during anti-angiogenic therapy. Patients with long term tumour control were included to correlate immune profiles and clinical responses. **Results:** Anti-angiogenic naive SFT lesions were heavily infiltrated by $CD163^{+}CD14^{+}CD68^{-}$ and $CD163^{+}CD14^{-}CD68^{-}$ myeloid cells but devoid of T cells. Conversely, post–SM tumours acquired a new subset of $CD68^{+}CD14^{+}$ myeloid cells and displayed traits of an on-going adaptive immunity, strongly enriched in activated $CD8^{+}$ and $CD4^{+}$ T cells. These changes at the tumour site paralleled the alleviation of systemic immunosuppression and the drop in the frequency of circulating mMDSCs and gMDSC. Rebound in the number of mMDSCs, but not of gMDSC occurred at disease progression, and a reduced percentages of mMDSCs, comparable to those found in healthy donors (HD), endured only in the SM-responsive patients. **Conclusion:** The immune contexture of SFT patients is heavily involved in anti-angiogenic therapy and it could be exploited to achieve more durable disease control through immune-based combination strategies.

Keywords: soft tissue sarcoma, solitary fibrous tumour, anti-angiogenic therapy, anti-tumour response, myeloid-derived suppressor cells, tumour-infiltrating lymphocytes, tumour microenvironment, immunohistochemistry.

Introduction

Solitary fibrous tumour (SFT) is a rare subtype of soft tissue sarcoma (STS) that can occur in several anatomical sites, most frequently in middle-aged patients. Whereas most SFTs have an indolent course and can be cured by surgery, 15-20% of SFTs progress with either local recurrence or distant metastases (Fletcher *et al*, 2013; Chan *et al*, 1997). In addition to the classical SFT (CSFT), two more aggressive clinical-pathological variants of SFTs are currently recognised: malignant (MSFT) and dedifferentiated (DSFT), the latter showing a high-grade sarcoma overgrowth (Mosquera *et al*, 2009; Collini *et al*, 2012). We and other groups have recently described the activity of sunitinib malate (SM) (Chow *et al*, 2007), in unresectable, progressive M/DSFT patients (Stacchiotti *et al*, 2010; Stacchiotti *et al*, 2012; George *et al*, 2009). Apart from being an anti-angiogenic drug, SM possesses immunomodulatory functions (Ozao-Choy *et al*, 2009; Ko *et al*, 2009). The role of the immune system in controlling tumour growth has long been recognised and the immune contexture, defined by the frequency, type, functional polarisation and local distribution of immunocompetent cells at the tumour site, has been shown to impact tumour prognosis (Fridman *et al*, 2012; Galon *et al*, 2014). Moreover, 'avoiding immune destruction' has been recently listed as an emerging hallmark of cancer (Schreiber *et al*, 2011; Hanahan *et al*, 2011) and among the immune suppression mechanisms active in cancer patients, those mediated by $Foxp3^+$ regulatory T cells (Tregs) and myeloid derived suppressive cells (MDSCs) strongly hinder the anti-tumour response in patients with cancer of different histology (Filipazzi *et al*, 2007; Diaz-Montero *et al*, 2009; Mougiakakos *et al*, 2010). Little is presently known about the nature and features of the immune response to SFT, and no accurate histological description of local immunity exists for this STS. Moreover, the impact of anti-cancer therapies on the immunological status of the SFT patients remains unexplored. Herein, we showed that the immunological profiles of CSFT, MSFT and DSFT patients, at the tumour site and in circulating PBMCs, revealed an immunosuppressive status. Our data demonstrated that SM treatment relieves systemic immunosuppression in PBMCs of M/DSFT patients, and at the tumour site it favoured the setting of an immune contexture with typical adaptive immunity traits. Altogether, these findings pave the way for the design of therapies that combine immune-based approach with anti-angiogenic treatment in SFT patients in order to achieve a more durable control of this aggressive disease.

Materials and methods

Immunohistochemistry (IHC) and confocal analysis

Serial sections of 5-μm thick formalin-fixed, paraffin-embedded (FFPE) SFT samples (n=15) were cut and processed for IHC or immunofluorescence staining as previously described (Stacchiotti *et al*, 2012) and as briefly summarised in the supplementary material (see Supporting information, Supplementary materials and methods). The clinical and pathological characteristics of each tumour are summarised in Supporting information, Table S1. All the tumour samples were analysed for the presence of the NAB2-STAT6 fusion as previously described (Mohajeri *et al*, 2013; Robinson *et al*, 2013) (see Supporting information, Supplementary materials and methods). The antibodies used for IHC and confocal analysis and their conditions of use are reported in Supporting information, Table S2. Confocal microscopy was carried out using a Radiance 2100 microscope (Bio-Rad Laboratories, Hercules CA) equipped with a krypton/argon laser and a red laser diode. Evaluation of all IHC stains was performed by the Pathologist (S.P.) who scored the intensity of the staining using a scale from (-) no staining to (++++) very strong staining.

Blood sample collection and patient characteristics

This study was conducted in compliance with the Declaration of Helsinki and approved by the Ethical Committee of Fondazione IRCCS Istituto Nazionale dei Tumori, and all of the patients signed a written informed consent for the collection of blood samples. Blood samples were collected from SFT patients before and at different time points after initiating continuous treatment with anti-angiogenic therapy. Blood was also collected at the time of disease progression. Blood from age-matched healthy donors (HDs) was also obtained for control. PBMCs were isolated by Ficoll/Paque^{TM} PLUS density gradient centrifugation within two hours of the blood draw, as described elsewhere (Casati *et al*, 2006). To avoid assay-to-assay variability, purified PBMCs were cryopreserved in liquid nitrogen for batch acquisition of Tregs and MDSCs based on phenotype and frequency. Immunological monitoring of circulating Tregs and MDSCs was conducted in a total of 17 SFT patients. The clinical characteristics of the immunologically monitored patients are reported in Table 1. Patients began anti-angiogenic treatments at disease progression and after

a minimum period of 15 days of washout if they had previously received chemotherapy agents. The mean duration of the anti-angiogenic treatment was 6 months (range, 1-20); patients underwent disease assessment at baseline and after approximately 1-2 months. Objective responses according to the Response Evaluation Criteria in Solid Tumors (RECIST) and tumour burden shrinkage were determined by physician assessment of radiographs. Patients were treated until they experienced RECIST-defined disease progression or unacceptable toxicity.

SFT tumour dissociation and tumour-infiltrating lymphocytes (TILs) analysis

TILs were obtained from tumour sample of patients who underwent surgery by enzymatic and mechanic digestion using the gentleMACS Dissociator (Miltenyi, Bergisch-Gladbach, Germany). Briefly, tumour specimens were minced under sterile conditions into small pieces and digested over 1 h following the gentleMACS Dissociator protocol (Miltenyi) at 37℃. The resulting cell suspension was filtered through a 70-μm mesh (BD Biosciences, San Jose, CA), the red blood cells (RBCs) were lysed, and the cells suspension was washed twice with RPMI. Cells were stored in liquid nitrogen until use. For intracellular cytokine staining, patients' TILs were seeded into 96-well round-bottomed plates at $1.5x10^5$ cells/well in RPMI + 10% human serum and stimulated overnight with PMA/Iono (50 ng/mL and 500 ng/mL, respectively) plus GolgiStop (4 uL/6 mL, BD Biosciences) at 37℃. TILs were stained for the cell surface markers CD3, CD4 and CD8. The cells were then washed, fixed and permeabilised with Fix/Perm reagents (eBioscience, San Diego, CA) following the manufacturer's protocol and then stained with a488-labelled anti-IFN-γ (BioLegend, San Diego, CA), PE-labelled anti-Tbet (eBioscience) or PE-labelled anti-granzyme B (BD Biosciences). Dead cells were identified using the LIVE-DEAD® Fixable Violet Dead Cell Stain Kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions and excluded from the analysis. The fluorescence intensity was measured using a Gallios™ (Beckman Coulter, Brea, CA) flow cytometer and analysed using Kaluza® software (Tree Star Inc, Ashland, OR).

Flow cytometry and intracellular cytokine staining

Treg and MDSC frequencies were determined by six-colour immunofluorescence staining of thawed PBMCs. The antibodies used are reported in Supporting information, Table S3. Dead cells were identified using the LIVE-DEAD® Fixable Violet Dead Cell Stain Kit (Life Technologies) according to manufacturer's instructions and excluded from the analysis. For surface staining, cells were incubated with antibodies for 30 minutes at 4℃ after blocking non-specific antibody binding to the Fc-receptors using FcR Blocking Reagent (Miltenyi). For Treg analysis, intracellular staining with APC-conjugated anti-Foxp3 (eBioscience) or the proper isotype control (rat IgG2a) was performed after fixation and permeabilisation of cells using an intracellular staining kit (eBioscience) according to the manufacturer's instructions. Intracellular staining was performed as follows. Lymphocytes activated overnight with anti-CD3/CD28 beads (DynaBeads® CD3/CD28 T cell Expander, Invitrogen Dynal AS, Oslo, Norway) in the presence of 1 µl/ml Golgi Plug (BD Biosciences) were stained for the cell surface marker CD3. The cells were then washed, fixed and permeabilised with Cytofix/Cytoperm buffer (BD Biosciences) and stained with a 488-labelled anti-IFN-γ (BioLegend), PE-labelled anti-IL2 (BD Biosciences). Data acquisition was performed using a Gallios™ (Beckman Coulter) flow cytometer, and the Kaluza® software (Tree Star Inc) was used for data analysis.

Intracellular protein kinase assay

Cryopreserved PBMCs were thawed, washed and rested 2h at 37℃ in RPMI containing 1%HS. Then, cells were incubated either without stimulation or stimulated with GMCSF 10ng/ml (Peprotech), IL-4 100ng/mL (Perprotech), VEGF 50ng/mL (Peprotech) and IFNα 10000 U/mL (Sigma –Aldrich, St Louis, MO, USA). Immediately after stimulation cells were fixed with pre-warmed BD Cytofix™ Buffer (BD Biosciences) for 10min at 37℃. After incubation cells were washed with PBS 1%FCS and then stained with anti-CD14 APC alexa750 (Beackman Coulter) and HLADR FITC (BD Biosciences) for 30 min and permeabilized with Perm Buffer III solution (BD Biosciences). Cell were then stained for intracellular expression of anti-pSTAT1 (Y701) Alexa Fluor 647, -pSTAT3 (Y705) Alexa Fluor 647, -pSTAT6 (Y641) PE and –pSTAT5(Y694) PE (all from BD Bioscences). Data were acquired on a

Gallios™ (Beckman Coulter) flow cytometer and analysed using the Kaluza® software (Tree Star Inc).

Arginase activity assay

Plasma from HD and SFT patients were tested for arginase activity by measuring the production of L-ornithine from L-arginine, as previously reported [Rodriguez PC et al Cancer Res 2004]. In brief, 25uL of plasma samples were added to 25uL of Tris-HCL (50nM; pH 7.5) containing 10mM MnCl2 (sigma). Arginase was then activated by heating the mix for 20 min at 55℃. Then a solution containing 150uL carbonate buffer (100mM; sigma) and 50uL L-arginine (100mM; sigma) was added and incubated at 37℃ for 20 min. The hydrolysis reacti on from L-arginine to L-ornithine was stopped with 750uL of glacial acetic acid and identified by a colorimetric assay after the addition of 250uL of ninhydrin solution (2.5g ninhydrin (sigma); 40mL H_3PO_4 6M; 60mL glacial acetic acid), followed by incubation at for 1h at 95℃. The amount (nmol) of L-ornithine was determined measuring the absorbance at 570 nm.

Statistical analysis

The two-tailed unpaired Student's t test (with a 95% confidence interval [Cl]) was used to compare groups, while the two-tailed paired Student's t test was used to analyse the effect of the treatments between different time points, as indicated in the figure legends. Statistical calculations were performed using the Prism5 software (GraphPad Software, La Jolla, CA, USA). P values < 0.05 were considered statistically significant. Error bars represent the standard error of the mean (SEM).

Results

Tumour-infiltrating immune cells in CSFT and M/DSFT

To gain insight into the immune contexture of SFTs, the presence and functional polarisation of tumour-infiltrating T cells and myeloid/macrophage cells were assessed by IHC analysis on a retrospective series of FFPE SFT specimens (n=11) collected from patients who did not receive anti-angiogenic therapy prior to surgery. This series of anti-angiogenic-naïve SFTs included 3 CSFTs, and 5 MSFTs and 3 DSFTs. Supporting information Table S1 reports the histopathological features of the studied tumours. The majority of the analysed samples were negative or very poorly infiltrated with $CD3^+$ T cells (Figure 1A). Only 2 cases (MSFT Tumour ID #8 and DSFT Tumour ID #9) displayed a remarkable positivity for CD3 staining (Figure 1A) that, however, was paralleled by a strong positivity for the Foxp3 nuclear marker, thus indicating enrichment in infiltrating Tregs (Figure 1A). In our samples, antibodies directed against the CD68 and CD163 markers did not stain tumour cells but did identify two different subgroups of tumour-associated macrophages (TAMs). All of the SFT cases displayed strong infiltration by $CD163^+$ cells intermingled with the tumour cells, and the frequency of this myeloid cell type was further enhanced in tumours with a worse prognosis, namely M/DSFT samples. Conversely, CD68 staining was completely negative or revealed only few/scattered positive cells (Figure 1A). Confocal analysis, performed to better clarify the nature of the myeloid cells present in the M/DSFT microenvironment, showed that the majority of the $CD163^+$ cells were positive for CD14, fitting with a pro-tumour, M2-TAM nature (Figure 1B (arrows)) (Mantovani *et al*, 2002). Moreover, a subset of CD163 single-positive cells was also detected (Figure 1B (circle)). According to the literature, these cells might represent a subset of myeloid progenitors or immature cells (de Vos van Steenwijk *et al*, 2013). Analysis of the granulocytic component was also performed and no evidence for a selective infiltration of this cell subset was evidenced. $CD66b^+$ cells were in fact only occasionally found inside the tumor, with some positivity detected only in perivascular areas (Figure S1). Collectively, the IHC and confocal analyses indicated poor T-cell infiltration and an immunological status skewed toward immune suppression in CSFT and in M/DSFT lesions.

Evidence of a distinct immune cell signature in M/DSFT lesions from SM-treated patients

Pro-angiogenic factors and abnormal tumour vasculature hamper the extravasation of immune cells into the tumour parenchyma and promote immune suppression (Dirkx *et al*, 2003). Conversely, anti-angiogenic treatments, while normalising blood vessels, enhance immune infiltration, as was recently shown in different animal models (Jain *et al*, 2013; Shrimali *et al*, 2010). In M/DSFT patients, we recently described the activity of SM, and we reported that SM led to vascular normalisation at the tumour site (Stacchiotti *et al*, 2012). We thus explored the immune contexture in 4 M/DSFT lesions surgically removed from patients who received SM in neo-adjuvant setting. All of these samples showed a high density of $CD3^+$ TILs (Figure 2A), which included both $CD4^+$ and $CD8^+$ T cells. The intratumoral lymphocytes were mainly HLA-DR positive, and a consistent fraction of them also stained positive for granzyme B (GZMB) and T cell-restricted intracellular antigen (TIA-1, a cytotoxic granule-associated protein expressed by cytotoxic T cells and involved in the induction of apoptosis in CTL sensitive targets) (Figure 2A). Thus, the $CD3^+$ infiltrating cells were mainly activated T cells endowed with cytolytic potential. Moreover, their positivity for the nuclear transcription factor T-bet (immune cell-specific member of the T-box family of transcription factor coordinating type 1 immune responses) suggested enrichment in functional, Th-1-polarised T cells (Figure 2A). No $Foxp3^+$ cells were detected (Figure 2A), indicating the absence of regulatory, suppressive T cells at the tumour site in post-SM M/DSFT patients. Concerning the monocyte/macrophage compartment, in addition to the $CD163^+CD68^-$ myeloid population (Figure 3), post-SM M/DSFTs displayed a strong positivity for intratumoral $CD68^+$ myeloid cells (Figure 3A). On confocal analysis, these $CD68^+$ cells co-expressed CD14 and represented a newly acquired population of macrophages rarely found in untreated tumours (Figure 3B Panel b). Moreover, these $CD68^+$ macrophages displayed a typical round morphology, and double-immunofluorescence staining revealed co-expression of both the CD68 and HLA-DR markers (Figures 3B Panel c). These features are compatible with the M1 phenotype of activated macrophages. On IHC evaluation, all of the cases treated with SM showed signs of a pathologic response. Extensive areas of necrosis and tumour regression were observed in the proximity of the immune and inflammatory infiltration. Of note, around the area of tumour regression, cytotoxic competent, Th1

$CD8^+$ and $CD4^+$ T cells were organised in clusters (Figure 2B). Altogether, the IHC analysis results were consistent with an ongoing adaptive immunity in post-SM M/DSFTs. To strengthen this conclusion, *ex vivo* TILs were isolated from excised naive and post-SM MSFT (Patient ID #13) specimens and tested *in vitro* for their immunological properties. T cells from post-SM lesions were found to contain functionally active $CD4^+$ T cells producing IFN-γ *ex vivo* and $CD8^+$ granzyme B-positive T cells, representing effector cytotoxic T lymphocytes (Figure 2C).

Standard treatment for M/DSFT patients includes different regimens of cytotoxic chemotherapy associated or not with radiotherapy. To verify whether modulation of the immune contexture at the tumour site also occurred in patients responding to chemotherapy, IHC analysis was performed in 4 M/DSFT tumour lesions surgically removed from patients who received chemo/radiotherapy in neo-adjuvant setting. Weak/moderate CD3 infiltration and only few, spared $CD68^+$ cells were detected in two tumours surgically removed from patients treated with chemotherapy (isofosfamide and/or epirubicin) plus radiotherapy (Figure S2, Tumour ID #14 and #15). Absence of $CD68^+$ cells and very week $CD3^+$ T cells infiltration characterised the post-epirubicin (monotherapy) tumour (Figure S2 Tumour ID #13). Of note, in the tumour sample from a patient treated with doxorubicin and dacarbazine, moderate $CD3^+$ infiltration, associated to a still week but more clusterised $CD68^+$ positive infiltrate was detected in the proximity of areas showing signs of necrosis and tumour regression likely suggesting a possible engagement of the immune response.

Accumulation of immunosuppressive cells in peripheral blood of solitary fibrous tumour patients

To evaluate the systemic immunological status of SFT patients, we explored the frequency of Tregs and mMDSCs in the peripheral blood of a prospectively collected series of 17 SFT patients who included 5 patients with tumours classified as CSFTs, 6 as MSFTs and 6 as DSFTs (Table 1). PBMCs of HDs, matched for gender and age, were included as controls. Tregs, defined as $CD25^{hi}Foxp3^{hi}$ within a live-gated $CD3^+CD4^+$ cell population, were significantly expanded in M/DSFT patients compared with age-matched HDs (n=11) ($p= 0.0008$, 1.04 ± 0.65 vs. 2.57 ± 1.05); conversely, no statistically significant difference existed between HDs and CSFTs patients (Figure 4A). The percentage of mMDSCs, first defined by our group as

$CD11b^+CD14^+HLADR^{-/low}$ (Filipazzi *et al*, 2007; Hoechst *et al*, 2008; Walter S *et al*, 2012), was significantly higher both in subjects with CSFTs and M/DSFTs than in HDs (p=0.0398, 6.19±4.03; p<0.0001, 13.88±6.56 vs. 3.23±1.31, respectively) (Figure 4B). No difference in the percentages of $Lin^-HLADR^-CD33^+$ MDSCs, were detected between patients and HDs (data not shown). The overall frequency of $CD3^+CD4^+$ T cells and myeloid/monocyte $CD14^+CD11b^+$ cells did not differ significantly between patients and HDs (Figures 4C and 4D). Moreover, circulating $CD3^+$ T cells from CSFT and M/DSFT patients were functionally impaired. Figures 4E and 4F show that the frequency of $CD3^+$ T cells that produced IFN-γ and IL-2 *ex vivo* was strongly reduced in patients' PBMCs compared with HDs. Altogether, these phenotypic and functional assays suggested a status of systemic immunosuppression in SFT patients.

Anti-angiogenic therapy modulates peripheral immunosuppressive cells in patients with M/DSFT

Our *in situ* analysis provided evidence that anti-angiogenic treatment reprogrammed the immune contexture of M/DSFTs and favoured the onset of an active T-cell immunity. To evaluate whether anti-angiogenic therapy also affected the systemic immunological status of patients with M/DSFT, we monitored the frequency of circulating Tregs and mMDSCs in PBMCs from patients with M/DSFTs collected at different time points during SM (n= 6 patients) or pazopanib (n=1 patient) therapy (Table 1). These anti-angiogenic drugs did not induce lymphopenia (data not shown). Interestingly, at the end of the second week of treatment (T15), the frequency of blood Tregs, evaluated within the $CD3^+CD4^+$ compartment (Figure 5A) or in the total number of live cells (Figure S3), was significantly reduced (p=0.0020, 2.63±1.12 vs. 1.41±0.75; p=0.0117, 0.57±0.37 vs. 0.36±0.26). This effect was long lasting, and it was maintained for the duration of the treatment (p=0.0204, 1.41±0.75 vs. 0.95±0.48; p=0.0403, 0.36±0.26 vs. 0.22±0.14). The frequency of circulating mMDSCs, within the monocytic compartment (Figure 5B) or in the total number of live cells (Figure S3), was significantly reduced at T15 (p=0.0040, 11.93±4.84 vs. 6.27±5.52; p=0.0295, 2.72±2.18 vs. 0.44±0.31). An increase in mMDSC frequency occurred in all of the patients at the time of disease progression although patients were still under drug treatment (p=0.0043, 6.27±5.52 vs. 13.13±5.86; p=0.0030 0.44±0.31 vs. 1.8±0.66) (Figures 5B). The MDSC population includes also

granulocytic MDSC (gMDSC). Contrary to the mouse gMDSC, definition of human gMDSC is still challenging due to the lack of a definitive marker that clearly differentiates this population from activated granulocytes. However, a consensus has been reached in considering gMDSC as $CD14^{-}CD66b^{+}$ and/or CD15+ activated neutrophils within total PBMC or inside the $Lin^{-}HLADR^{-}$ fraction, displaying low density (thus being co-purified with PBMC during blood centrifugation) and releasing arginase I in the circulation (Brandau *et al*, 2011, Rodriguez *et al*, 2009; Zea H *et al*, 2005). Since gMDSC have been found expanded in the blood of patients with tumours of different histology (Filipazzi *et al*, 2012), we assessed the presence and frequency of this MDSC subtype in PBMC of D/MSFT by monitoring the presence of $CD66b^{+}CD15^{+}$, or $CD66b^{+}Lin^{-}$HLA-DR^{-} cells as done in others published studies (Brandau *et al*, 2011, Rodriguez *et al*, 2009; Zea H *et al*, 2005) (Figure 5D). Moreover, for each M/DSFT patient, the arginase activity was quantified in the plasma and plotted along the absolute number of blood neutrophils (Figure 5E). As respect to HD, M/DSFT patients displayed an enhanced frequency of gMDSC (Figure 5D) and an increased number of neutrophils in the blood (Figure 5E), number that matched the higher plasma arginase activity. Frequency of gMDSC, number of neutrophils and arginase activity were co-ordinately down-modulated by SM. However, similarly to Treg and at difference from mMDSC, gMDSC remained low all along the duration of sunitinib treatment including at the time of progressive disease (Figure 5D and 5E)..

The functional assessment of the circulating $CD3^{+}$ T cells, which was based on their capacity to produce IFN-γ and IL-2 *ex vivo* (Figures 5F and 5G), revealed that immunosuppression, present in patient PBMCs prior to anti-angiogenic treatment (Figures 5Fand 5G, PRE), was quickly relieved at T15. At progression, with the increase in mMDSCs, T cells displayed again an impaired function characterized by a limited IFN-γ and IL-2 production, similarly to what was found for the pre-treatment T cells.

Three patients displaying a long lasting response to SM treatment (Table 1, Pts #6a,8a,10a; SD or PR according RECIST evaluation after ≥10 months) consistently showed a low level of mMDSCs, with values comparable to HDs (Figure 6A Panel *a*) and no evidence of $CD3^{+}$ T cell dysfunction in the peripheral blood could be detected in these SM-responsive patients (Figure 6A Panel *b* and *c*). mMDSCs from patients at time of disease progression were assessed for the activation of STAT-1, 3, 5 and

6 in response to the *ex vivo* cytokine stimulation. VEGF was unable to trigger STAT-3 phophorylation in mMDSCs, likely as a result of the blocking activity exerted by SM. The other cytokines tested, namely GM-CSF and IL-4, exerted their canonical activation pathways and induced STAT-5 and STAT-6 activation respectively (data not shown). Conversely, these mMDSCs stimulated with IFNα, in addition to STAT-1 (data not shown), displayed a consistent phophorylation of STAT-3. IFNα-dependent STAT-3 activation did not occurred in mMDSCs of HD and SM-responsive patients (Figures 6B and 6C).

In summary, the analysis of the circulating immune cells in PBMCs from M/DSFT patients provided phenotypic and functional evidence of an immunosuppressive status that was quickly but temporary relieved by anti-angiogenic treatment. Suspension in the immunosuppression correlated with response to treatment.

Discussion

To our knowledge this is the first report that performed a detailed characterisation of the immunological status in the peripheral blood and at the tumour site of SFT patients and that considers the immune contexture of SFTs as a possible player in the response to therapy as well as in disease progression. SM exerts a significant anti-tumour activity in M/DSFT (Stacchiotti *et al*, 2010; Stacchiotti *et al*, 2012; George *et al*, 2009) and patients achieving long term tumour control have been reported (Levard *et al*, 2013; Domont *et al*, 2010). Besides the notion that immunity is emerging as a critical player in the response to treatment in cancer patients (Zitvogel *et al*, 2013), the rationale of assessing the role of the immune system in the efficacy of SM in SFTs stems from our previous observation that the levels of PDGFRβ and/or VEGFR2 activation, evaluated by IHC in tumour lesions, did not fully account for the therapeutic response to treatment (Stacchiotti *et al*, 2012).

The first interesting observation of our analysis was the presence of an immunosuppressed environment at the tumour site, characterized by a very dense infiltrate of myeloid cells. These cells, mostly CD68 negative, included $CD163^{+}CD14^{+}CD68^{-}$ TAMs, interdispersed among cancer cells and with an elongated, ramified morphology compatible with M2-type macrophages (Mantovani *et al*, 2002; Ino *et al*, 2013; Jensen *et al*, 2009; Caillou *et al*, 2011), together with $CD163^{+}CD14^{-}CD68^{-}$ cells likely representing immature myeloid-derived cells (de Vos van Steenwijk *et al*, 2013; Jensen *et al*, 2009). Conversely, $CD3^{+}$ lymphocytes were mainly absent, and when present, they were enriched in suppressive $Foxp3^{+}$ Tregs. An immunosuppressive status was also detectable in the peripheral blood of SFT patients. In fact, circulating T cells were consistently functionally impaired, and a significant accumulation of mMDSCs and gMDSCs was observed in all the patients analysed. Notably, the increased frequency of circulating mMDSCs (Figure 4B) and gMDSCs (data not shown) seemed to correlate with tumour grade and disease aggressiveness, being already detectable in CSFT patients and reaching the highest level in M/DSFT patients. The more compromised immune status of these patients with advanced SFTs was further confirmed by the additional accumulation of circulating Tregs, which instead showed frequency close to normal values in patients with CSFTs. Altogether, this scenario reveals a previously unappreciated tumour-mediated immunosuppression in SFT patients and particularly in patients with M/DSFTs. This observation opens the question whether this immunosuppressive

signature can be reversed by anti-angiogenic treatments and whether re-activated tumour immunity could be part of the response to treatment. Noteworthy, no information about the immunological effect of SM, as detected *in situ* at the tumour site in human setting, are available to date. Our IHC analysis showed that, as opposed to SM-naïve tumours, SM-treated lesions were all characterised by a remarkable $CD3^+$ T cell infiltration, with no $Foxp3^+$ Treg, but that included Th1 and cytotoxic-competent $CD4^+$ and $CD8^+$ T cells. Moreover, TILs purified from a SM-treated MSFT lesion released e*x vivo* Th1-related cytokines and cytotoxic Granzyme B, thus supporting the local engagement of a functionally active host immune response. Activated T cells at the tumour site correlated with the concomitant presence of a new subset of $CD68^+$ myeloid cells rarely found in untreated tumours. These $CD68^+$ macrophages displayed the round-shape morphology typical of the M1 polarization (McWorther *et al*, 2013), and expressed high level of HLA-DR. The pro-inflammatory and anti-tumour activity of $CD68^+$ macrophages infiltrating post-therapy SFTs is also supported by their organization in clusters around the areas of tumour regression and in close proximity with activated T lymphocytes. Since macrophages display an elevated grade of plasticity in response to external stimuli (Mantovani *et al*, 2013), we may hypothesise that in SFTs, SM re-educated tumour-resident myeloid cells toward a more M1 related phenotype, or, alternatively, it recruited *ex novo* a new subset of monocytes/macrophages from peripheral blood.

Standard treatment for M/DSFT patients includes cytotoxic chemotherapy and/or radiotherapy. Evidence from the literature indicates that some chemotherapy regimens can be endowed with immunomodulatory activities (Bracci *et al*, 2014). Analysis of the immune contexture was thus performed in M/DSFT tumours obtained from cytotoxic responsive patients. Only very mild modulation of the immune infiltration was observed and this occurred mainly in those cases receiving radiotherapy, thus confirming previously published data (Sharma *et al*, 2013). Of note, two of the analysed tumours, one that did not show any infiltration of T or myeloid cells (Tumour ID #13) and one treated with chemotherapy plus RT (Tumour ID #14), were surgically removed from patients who were subsequently treated and responded to SM and whose post-SM tumours were here analysed (Figure 2A and 2B). Indeed, at difference from the autologous post-chemotherapy counterpart, post-SM tumours displayed a profound change in the tumour immune microenvironment with huge $CD3^+$ and $CD68^+$ infiltrating cells organised in cluster and intermingled

with tumour cells and areas of tumour necrosis. A lymphocyte infiltration, with a lower density but with distribution similar to that observed in post-SM tumours, was only found in the sample treated with adriamycin and dacarbazine, drugs reported as strong immunomodulators (Bracci *et al*, 2013). In this case a coordinated increase in $CD3^+$ and $CD68^+$ cell infiltration could be observed together with areas of tumor regression, thus possibly testifying an involvement of the immune system in the response to this chemotherapy regimen. In summary, a coordinated immune modulation, involving both myeloid and lymphoid immune infiltrate occurred in all the 4 post-SM tumours, thus indicating a tight association between immune modulation and response to SM. This was not the case for the post-chemotherapy samples. However, the here analysed post-chemotherapy tumours were derived from patients who underwent heterogeneous chemotherapy treatments. So, definitive conclusions on the involvement of immune system into chemotherapy-induced response deserve further investigation. This is particularly true for the adriamycin and dacarbazine regimen, since a single case was available for analysis and in view of the fact that a retrospective study recently reported this chemotherapy regimen as effective in M/DSFT (Stacchiotti *et al*, 2013).

The ability of SM to interfere with myeloid cells, a property already suggested in other cancer patients (van Cruijsen *et al*, 2008; Ko *et al*, 2009), was further supported by our evidence that circulating $CD14^+CD11b^+HLADR^{-/low}$ mMDSCs and gMDSC were significantly decreased in M/DSFT patients upon SM administration. In fact, the frequency of gMDSC remained low and similar to frequency found in HD all along the duration of the treatment, including the time of progressive disease. gMDSC behaviour thus overlapped that of Tregs. Conversely, the SM effect on mMDSC appeared to be associated with disease control, as a rebound in the number of mMDSCs was observed at disease progression. Moreover, SM-responsive patients (n=3, 1 SD and 2 PR according RECIST evaluation, mean duration time of response ≥10 months, Table 1) consistently displayed percentages of mMDSCs comparable to HDs. Initial functional characterization of $CD14^+CD11b^+HLADR^{-/low}$ mMDSCs analyzed *ex vivo* from peripheral blood of patients progressing during SM treatment revealed the capacity of these cells to promote STAT3 phophorylation upon IFNα stimulation. IFNα-mediated STAT3 phophorylation did not occur in mMDSCs of SM-responding patients. The role of STAT3 in the development and effector functions of MDSCs is well documented in

murine setting (Gabrilovich *et al*, 2009) and recently suggested as crucial also for human monocytic MDSCs (Poschke *et al*, 2010). However, the functional implication of this alternative STAT3 activation in mMDSC biology, and most importantly, the effects of anti-angiogenic therapy on such a signalling pathway deserve to be further explored. Nevertheless, these data together with the mMDSC boost at progression suggest that SM-treatment might induce/select a qualitatively different mMDSCs population, possibly representing an immune-mediated mechanism of acquired resistance. This 'immunological resistance to treatment' occurred only for the mMDSC compartment, as the SM-induced modulation of Treg and gMDSC frequency was detected in all the treated patients and it lasted for all the duration of the treatment.

In conclusion, our results shed light on a previously unappreciated phenomenon of immune dysfunction in this STS subtype and demonstrate that anti-angiogenic therapy opens a temporal window during which SFT patients regain normalisation in systemic myeloid differentiation status and T-cell functions. Our data indicate that a reduced frequency in circulating mMDSC, gMDSC and Tregs, paralleled by a regained T cell functions occurred in association to disease control, thus suggesting a contribution of the host immunity to the drug efficacy. Moreover, the rebound of circulating mMDSCs and impaired T cell functions at tumour progression suggest that therapeutic strategies aimed at limiting potential residual myeloid suppressor activities (Nagaraj *et al*, 2010; Iclozan *et al*, 2013; Mok *et al*, 2013) and boosting tumour-specific immune responses represent a promising approach to improve the activity of anti-angiogenic treatment in SFT patients and to achieve a more durable control of this aggressive disease. Of note, the recent discovery that SFTs are marked by a tumour-specific chromosomal translocation (NAB2-STAT6) makes this tumour type an attractive target for active immunotherapy (Mohajeri *et al*, 2013; Robinson *et al*, 2013). In fact, the chimeric protein encoded by the recombinant *NAB2-STAT6* gene is a potential reservoir of unique tumour-specific antigens that are now considered crucial in the design of an efficient personalised immunotherapy (Robbins *et al*, 2013; Nadler *et al*, 2002; Tran *et al*, 2014).

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Author contributions

MT, TN, BV, SS, LR, SP and CC made intellectual contributions to the conception and/or design of the study. MT and FR conducted the experiments. BV and AV designed and performed the immunohistochemical and confocal analysis. MT, TN, BV, AV, SS, LR, SP and CC were involved in the interpretation of data. All of the authors were involved in drafting and/or critical revisions of the manuscript and approved the final submitted version.

Conflict of interest

SS: Pfizer coverage for medical meetings, research funding. CPG: Pfizer advisory honoraria, research funding. All of the remaining authors have no conflicts of interest.

List of online Supporting Information

Table S1. Summary of the retrospective series.

Table S2. Panel of antibodies used and immunohistochemical methodological conditions.

Table S3. Flow cytometry antibodies.

Supplementary Materials and methods

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Figure legends

Figure 1. Analysis of tumour-infiltrating immune cells in M/DSFTs not treated with anti-angiogenic therapy. Representative IHC stainings of two targeted therapy-naïve MSFT lesions (Tumour IDs #5 and #8). (A) (H&E) Haematoxylin and eosin staining. Images show MSFT (ID #5) with no or (ID #8) moderate CD3 infiltration. $CD3^+$ T cells, when present, showed positivity for the Foxp3 nuclear marker. Presence of a very high density of CD163-positive macrophages diffusely dispersed among the cancer cells. (ID #5) sparse or (ID #8) absence infiltration of $CD68^+$ macrophages. (B) Double-label immunofluorescence staining for CD14 (red) and CD163 (green) macrophage markers (Tumour ID #4). The arrow indicates $CD163^+CD14^+$ cells. The circle identifies $CD163^+$ cells that do not express CD14.

Figure 2. Analysis of infiltrating immune T cells in SM-treated M/DSFT lesions. (A) Representative IHC stainings of a SM-treated MSFT lesion (Tumour ID #13). (H&E) Haematoxylin and eosin stain. Staining for $CD3^+$ T cells (lower and higher magnification, respectively), $CD4^+$ T cells and $CD8^+$ T cells are showed. Representative images of the expression of T-cell associated markers HLA-DR, granzyme B (GZMB), T-bet and Foxp3. (B) IHC analysis of a SM-treated DSFT lesion (Tumour ID #14) with evidence of tumour regression. In areas of tumour regression T cells (CD3, CD4 and CD8) are organised in clusters. (C) Multiparametric flow cytometry analysis of live lymphocytes from freshly dissociated naive and SM-treated MSFT tumours (Tumour ID #13). Expression levels of T-bet, IFN-γ and granzyme B were evaluated by intracellular flow cytometry in $CD3^+$ T cells. The gating strategy is reported.

Figure 3. Analysis of infiltrating myeloid cells in SM-treated M/DSFT lesions. Stainings representative of SM-treated MSFT lesion (Tumour ID #13). (A) IHC staining for the macrophage-associated markers CD163 and CD68. Higher magnifications of the identified area are shown. (B) Double-label immunofluorescence staining and confocal analysis for (a) CD163 (green) and CD68 (red), (*b*) CD68 (green) and 14 (red), and (*c*) CD68 (green) and HLA-DR (red).

Figure 4. Accumulation of immunosuppressive cells in the peripheral blood of SFT patients. The frequencies of circulating Tregs and mMDSCs were monitored in the peripheral blood of CSFT (n=5) and M/DSFT (n=9) patients compared with healthy donors (HDs) (n=11). (A, B) Percentages of $CD25^{hi}Foxp3^{hi}$ cells (Tregs) and $CD11b^{+}CD14^{+}HLADR^{-/low}$ cells (mMDSCs) defined within $CD3^{+}CD4^{+}$ T cells and $CD14^{+}CD11b^{+}$ cells, respectively. (C, D) Analysis of peripheral $CD3^{+}CD4^{+}$ T lymphocytes and $CD11b^{+}CD14^{+}$ myeloid cells within live-gated PBMCs. (E, F) Frequency of $CD3^{+}$ T cells producing (E) IFN-γ and (F) IL-2 after anti-CD3/CD28 overnight stimulation. Each dot represents one patient. Statistical analysis: two-tailed unpaired Student's t test (95% confidence interval [CI]); only significant P values are shown; bars indicate SEM.

Figure 5. Anti-angiogenic therapy modulates immunosuppression in M/DSFT patients. (A,B,D and E) Anti-angiogenic therapy modulates the frequencies of immunoregulatory cells in M/DSFT patients. PBMCs of M/DSFT patients collected at three time points during anti-angiogenic treatment were analysed for the frequency of (A) Tregs in $CD3^{+}CD4^{+}$ T cells (B) mMDSCs in $CD14^{+}CD11b^{+}$cells. (C) Gating strategy for gMDSC determination; (D) gMDSC detected as CD15+CD66b+ in live gated PBMC (black) or as CD66b+ cells within the $Lin^{-}HLA\text{-}DR^{-}$ fraction (light blue). (E) significantly higher neutrophil absolute count (black dots) and arginase activity (red dots) were found in M/DSFT PRE compared to HD and both decreased during SM treatment. Grey rectangle indicate reference ranges; each dot represent one patient. PRE, PBMCs collected prior anti-angiogenic therapy; T15, PBMCs collected at day 15 during therapy; at progression, PBMCs collected at the time of disease progression, (F, G) Increased levels of circulating mMDSCs correlated with decreased T-cell functionality. PBMCs from M/DSFT patients (n=7) collected at different time points during anti-angiogenic treatment (PRE; T15; at progression) were assayed for (F) IFN-γ and (G) IL-2 secretion in response to overnight activation with anti-CD3/CD28-coated beads. The box plot depicts the median percentages of cytokine-producing $CD3^{+}$ T cells. Statistical analysis: two-tailed paired Student's t test (95% confidence interval [CI]); only significant P values are shown; bars indicate SEM.

Figure 6. Modulation of mMDSC in SM-treated M/DSFT patients. (A) Patients responding to SM-treatment had normal levels of mMDSCs and did not display dysfunctional T cells. (*a*) Frequency of $CD11b^{+}CD14^{+}HLADR^{-/low}$ mMDSCs in PBMCs from M/DSFT patients treated with SM and displaying disease progression (Progression) or responsive to SM-treatment (Response: 2 PR and 1 SD, duration of the response ≥10 months). The same PBMCs as in (*a*) were evaluated for the (*b*) frequency of $CD3^{+}$ T cells producing IFN-γ and (*c*) IL-2 after anti-CD3/CD28 overnight stimulation. (*a*) Each dot represents the data of a single patient. (*b* and *c*) Dot represents the mean value. (B) Representative histograms of pSTAT3 analyses in $CD11b^{+}CD14^{+}HLADR^{-/low}$ cells (mMDSCs) with (black) and without (gray) IFNα stimulation (10000U/mL for 15min at 37℃). (C) Colo umns represent the IFNα-induced STAT3 activation in $CD11b^{+}CD14^{+}HLADR^{-/low}$ cells of HDs (n=4), SM-responsive (n=3) and SM-progressive patients (n=6). Δ%pSTAT3 was calculated as: %pSTAT3 (IFNα)-%pSTAT3 (basal). Columns represent mean values; bars indicate SEM.

Table1. Clinicopathologic characteristics of patients

Patient ID	Tumour Site	Diagnosis	Drug treatment**	Reponse to SM: RECIST evaluation	PFS (months)
1a	Thigh	CSFT	-	-	-
2a	Abdomen	CSFT	-	-	-
3a	Abdomen	CSFT	-	-	-
4a	Pelvis	CSFT	-	-	-
5a	Thigh	CSFT	-	-	-
6a[¥]	Pleura	MSFT	+	PR	12+
7a[#]	Pleura	MSFT	+	SD	6
8a[¥]	Pleura	MSFT	+	SD	10
9a	Abdomen	MSFT	+	PD	1.5
10a[¶¥]	Pelvis	MSFT	+	PR	20
11a	Pleura	MSFT	+	SD	5
12a[*#]	Pleura	DSFT	+[§]	Not assessable[γ]	-
13a[#]	Pleura	DSFT	+	PD	2
14a[#]	Meninges	DSFT	+	PD	1.5
15a	Pleura	DSFT	+	PD	2
16a	Peritoneum	DSFT	-	-	-
17a	Cerebellum	DSFT	-	-	-

Age (median; range): 56; 35-76; Gender (n and %): M 29%, F 71%; Male 5, Female 12. Abbreviations: CSFT, classical solitary fibrous tumour; MSFT, malignant solitary fibrous tumour; DSFT, dedifferentiated solitary fibrous tumour; PFS, progression-free survival.

** Patients received 37.5 mg/die of SM
§ This patient received 800 mg/die of pazopanib
* PBMCs at the time of progression were not available for analysis.
¥ PBMCs from the pre-treatment (PRE) period were not available for analysis.
¶ For this patient, tumour removed after SM treatment was analysed by IHC and corresponded to Tumour ID #13 in TableS1. TILs from Tumour ID #13 were analysed *ex vivo* for their functional activity.
# Patients had undergone a previous chemotherapy regimen. A washout period of at least 15 days was respected before entering SM treatment and beginning blood draws.
γ therapy interrupted due to toxicity.

Figures (Manuscript I)

		marker expression			
Tumour ID	Diagnosis	CD3	Foxp3	CD163	CD68
1	CSFT	-	-	++	±
2	CSFT	±	-	++	-
3	CSFT	±	-	+	±
4	MSFT	-	/	+++	±
5	MSFT	-	/	++	±
6	MSFT	±	+	+	-
7	MSFT	-	/	+++	-
8	MSFT	++	+	++++	-
9	DSFT	+++	++	++++	±
10	DSFT	-	/	++	-
11	DSFT	-	/	+++	-

Summary of IHC results for anti-angiogenic naïve SFT lesions
Abbreviations: CSFT, classical solitary fibrous tumour; MSFT, malignant solitary fibrous tumour; DSFT, dedifferentiated solitary fibrous tumour; /, not done; -, no staining; ±, very weak; +, weak; ++ moderate; +++, strong; ++++, very strong.

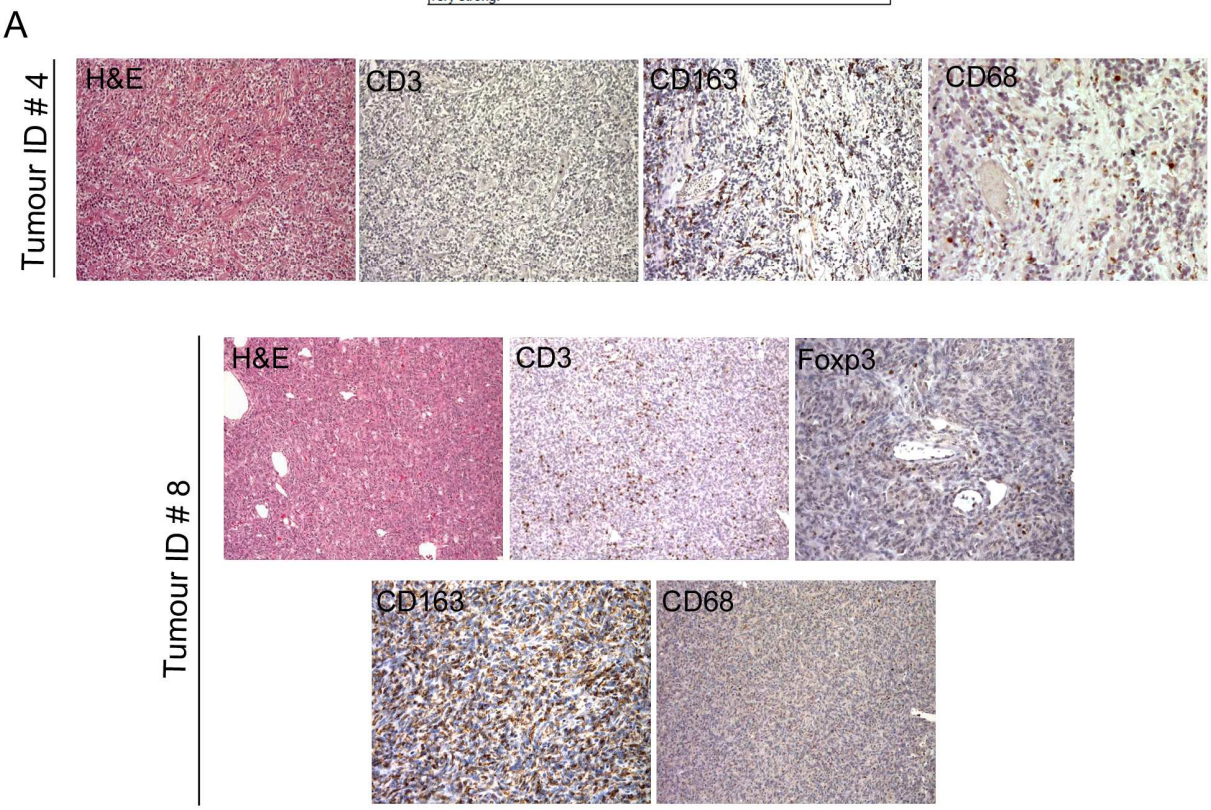


Figure 1

Tumour ID	Diagnosis	T cell marker expression						
		CD3	CD4	CD8	Granzyme B	T-bet	TIA-1	Foxp3
6	MSFT	++	++	++	-	+	/	-
12	DSFT	++	++	++	++	+	+	-
13	MSFT	++++	+++	++	+	++	-	-
14	DSFT	++++	++	++	-	++	++	-

Summary of IHC results for post-SM M/DSFT lesions

Abbreviations: MSFT, malignant solitary fibrous tumour; DSFT, dedifferentiated solitary fibrous tumour; /, not done; -, no staining; ±, very weak; +, weak; ++ moderate; +++, strong; ++++, very strong.

The expression of TIA-1,T-bet and Granzyme B was evaluated inside the T-cell area.

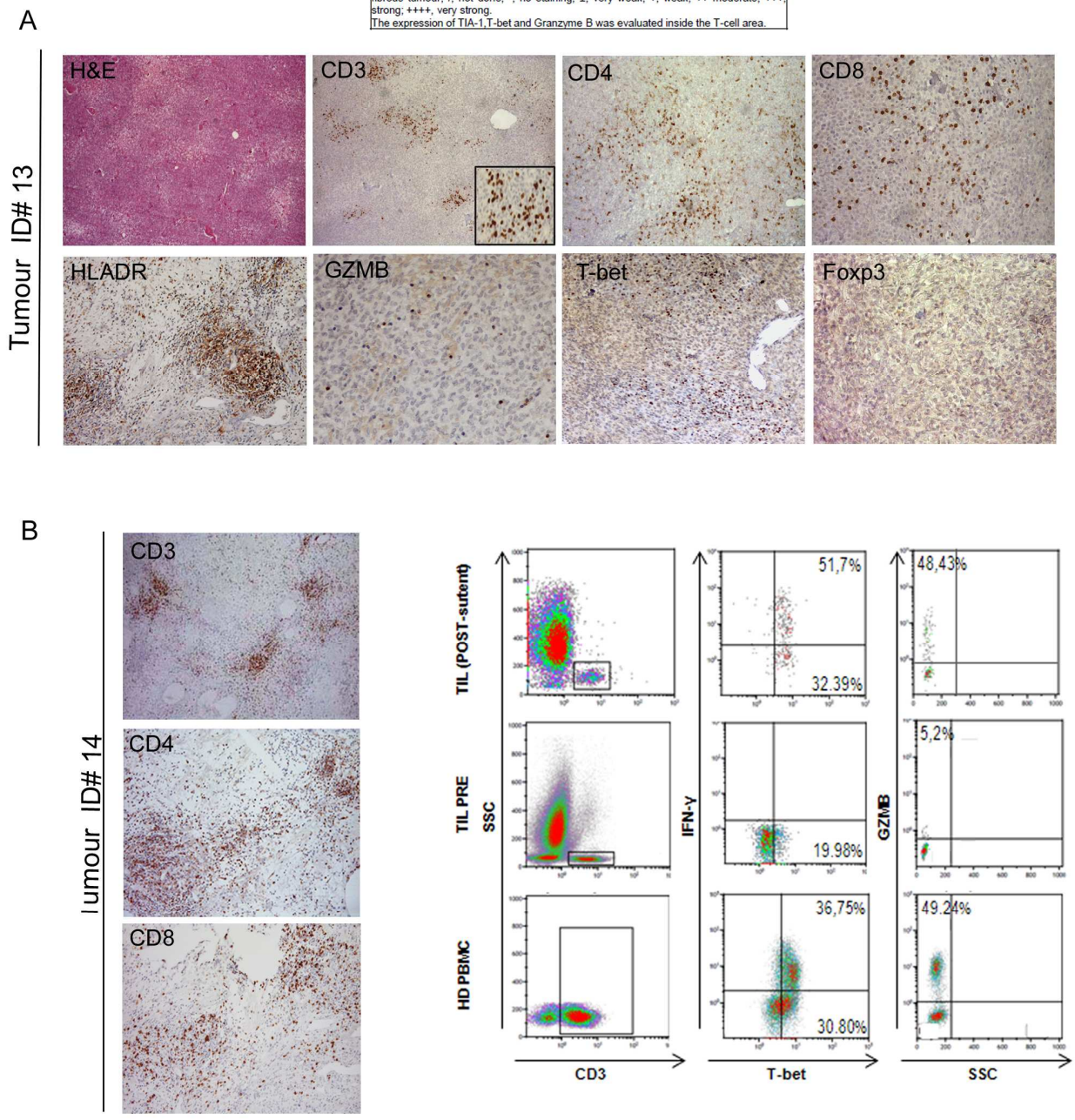
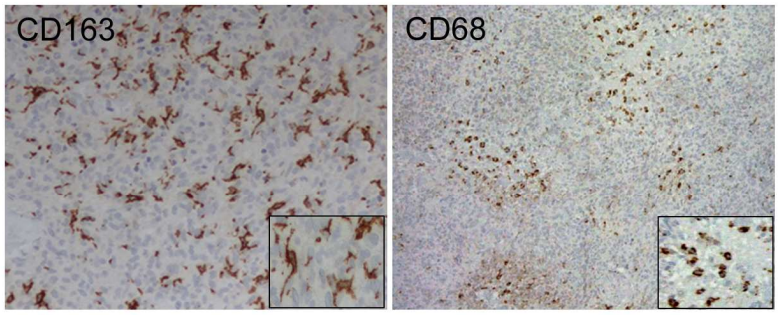


Figure 2

A

Tumour ID	Diagnosis	myeloid marker expression CD163	CD68
6	MSFT	+++	+++
12	DSFT	+++	+++
13	MSFT	++	++++
14	DSFT	+++	+++

Summary of IHC results for post-SM M/DSFT lesions
Abbreviations: MSFT, malignant solitary fibrous tumour; DSFT, dedifferentiated solitary fibrous tumour; ++ moderate; +++, strong; ++++, very strong.



B

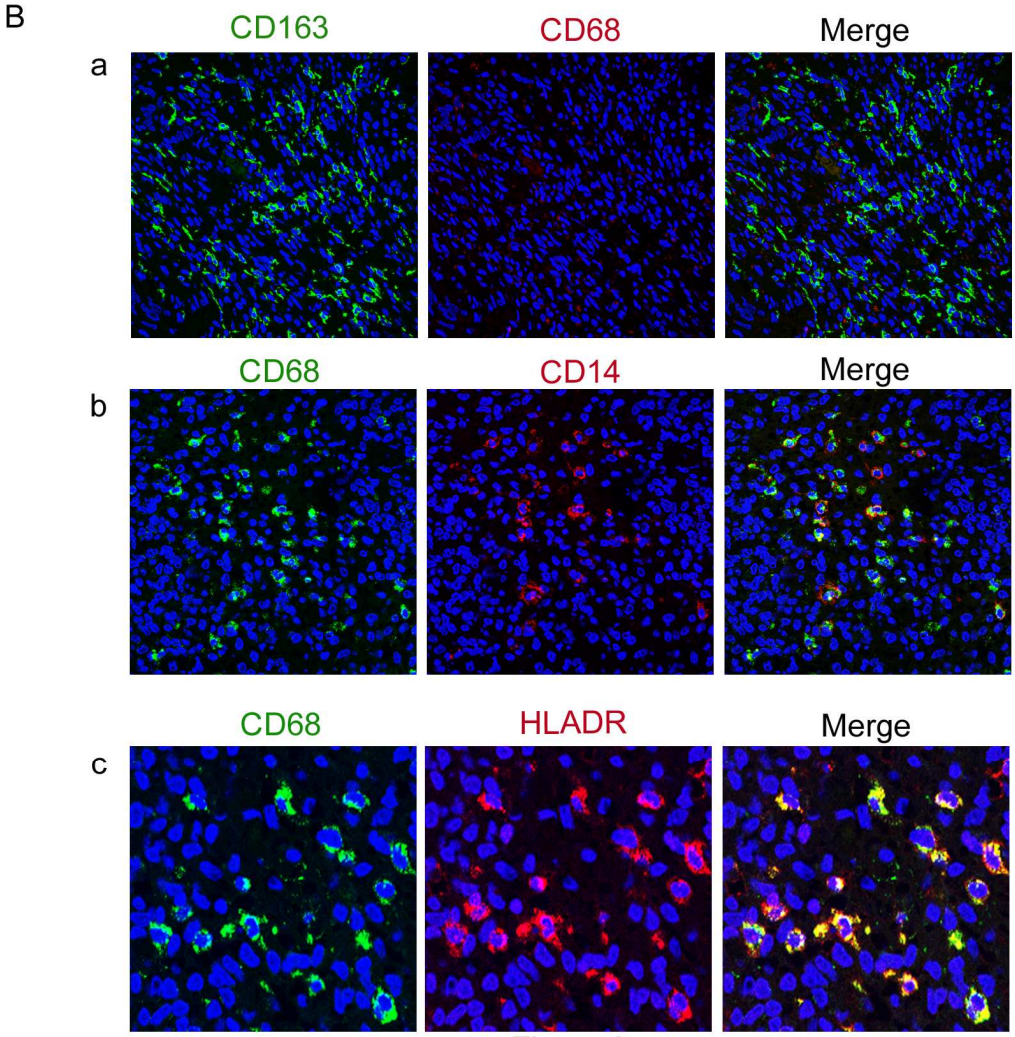


Figure 3

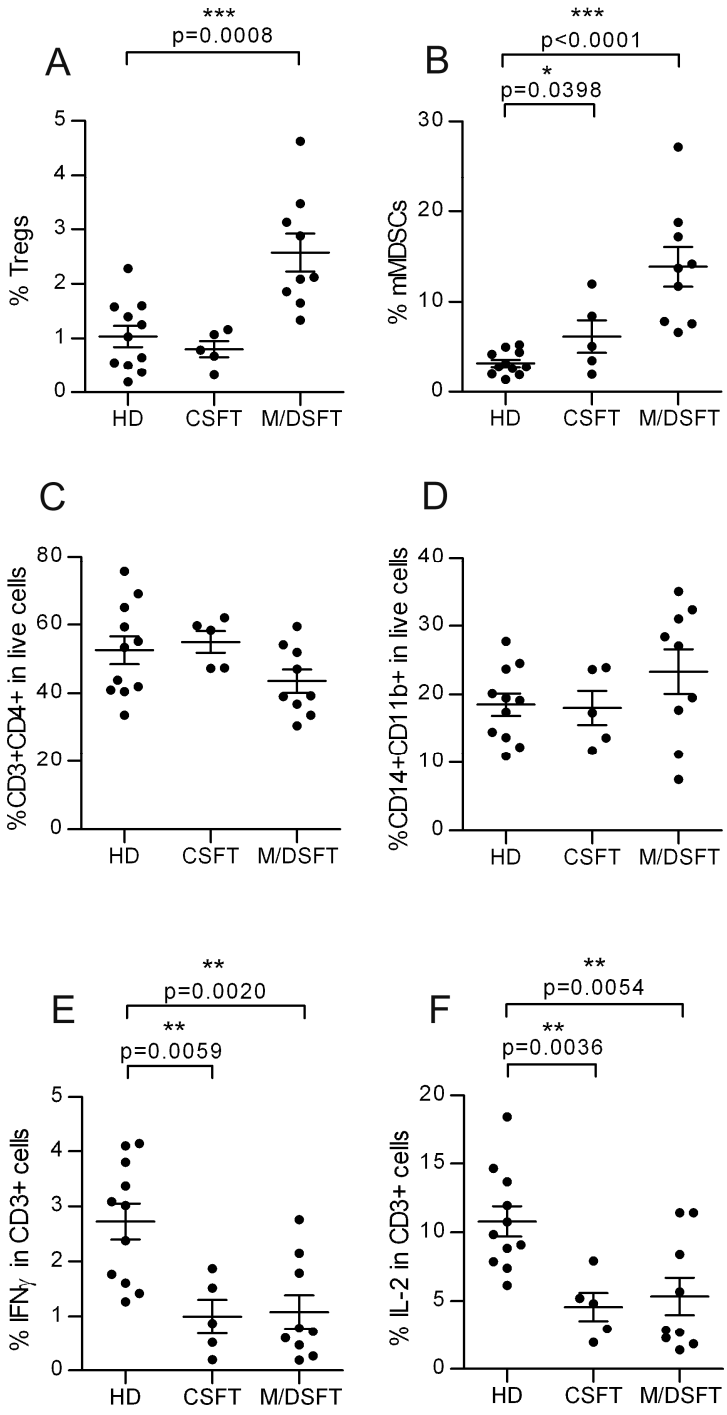

A
***
p=0.0008
% Tregs
HD
CSFT
M/DSFT
B
***
p<0.0001
*
p=0.0398
% mMDSCs
C
%CD3+CD4+ in live cells
D
%CD14+CD11b+ in live cells
E
**
p=0.0020
**
p=0.0059
% IFNγ in CD3+ cells
F
**
p=0.0054
**
p=0.0036
% IL-2 in CD3+ cells

Figure 4

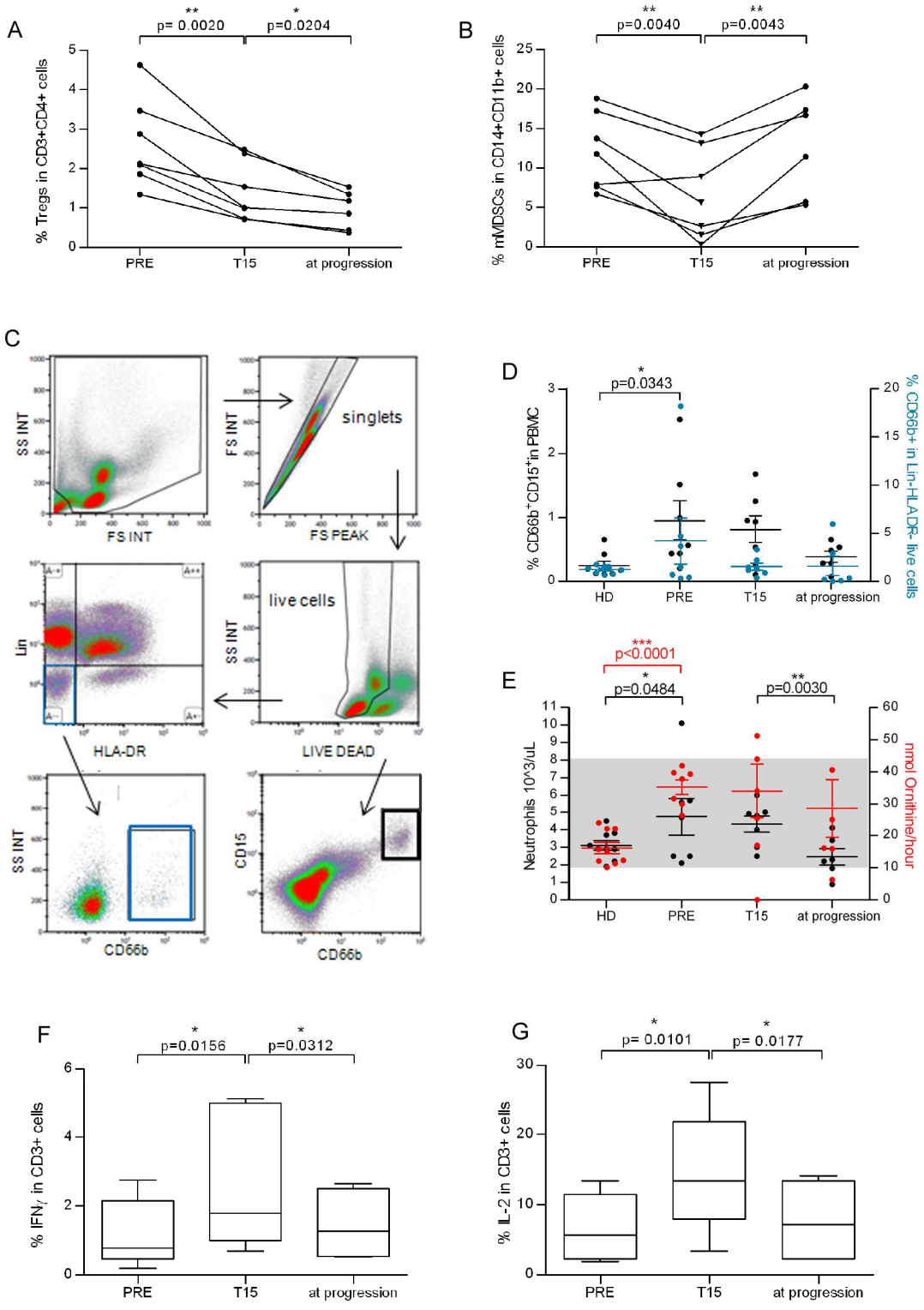

A
** p= 0.0020
* p=0.0204
% Tregs in CD3+CD4+ cells
PRE
T15
at progression
B
** p=0.0040
** p=0.0043
% mMDSCs in CD14+CD11b+ cells
PRE
T15
at progression
C
SS INT
FS INT
singlets
FS INT
FS PEAK
Lin
HLA-DR
live cells
SS INT
LIVE DEAD
SS INT
CD66b
CD15
CD66b
D
* p=0.0343
% CD66b+CD15+ in PBMC
% CD66b+ in Lin-HLADR- live cells
HD
PRE
T15
at progression
E
*** p<0.0001
* p=0.0484
** p=0.0030
Neutrophils 10^3/uL
nmol Ornithine/hour
HD
PRE
T15
at progression
F
* p=0.0156
* p=0.0312
% IFNγ in CD3+ cells
PRE
T15
at progression
G
* p= 0.0101
* p= 0.0177
% IL-2 in CD3+ cells
PRE
T15
at progression

Figure 5

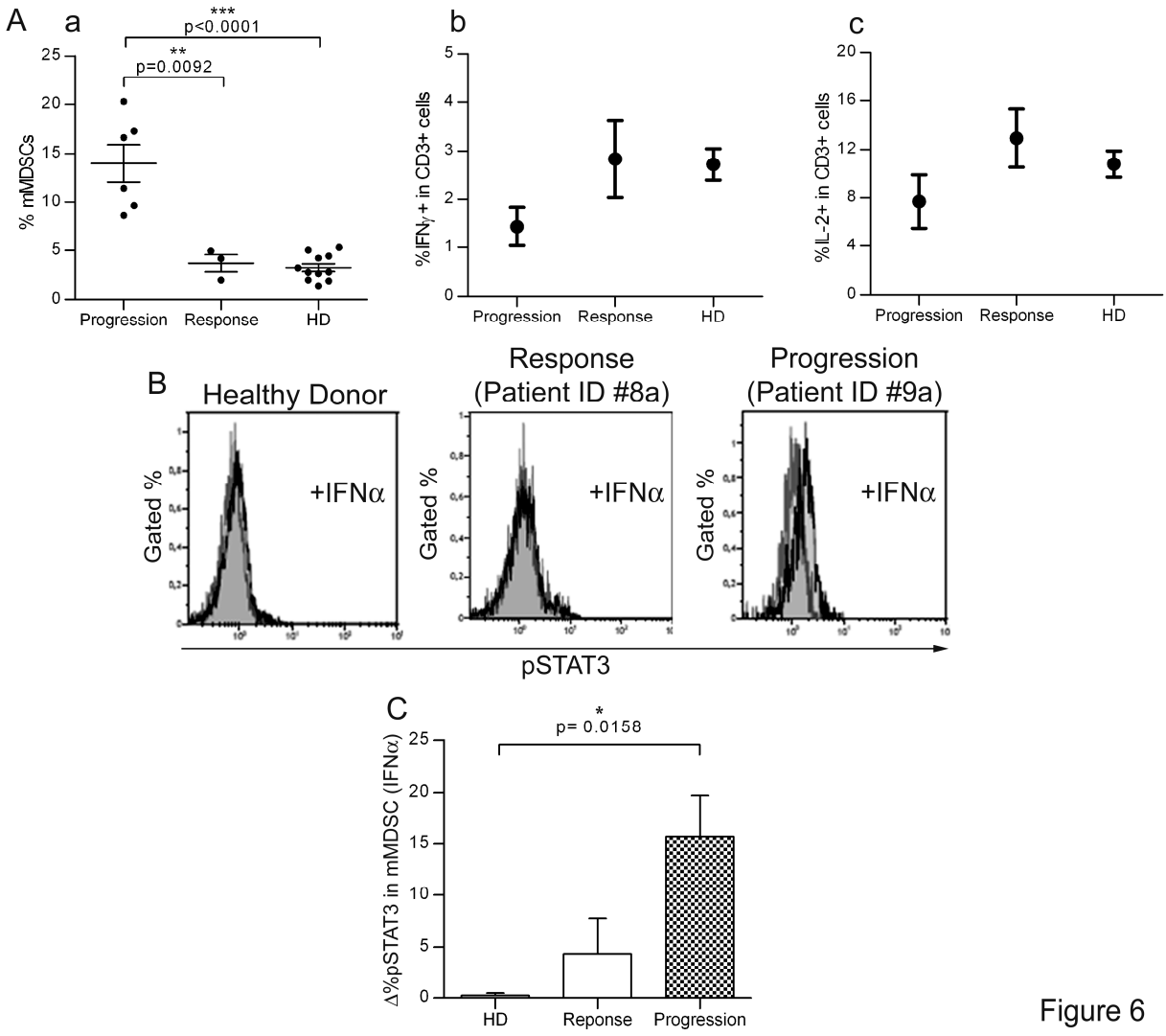

A
a
***
p<0.0001
**
p=0.0092
% mMDSCs
Progression
Response
HD
b
%IFNγ+ in CD3+ cells
Progression
Response
HD
c
%IL-2+ in CD3+ cells
Progression
Response
HD
B
Healthy Donor
Response
(Patient ID #8a)
Progression
(Patient ID #9a)
Gated %
+IFNα
pSTAT3
C
*
p= 0.0158
Δ%pSTAT3 in mMDSC (IFNα)
HD
Reponse
Progression

Figure 6

Supplementary Materials and methods

Immunohistochemistry and confocal analysis

After xylene deparaffinisation and rehydration, the sections were incubated in a 3% H_2O_2 solution for 10 minutes to block endogenous peroxidase. Antigen retrieval was performed by heating the sample in a 5-mM EDTA (pH 8) or Tris-EDTA buffer or 5-mM citrate buffer solution in a high-pressure cooker for 10-15 or 20 minutes and cooled for 15 minutes prior to immunostaining. A peroxidase-labelled polymer was used for the detections according to the manufacturer's instructions and visualised using 3,3′-diaminobenzidinetetra hydrochloride (DAB)/H_2O_2. Sections were assessed using a semi-quantitative scoring system. Double immunofluorescence staining was performed as follows: after deparaffinisation and antigen retrieval, sections were treated briefly with 0.1 M glycine in PBS (pH 7.4) followed by 0.3% Triton X-100 buffer and incubated overnight at 4℃ with the primary antibodies. The samples were washed and incubated for 1 h at RT with appropriately conjugated secondary antibodies. Following a final wash, they were mounted on glass slides with 95% glycerol in PBS.

RT-PCR

Total RNA was extracted from frozen specimens, reverse transcribed and amplified using two sets of primers for the NAB2-STAT6 fusion construct, as described previously [19,20]. The PCR products were directly sequenced using the Big Dye v1.1 cycle sequencing kit (Applied Biosystems) on a 3500Dx Genetic Analyzer (Applied Biosystems).

Legends to supplementary Figures

Figure S1. Representative example of intratumoral CD66b immunostaining. The top and the middle panels display sparse $CD66b^+$ cells infiltration in one of the analysed lesion (Tumour ID #4). High expression of the CD66b marker was detected inside the spleen (top panel, right) and was used as positive control of the staining. The bottom panel shows positivity of the analysed marker in a perivascular area (Tumour ID #8).

Figure S2. Analysis of infiltrating $CD68^+$ myeloid and $CD3^+$ T cells in chemotherapy (CT) and radiotherapy (RT)-treated M/DSFT lesions.

Figure S3. Anti-angiogenic therapy modulates immunosuppression in M/DSFT patients. Modulation of Tregs (top graph) and mMDSCs (bottom graph) detected within total live cells. Student's t test (95% confidence interval [CI]); only significant p values are shown.

Supplementary Tables (Manuscript I)

	Tumour ID	Diagnosis	Duration of treatment before surgery	Tumor Site	Tumor Material (Primary-Recurrence-Metastasis);Assessment
Pretherapy lesions	1	CSFT		Retroperitoneum	primary; surgical specimen
	2	CSFT		Thigh	primary; surgical specimen
	3	CSFT		Trapezium	primary; surgical specimen
	4	MSFT		Thigh	primary; surgical specimen
	5	MSFT		Abdomen	primary; surgical specimen
	6*	MSFT		Peritoneum	metastasis; surgical specimen
	7	MSFT		Retroperitoneum	primary; surgical specimen
	8	MSFT		Pleura (multiple lesions)	Local Recurrence; surgical specimen
	9	DSFT		Retroperitoneum	primary; surgical specimen
	10	DSFT		Lung	metastasis; surgical specimen
	11	DSFT		Retroperitoneum	primary; surgical specimen
post-SM lesions	6*	MSFT	10 months (SM)	Peritoneum	metastasis; surgical specimen
	12	DSFT	5 months (SM)	Lung	metastasis; surgical specimen
	13*	MSFT	6 months (SM)	Pelvis (multiple lesions)	Local Recurrence; surgical specimen
	14*	DSFT	3 months (SM)	Peritoneum (multiple lesions)	metastasis; surgical specimen
post-CT/RT lesions	13*	MSFT	6 months (CT) §	Pelvis	Local Recurrence; surgical specimen
	14*	DSFT	5 months (CT/RT)#	Peritoneum (multiple lesions)	metastasis; surgical specimen
	15	DSFT	7 months (CT/RT)¶	Pelvis	Local Recurrence; surgical specimen
	16	MSFT	5 months (CT) ¥	Lung	metastasis; surgical specimen

Table S1. Summary of the retrospective series

Abbreviations: CSFT, classical solitari fibrous tumour ; MSFT, malignant solitary fibrous tumour; DSFT, dedifferentiated solitary fibrous tumour; SM, sunitinib; CT, chemotherapy; RT, radiotherapy

Note :*paired autologous samples; §second-line treatment with epirubicin; #high-dose ifosfamide plus readiotherapy; ¶epirubicin and ifosfamide plus radiotherapy; ¥doxorubicine plus dacarbazine.

Antibody	Clone	Isotype	Company	Dilution	Antigen Retrieval
Primary antibodies:					
CD3	PS1	mouse monoclonal IgG2a	Novocastra	1:50 IHC	citrate buffer 15min
CD4	4B12	mouse monoclonal IgG1	Novocastra	1:50 IHC	EDTA 15min
CD8	C8/144B	monocolonal mouse IgG1,k	Dako	1:20 IHC	citrate buffer 15min
Foxp3	259D/C7	monocolonal mouse IgG1	BD	1:100 IHC	EDTA 15min
CD163	10D6	monocolonal mouse IgG1	Novocastra	1:100 IHC	citrate buffer 15min
CD68	kp1	monocolonal mouse IgG1,k	Dako	1:200 IHC/ 1:20 IF	EDTA 10min
CD68	PGM1	Monoclonal mouse IgG3,k	Dako	1:200 IHC/ 1:40 IF	EDTA 15min
HLADR	LN3	monoclonal mouse IgG2b	ThermoScientific	1:400 IHC/ 1:40 IF	citrate buffer 15min
Tbet	polyclonal H-210		S.Cruz Biotecnology	1:20 IHC	EDTA 20min
Secondary antibodies:					
Alexa Fluor® 488 goat anti mouse IgG1			Life Technologies	1:150	
AlexaFluor® 568 goat anti mouse IgG2b			Life Technologies	1:150	
Rat biotin anti- mouse IgG3 + Streptavidin AlexaFluor® 568			Biolegend (biotin)+Immunological science (streptavidin)	1:100 biotin 1:300 strept.	

Table S2. Panel of antibodies used and immunohistochemical methodological conditions

Abbreviations: IHC, Immunohistochemistry; IF, Immunofluorescence

Marker	Clone	Isotype	Fluorochrome
CD3[1]		$IgG1_k$	PE
CD4[1]	RPA-T4	$IgG1_k$	APCH7
CD8[2]	B9.11	$IgG1_k$	Krome Orange
CD11b[2]	Bear1	$IgG1_k$	PECy7
CD14[1]	MoP9	IgG2b	APCH7
CD19[1]	HIB19	IgG1	FITC
CD20[1]	2H7	IgG2b	FITC
CD25[1]	M-A251	$IgG1_k$	PC7
CD33[1]	WM53	$IgG1_k$	Alexa700
CD45RA[1]	HI100	IgG2b	FITC
CD56[1]	NCAM16.2	IgG2b	FITC
HLADR[1]	G46.6	IgG2a	APC
FOXP3[3]	PCH101	Rat $IgG2a_k$	APC
T-bet[3]	4B10	$IgG1_k$	PE
IL2[1]	MQ1-17H12	$IgG2a_k$	PE
IFNγ[4]	4S-B3	$IgG1_k$	a488
Granzyme B[1]	GB11	$IgG1_k$	PE
isotype controls			
	G155-178[1]	IgG2a	APC
	MOPC-21[1]	$IgG1_k$	PE
	G155-178[1]	IgG2b	FITC
	eBR2a	Rat $IgG2a_k$	APC

Table S3. Flow cytometry antibodies

Notes: [1]BD Biosciences, San Jose, CA ; [2]Beckman Coulter , Fullerton, CA; [3]eBioscence, San Diego, CA; [4]Biolegend,San Diego, CA.

Prior to staining, each antibody was titrated to determine its optimal dilution using PBMC obtained from HD

Supplementary Figures (Manuscript I)

Tumour ID	Diagnosis	CD66b
4	MSFT	±
5	MSFT	-
6	MSFT	-
8	MSFT	-*

Summary of CD66b expression in anti-angiogenic naïve SFT lesions

Abbreviations: MSFT, malignant solitary fibrous tumour -, no staining; ±, very weak.

* only perivascular expression

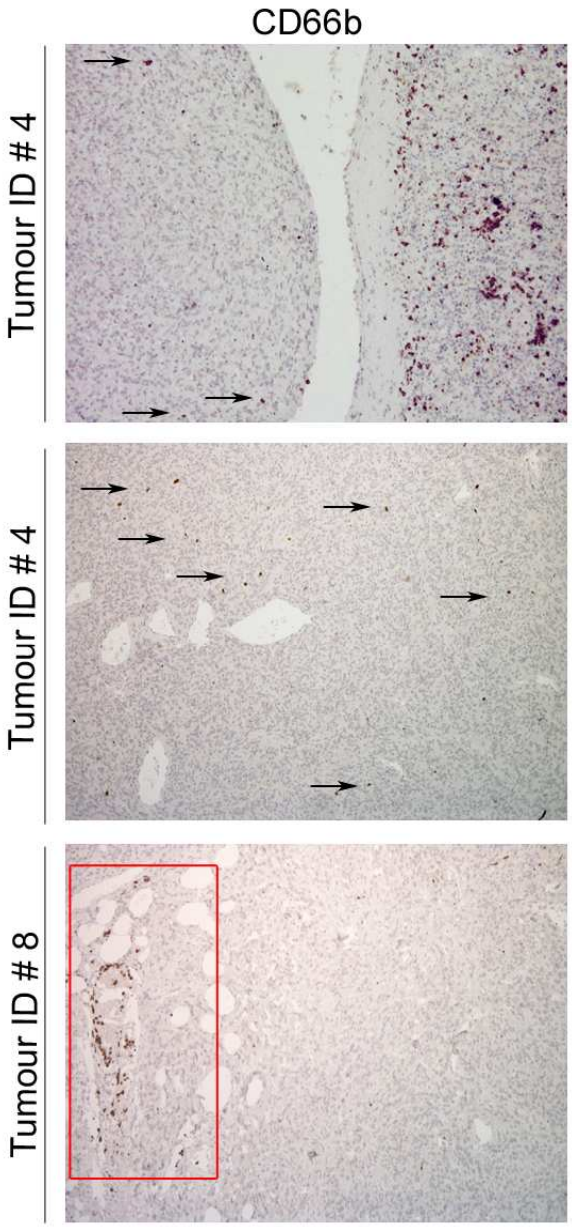


Figure S1

		marker expression	
Tumour ID	**Diagnosis**	**CD3**	**CD68**
13	MSFT	±	-
14	DSFT	++	±
15	DSFT	+	±
16	MSFT	++	+

Summary of IHC results for post-chemo/radiotherapy M/DSFT lesions

Abbreviations: MSFT, malignant solitary fibrous tumour; DSFT, dedifferentiated solitary fibrous tumour; ±, very weak; +, weak; ++ moderate.

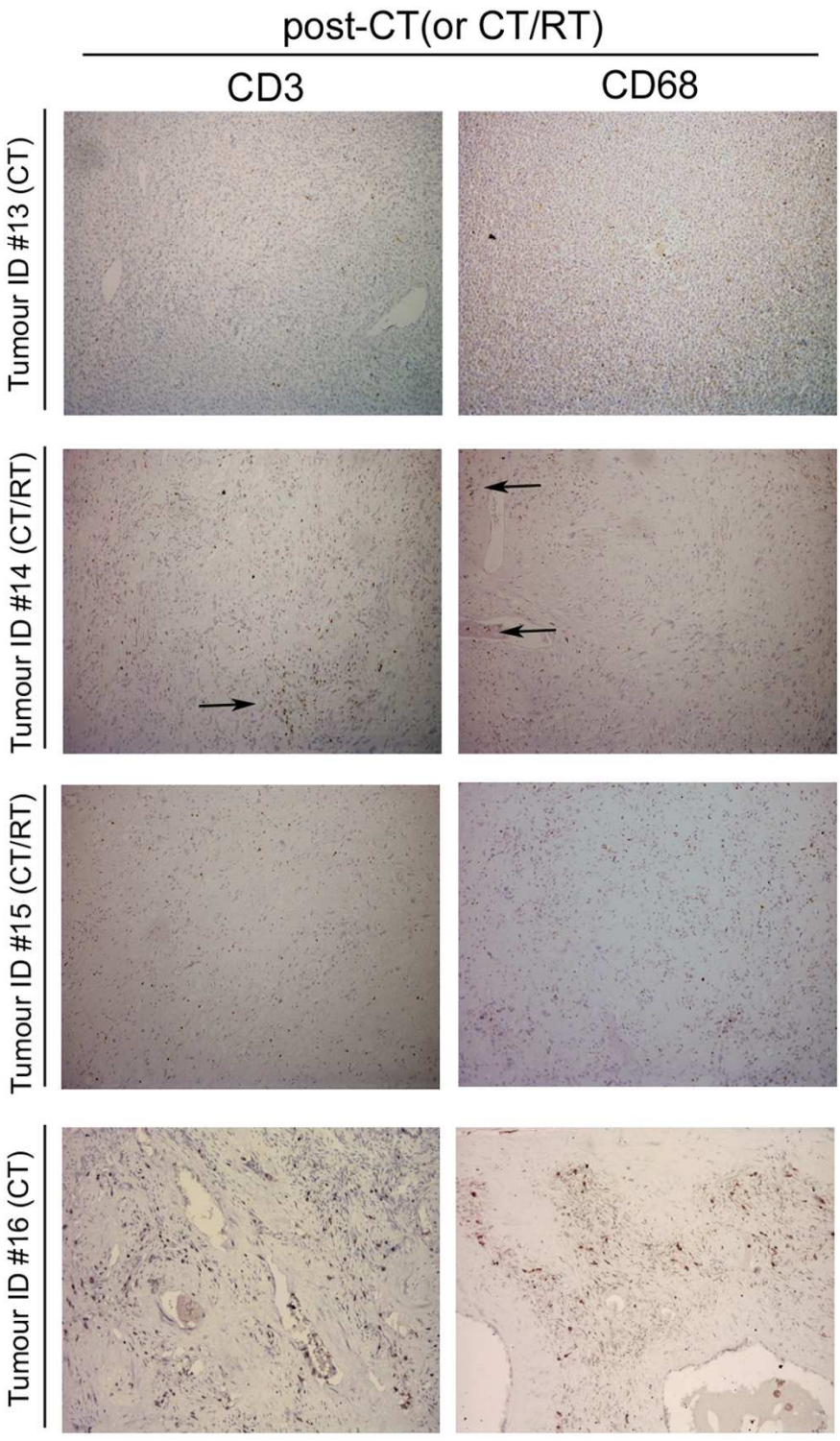


Figure S2

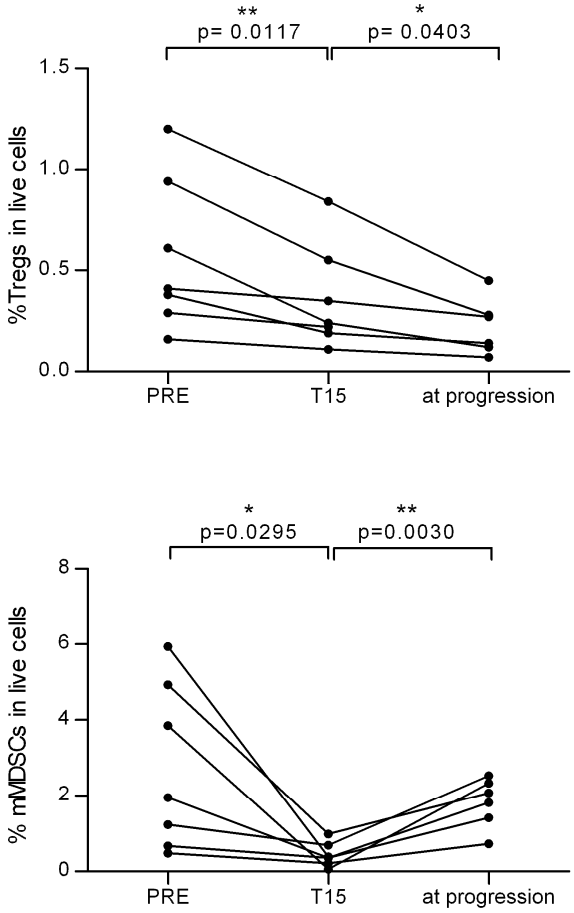

**
p= 0.0117
*
p= 0.0403
% Tregs in live cells
1.5
1.0
0.5
0.0
PRE
T15
at progression
*
p=0.0295
**
p=0.0030
% mMDSCs in live cells
8
6
4
2
0
PRE
T15
at progression

Figure S3

Manuscript II

[Manuscript in preparation]

Target Journal: BMC Cancer

Title: Melan-A/MART-1 immunity in a clear cell sarcoma patient treated with sunitinib: a case report

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Abstract

Background: Clear cell sarcoma (CCS), initially named malignant melanoma of soft parts, is an aggressive soft tissue sarcoma that, due to MITF activation, shares with melanoma the expression of melanocyte differentiation antigens. CCS is poorly sensitive to chemotherapy and, based on the presence of activated PDGFRβ and c-Met, multikinase inhibitors have been used as therapeutic agents. In the case we report here, treatment with sunitinib induced a long-lasting clinical response that was associated with an immune activation directed against Melan-A/MART-1 antigen.

Case presentation: Here we describe a case of a 28 years old female patient with an advanced molecularly confirmed CCS, initially arising from the deep soft tissue of the left foot removed in 2007. Sunitinib was started in January 2012 at the dose of 37.5 mg/day, due to disease progression with radiologic response. In April 2012 residual tumor was removed with evidence of pathologic response and loss of the Melan-A/MART1 antigen on surgical specimen. Immunological monitoring during treatment with sunitinib showed a reduce frequency of immunosuppressive cells and the presence of a systemic immunity directed against the Melan-A/MART-1 antigen in the patient' blood. Patient relapsed and sunitinib was restarted in May 2012, with a new response, and continued for 4 months although with repeatedly interruptions due to toxicity. Disease progression and new responses were documented at each treatment interruption and restart. Sunitinib was definitively interrupted in April 2013 for disease progression.

Conclusion: The analysis of this case proves that antigens expressed by CCS, as for melanoma, can be immunogenic *in vivo* and that tumor-antigen specific T cells may exert anti-tumor activity in CCS patient. Thus, manipulation of the immune response may have therapeutic potential for this soft tissue sarcoma (STS) subtype and immunotherapy approaches, such as those using the antibodies ipilimumab and nivolumab, directed to the inhibitory immunological checkpoints, can be promising therapeutic options for these patients.

Keywords: sarcoma**,** sunitinib, clear cell sarcoma, antigen specific T cell, immunotherapy, chemotherapy.

Background

Clear cell sarcoma (CCS) is a very rare and aggressive soft tissue sarcoma (STS), usually arising from deep soft tissue or viscera [1], and marked by a very high metastatic risk resulting in a 5-year overall survival of about 50% [2-4]. In contrast with other STS, and similarly to melanoma, its metastatic sites include lymph nodes. CCS, initially named malignant melanoma of soft parts [5], are molecularly characterized in most cases by a specific translocation, t(12;22)(q13;q12), which results in fusion of the Ewing's sarcoma gene, EWS, with the cyclic AMP (cAMP) regulated transcription factor, ATF1, a member of the cAMP-responsive element binding protein (CREB) family [6]. The EWS-ATF1 chimeric fusion protein interacts with the MITF (melanocyte master transcription factor) promoter, thus it directly and aberrantly activates MITF expression. Consequently, CCS is characterized by the expression of the melanocytic differentiation markers HMB45/gp100 and melan-A/MART-1 [7]. Overall, several immunophenotypic and molecular features are shared between CCS and malignant melanoma. Importantly, a proportion of CCS cases lack specific translocation and thus, clinical presentation as well as FISH analysis and RT-PCR for the specific translocation are crucial to distinguish the two entities. Receptor tyrosine kinase expression/activation [8] and gene expression analysis [9] indicate that MITF drives the same down-stream pathways in CCS and in melanoma and that PDGFRβ and c-Met, are expressed by CCS [10,11]. Moreover, BRAF activating mutations have been occasionally detected in both EWS-ATF1 positive and negative CCS [8,12,13]. CCS is poorly sensitive to chemotherapy and anecdotal responses to regimens containing dacarbazine, vincristine, anthracycline, and cyclophosphamide and to interferon-alpha-2b [14] have been reported. Based on the molecular features described above, multi-kinase inhibitors have been used as therapeutic agents in this STS and objective responses to sunitinib, and sorafenib treatments have been recently reported [15-16]. Here we describe a case of a 28 years old female patient with a metastatic, translocated CCS who experienced a prolonged, objective response to sunitinib. We consider this case of interest as objective response to sunitinib paralleled the down-modulation in the frequency of immunosuppressive cells in the periphery, the presence of a systemic immunity directed against the CCS associated antigen Melan-A/Mart-1 and the *in vivo* immune selection of post-sunitinib, MART-1 negative tumor. The analysis of this case proves that antigens expressed by CCS, as for the melanoma, can be immunogenic *in vivo* and that tumor-antigen specific T cells may exert anti-tumor activity *in vivo* in CCS patient. Thus immunotherapy approaches, such as those using the antibodies ipilimumab and nivolumab, directed to the inhibitory immunological checkpoints, alone or in association with anti-angiogenic therapy, are promising therapeutic options for these patients.

Case presentation

A female patient aged 28 years presented in 2007 with a lesion arising from the deep soft tissue of the left foot, covered by a healthy skin. Prior clinical history was negative for melanoma. The lesion was removed together with loco-regional lymph nodes (LN) with diagnosis of clear cell sarcoma (CCS) (surgery 2007), and confirmed by the positivity of the FISH analysis for EWS-ATF1. Three of five LN were also positive for disease. A loco-regional and inguinal LN relapse was detected in July 2011 and treated with chemotherapy with doxorubicin plus dacarbazine for 5 cycles with response. Given the evidence of a new disease progression and based on preliminary evidence of sunitinib possible activity in CCS [15], in January 2012 sunitinib was started at the dose of 37.5 mg/day, with a tumor partial response to the lesion located on left foot and a complete response to metastasis on upper left leg. The response was confirmed by PET and CT scan (Figure 1). In April 2012, patient underwent left leg amputation, with evidence of pathologic response to sunitinib in the surgical specimen. In May 2012, sunitinib was restarted and maintained at the same dosage. During these months of treatment, sunitinib was repeatedly stopped due to toxicity, with evidence of rapid disease progression following treatment interruption and of a new response after restoring treatment. From January 2013, sunitinib was finally reduced to 12.5 mg/day due to Grade 3 cardiac toxicity. After initial disease stabilization, disease progression occurred and sunitinib was definitively interrupted in April 2013. Patient died of disease in February 2014.

The expression of the MITF regulated melanocytic antigens (HMB-45/gp100 and Melan-A/MART-1) and S-100 was assessed by immunohistochemistry on pre- and post-sunitinib tumor specimens (surgery dic-2010/nov-2011and apr-2012, respectively). Pre-treatment tumor lesions displayed a clear positivity for all of the analyzed antigens. Conversely, tumor specimen removed after treatment with sunitinib (surgery april-2012) displayed a selective loss of MART-1 expression, while it retained the positivity for HMB-45 and S-100 (Figure 2A). Post-sunitinib tumor was heavily infiltrated by $CD3^+$ T cells that contained a significant proportion of $CD8^+$T cells. Areas with pathological regression were clearly evident in association with lymphocyte infiltration (Figure 2B). The *in vivo* generation of the MART-1 loss antigen variant was associated with the presence of anti-MART-1 systemic immunity in the blood of this CCS patient. Patient's peripheral blood mononuclear cells (PBMCs) isolated in the course of sunitinib treatment and before surgery (surgery april-2012), sensitized *in vitro* with the immunogenic HLA-A*0201 restricted peptide Melan-A/MART-$1_{[27L]}$ displayed the presence of a remarkable frequency of MART-1 specific $CD8^+$T cells (7,72%), as monitored by pentamer staining (Figure3). These anti-MART-1 specific T cells were functionally active. MART-1 sensitized PBMC released IFNγ when stimulated with the target

cells loaded with Melan-A/MART-1-epitope (modified and native) and, importantly, they recognized in a MHC restricted fashion HLA-A*0201^{+}MART1^{+}, but not HLA-A*0201^{+}MART1^{-} and HLA-A*0201^{-}MART1^{+} tumor cells as evaluated by ELIspot assay (Figure 3). Conversely, no T cells specific for the HLA-A*0201- gp100$_{[210M]}$, peptide was evidenced in post-sunitinib PBMCs of the patient applying the same procedure . All together these evidences strongly support the conclusion that the post-sunitinib MART-1 negative tumor variant was the *in vivo* outcome of a T cell-mediated immune selection occurring in CCS patient during sunitinib treatment. The anti-MART-1 systemic immunity in CCS patients was associated with the release of immune suppression in post-sunitinib PBMC of the patient. Multi-parametric flow cytometry indicate that the peripheral frequency of CD3^{+}CD4^{+}CD25^{hi}Foxp3^{hi} regulatory T cells (Tregs) and CD14^{+}CD11b^{+}HLADR$^{neg/low}$ monocytic myeloid-derived suppressor cells (mMDSCs), expanded in cancer patients, including melanoma [17-20] , was down-modulated in PBMCs collected during sunitinib treatment (Fig. 4A and 4B). Down-modulation of suppressive cells correlated with a generalized boost in the functional activity of peripheral T cells measured as IL-2 and IFN-γ produced *ex vivo* upon TCR stimulation by CD3^{+} cells of post-sunitinib patients' PBMCs (Figure 4C).

Conclusions

We described herein the case of a CCS (HLA-A*0201) patient with advanced disease that displayed a long-lasting response to treatment with the anti-angiogenic drug sunitinib. In this patient, objective response, obtained during sunitinib treatment, was associated with anti-tumor immunity evidenced in the periphery by the high frequency of cytokine competent anti-MART-1 T cells and, at the tumor site, by signs of pathological regression associated with $CD3^+/CD8^+$T cell infiltration and with the *in vivo* immune selection of MART-1 negative antigen loss tumor variant. The study of this clinical case shows that antigen expresses by CCS can be immunogenic *in vivo* and indicates that manipulation of the immune response may have therapeutic potential in this STS subtype. As melanoma, CCS express the MITF-regulated genes including gene encoding for the melanoma differentiation antigens. Thus we look at the presence of antigen-specific response in this CCS patient. Interestingly, we observed that tumor specimen resected after treatment with sunitinib had lost the expression of MART-1 antigen. The *in vivo* generation of MART-1 loss variant was associated to a $CD3^+CD8^+$ T cell infiltration and to the presence of areas of pathologic regression thus suggesting the *in vivo* occurrence of MART1-specific response. This hypothesis was further supported by the finding that functionally active anti-MART-1 T cells were detectable in the blood of this patients collected during sunitinib treatment. Altogether, these evidences strongly support the conclusion that the post-sunitinib MART-1 negative tumor variant was the *in vivo* outcome of a T cell-mediated immune selection occurring in CCS during sunitinib treatment and demonstrate the immunological response toward a melanocyte differentiation antigen, shared with melanoma, in this patient. To our knowledge this is the first report documenting the *in vivo* immunogenicity of CCS tumor. However, this response was limited to Melan-A/MART-1, the most immunogenic antigen in melanoma and no specific $gp100^+$ T cells were detected in the blood of this patients and reactivity for HMB45/gp100 was maintained in post-sunitinib surgical specimen. In the peripheral blood of this patient, we observed that sunitinib treatment induced a sustained down-modulation of the frequency of immune suppressive cells, Treg and mMDSC, and a parallel reactivation of a generalized T cell function evaluated as the capacity of $CD3^+$T cells to release Th1 cytokines in response to a polyclonal stimulation. The immunomodulatory function of sunitinib has been clearly documented in other human tumors and we confirmed this activity in the setting of CCS [21, 22]. However, our observations also suggest that the release in the immune suppression induced by sunitinib, may have unleashed anti-tumor immunity in this CCS patient. Indeed, this hypothesis is in agreement with recent findings showing that, in melanoma patients, antigen- specific responses are prevented by the presence of high frequency of circulating

mMDSCs [23], while decrease of their number is favoring the clinical response in patients treated with immunotherapy [24].

In conclusion, the study of this case shed light on immune-similarities between CCS and melanoma, and indicates that manipulation of the immune response in this STS subtype likely evokes antigen-specific response. In addition to T cells specific for MITF- regulated antigens, this response may potentially include also T cells recognizing unique, mutation - specific determinants. In fact, as previously shown by *in vitro* immunological assays [25], the chimeric protein encoded by the specific chromosome translocation of CCS is certainly a source for these type of antigens and it is well known that immune response directed to mutated antigens plays a crucial role in determining tumor rejection and clinical responses in cancer patients under immunotherapy regimens [26,27]. Although generalized conclusion cannot be depict from a single case, these findings suggest that immunotherapy, exploiting antibodies directed to immunological checkpoints such as ipilimumab (anti-CTLA4) or nivolumab (anti-PD1) now in use for melanoma patients, may offer, alone or in association with targeted-therapies, a new therapeutic option for advanced CCS patients, for which no successful therapies are currently available.

Materials and methods

PBMCs and cell lines

PBMCs were obtained by Ficoll density gradient centrifugation followed by cryopreservation. The A375mel and the lymphoblastoid cell line T2 were obtained from the American Type Cell Culture (ATCC). All these cell lines were cultured in RPMI 1640 (Lonza) supplemented with 10%FCS (Lonza), Hepes and antibiotics. For tumor cell line immuno-phenotyping, the FITC–labeled BB7.2 monoclonal antibody (BD Bioscence) was used.

Immunohistochemical analysis of antigen expression in tumor biopsies

5-µm thick formalin-fixed, paraffin-embedded (FFPE) tissue sections were processed for IHC staining. The monoclonal antibodies used were directed against the following antigens: anti-S100, anti-Melan A, anti-HMB45, anti-CD8 (DAKO) and anti-CD3 (Novocastra).

Lymphocyte stimulation and ELISPOT assay

PBMCs isolated from the patient were thawed and cultured in the presence of the HLA2-A*0201 restricted-modified peptides (Melan-A/MART-$1_{[27L]}$ or $gp100_{[210M]}$) (2umol/L) plus 60IU/mL IL-2 (Proleukin). The cells were tested every 10 to 14 days by flow cytometry analysis for the enrichment of $CD8^{+}$pentamer^{+} T cells. To assess their reactivity against tumor cells, IFN-γ release was determined by ELISpot assay (Mabtech) in the presence of

MART1 (modified or native)-pulsed (2umol/L) T2 or HLA-A*0201$^{+/-}$ (MART$^{+/-}$) melanoma cell lines. HLA class I-blocking experiments required preincubation of target cells with the W6/32 mAb.

Flow cytometry analysis of antigen specific T cells and immunosuppressive cells

Phenotypic characterization of T cell cultures was done by the multiparametric flow cytometry analysis using the following mAbs: anti-CD8-Krome Orange (Beckman Coulter), anti-CD4-APC (BD Bioscence), the HLA-A*0201 multimers were provided by Proimmune Ltd. Tregs and MDSCs frequencies were determined by six-colour immunofluorescence staining of thawed PBMCs, excluding dead cells using the LIVE-DEAD® Fixable Violet Dead Cell Stain Kit (Life Technologies). For surface staining, cells were incubated with the following antibodies for 30 minutes at 4℃ after bl ocking non-specific antibody binding to the Fc-receptors using FcR Blocking Reagent (Miltenyi). For Treg analysis, intracellular staining with APC-conjugated anti-Foxp3 (eBioscience) or the proper isotype control (rat IgG2a) was performed. Lymphocytes activated overnight with anti-CD3/CD28 beads (DynaBeads® CD3/CD28 T cell Expander, Invitrogen Dynal AS, Oslo, Norway) in the presence of 1 µl/ml Golgi Plug (BD Biosciences) were stained for the cell surface marker CD3. The cells were then washed, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) and stained with a 488-labelled anti-IFN-γ (BioLegend), PE-labelled anti-IL-2 (BD Biosciences). Data acquisition was performed using a Gallios™ (Beckman Coulter) flow cytometer, and the Kaluza® software (Tree Star Inc) was used for data analysis.

Figure Legends

Figure 1. Radiologic response in metastatic lesions after sunitinib treatment.

Figure 2. (A) Immunohistochemical analysis of antigen expression in pre-treated and post-suntinib tumor lesions. (B) Analysis of infiltrating immune T cells (CD3 and CD8) in sunitinib-treated tumor.

Figure 3. (A) Phenotypic analysis of CD8$^+$pentamer$^+$ T cells after sensitization with the HLA2-A*0201 restricted-modified peptides (Melan-A/MART-1$_{[27L]}$ or gp100$_{[210M]}$). (B) Following 10days stimulation, the tumor specificity of T cells was assessed by measuring IFN-γ secretion (ELISpot assay) in the presence of HLA-A*0201-restricted Melan A/MART-1 (modified or native)-pulsed (2umol/L) T2 or HLA-matched HLA-A*0201$^+$MART1$^+$ tumor cells ((#501mel and #624.38mel) pretreated or not with the anti-HLA class I (W6/32) mAb. Moreover T cells were also incubated with HLA-mismatched allogeneic HLA-A*0201$^-$

MART1^{+} (#624.28mel) or HLA-A*0201^{+}MART1^{-} melanoma cells (#A375mel). The irrelevant peptide $NEF_{[180–189]}$ was used as negative control. Statistical analysis of differences between means of IFN-γ released by T cells was done by two-tailed *t* test.

Figure 4. Sunitinib (SM) treatment modulates peripheral immunoregulatory cells. SM modulates the frequencies of (A) CD14^{+}HLADR$^{-/low}$ (mMDSCs) in (B) CD4^{+}CD25^{hi}Foxp3^{hi} (Tregs) in live gated PBMC. (C) Increased levels of circulating mMDSCs and Tregs correlated with decreased T cell functionality. PBMCs collected during SM treatment (during SM) or at time of disease progression (at progression) were assayed for IFN-γ (red line) and IL-2 (blue line) secretion in response to overnight activation with anti-CD3/CD28-coated beads.

Competing interests

SS: Pfizer coverage for medical meetings, research funding. CPG: Pfizer advisory honoraria, research funding. All of the remaining authors have no conflicts of interest.

Authors' contributions

MT and FR conducted the experiments. BV performed the immunohistochemical analysis. MT, SS, LR and CC wrote the paper. All authors read and approved the final manuscript.

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Figures (Manuscript II)

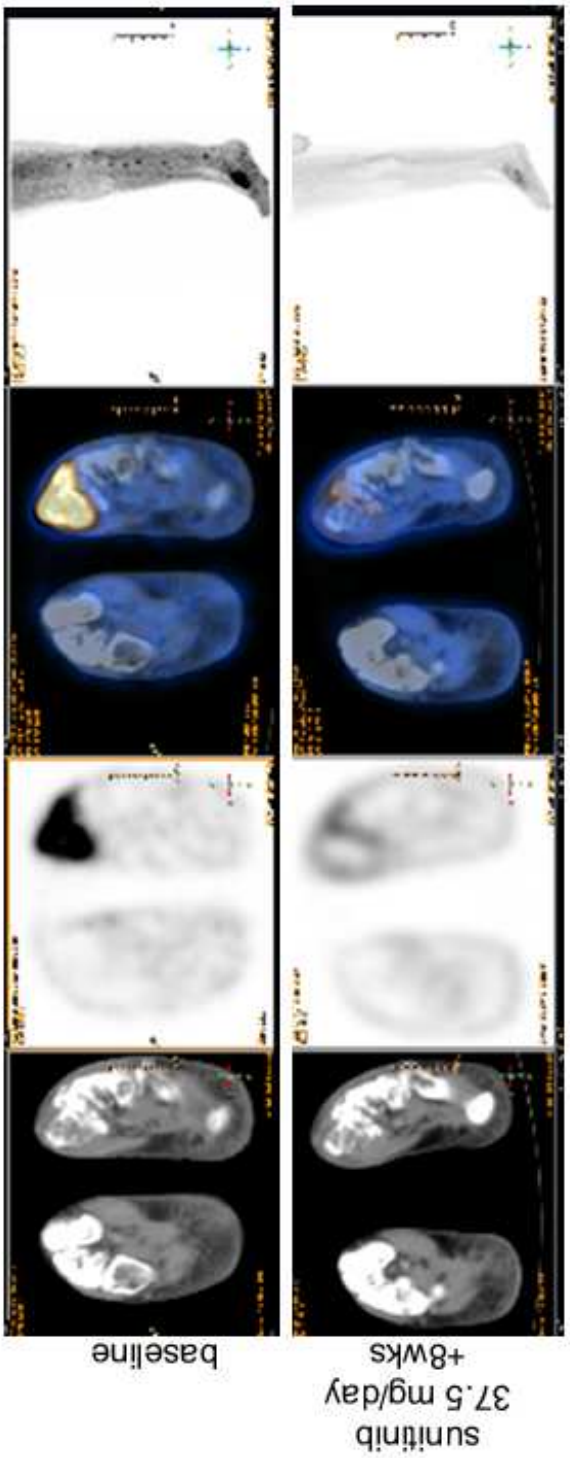
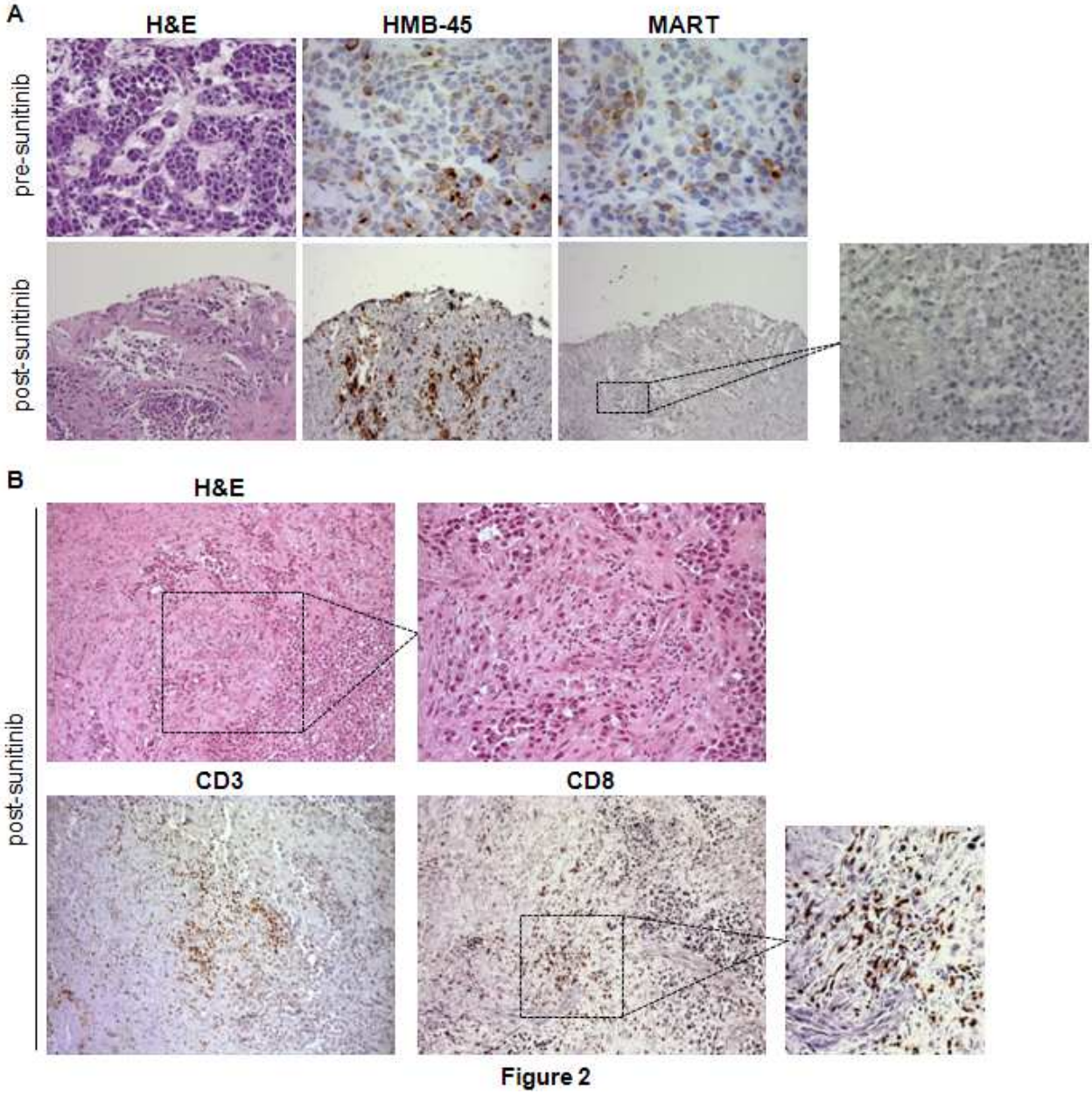


Figure 1


A
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HMB-45
MART
pre-sunitinib
post-sunitinib
B
H&E
post-sunitinib
CD3
CD8

Figure 2

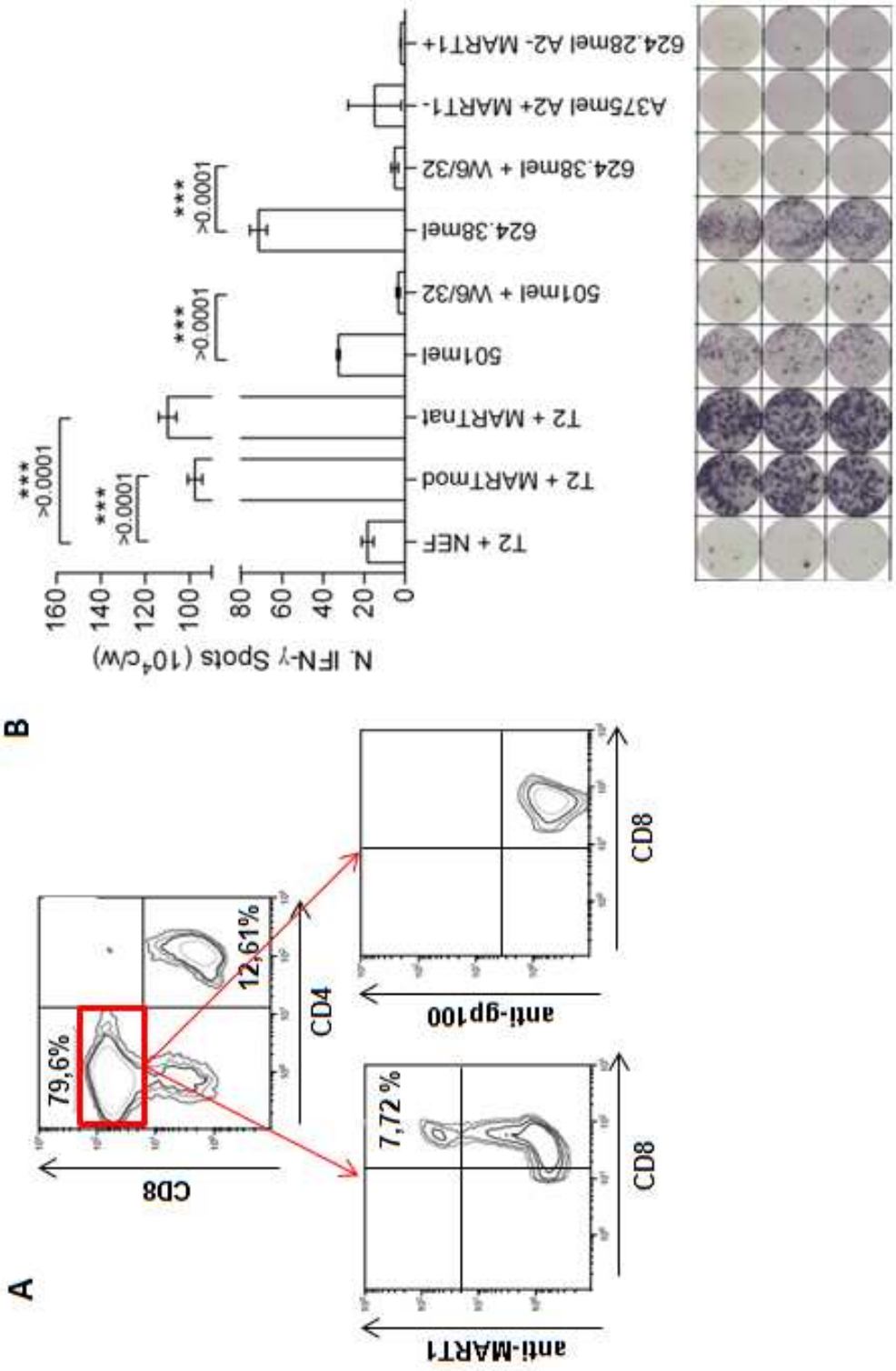
A
CD8
79,6%
12,61%
CD4
anti-gp100
CD8
7,72 %
anti-MART1
CD8
B
N. IFN-γ Spots (10⁴c/w)
160
140
120
100
80
60
40
20
0
T2 + NEF
T2 + MARTmod
T2 + MARTnat
501mel
501mel + W6/32
624.38mel
624.38mel + W6/32
A375mel A2+ MART1-
624.28mel A2- MART1+
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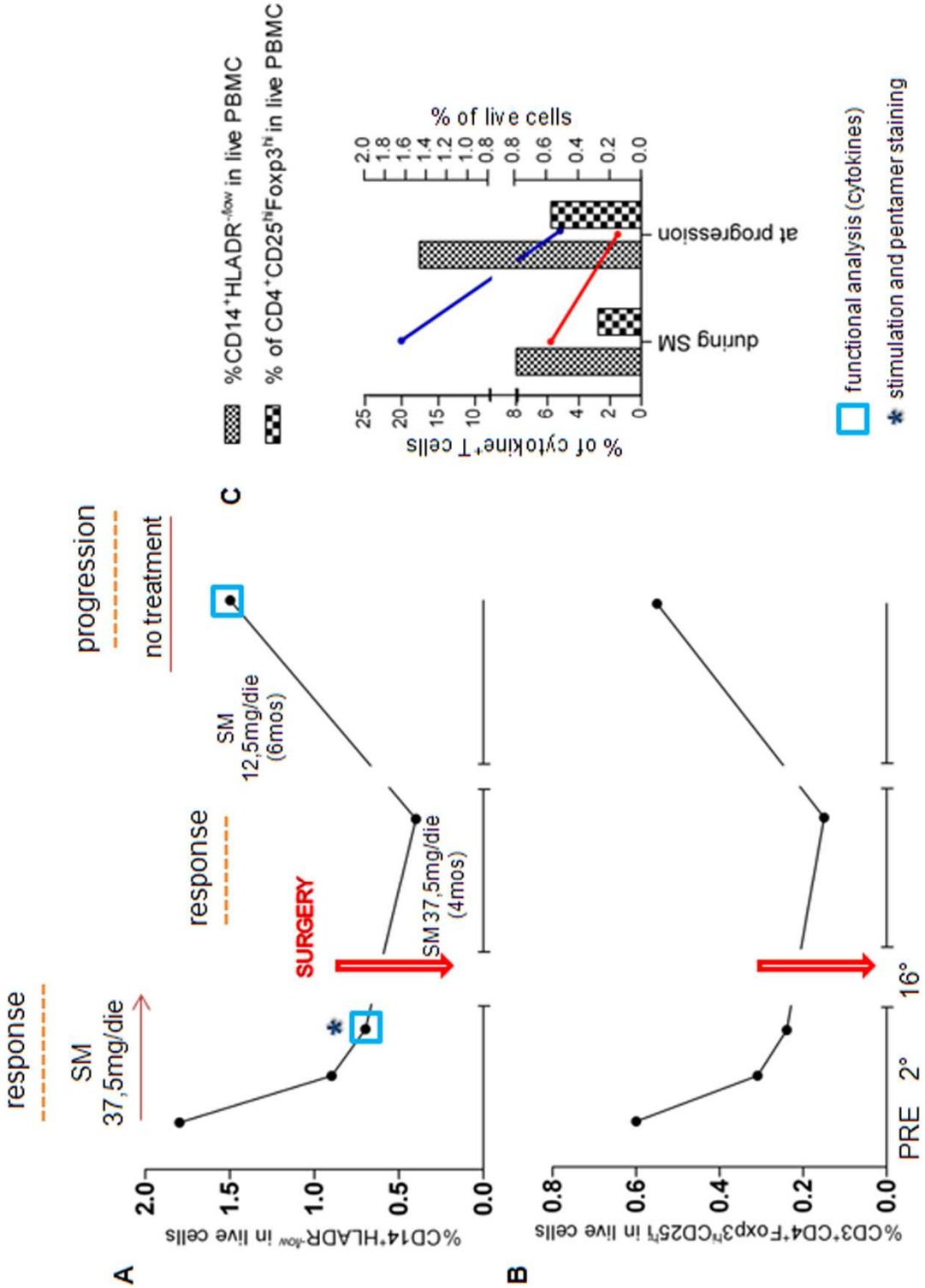
Figure 3


response
SM
37,5mg/die
A
%CD14+HLADR-/low in live cells
2.0
1.5
1.0
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*
SURGERY
response
progression
no treatment
SM
12,5mg/die
(6mos)
SM 37,5mg/die
(4mos)
B
%CD3+CD4+Foxp3hiCD25hi in live cells
0.8
0.6
0.4
0.2
0.0
PRE
2°
16°
weeks of treatment
C
%CD14+HLADR-/low in live PBMC
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2.0
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0.0
at progression
during SM
% of cytokine+ T cells
25
20
15
10
8
6
4
2
0
functional analysis (cytokines)
* stimulation and pentamer staining

Figure 4

Published Paper III

[Commentary]

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JOURNAL OF TRANSLATIONAL MEDICINE

COMMENTARY Open Access

Structured myeloid cells and anti-angiogenic therapy in alveolar soft part sarcoma

Chiara Castelli[1], Marcella Tazzari[1], Tiziana Negri[2], Barbara Vergani[3], Licia Rivoltini[1*], Silvia Stacchiotti[4] and Silvana Pilotti[2]

Abstract

Alveolar soft part sarcoma (ASPS) is a rare soft tissue sarcoma and the clinical management of patients with unresectable, metastatic disease is still challenging. ASPS expresses an array of potentially therapeutically targetable, angiogenesis-related molecules and, importantly, it has a distinctive angiogenic phenotype marked by a peculiar tumor-associated vasculature. Several studies, conducted in transgenic mouse models and in a large variety of human tumors of different histotype, clearly proved the substantial contribution of tumor-infiltrating myeloid cells, such as myeloid derived suppressor cells, monocytes and macrophages, in the formation and maintenance of abnormal blood vessels in tumors. By immunohistochemistry we thus explored the presence and the distribution of cells expressing myeloid markers in the inflammatory infiltrate of surgical treated metastatic ASPS. Indeed, we found that myeloid cells expressing CD14 and CD163 markers constitute the prominent cells in the inflammatory infiltrate of ASPS. These macrophage-like cells form a network surrounding the endothelial cells, or, interspersed in the tumor nest, they keep deep contact with tumor cells. In this commentary, we discussed our findings in relation to the recently published paper by Kummar and colleagues reporting the clinical and molecular results of a phase II clinical trial in patients with unresectable, metastatic ASPS treated with the anti-angiogenic drug cediranib, targeting the VEGFR-1,-2,-3 tyrosine kinases.

Keywords: Immune infiltrating cells, Inflammation, Myeloid cells, Soft tissue sarcoma, Anti-angiogenic therapy

Commentary

We read with great interest the paper by Kummar et al. on cediranib in metastatic alveolar soft part sarcoma (ASPS) [1]. The study evaluated the antitumor activity of cediranib in 43 patients. The disease control rate (partial response plus stable disease) of patients who completed the therapy course was 84%. Tumor biopsies prior and after one week of cediranib were obtained from a subset of patients. Thus, the authors investigated for the first time the gene expression changes in ASPS after anti-angiogenic treatment, giving a comprehensive overview of the micro-array/qRT-PCR profiles of significantly modulated genes in 7 validated cases. Included in this list are genes playing a direct role in the cancer driven neo-angiogenesis. Tumor lesions from patients treated with cediranib displayed a selective down-regulation of angiopoietin 2 (ANGPT2) and the up-regulation of its receptor TIE2. This opposite gene modulation may favor the angiopoietin 1 (ANGPT1)-TIE2 interaction and, together with the down-modulation of VEGFR1 (FLT1) and VEGFR2 (KDR), drive a physiological normalization of the vasculature at tumor site [2]. This hypothesis is also supported by the findings that genes encoding for proteins expressed by endothelial precursor cells or by the tumor-associated neo-vasculature such as Folate receptor 1 (FOLH1, known also as PMSA), CXCR7 and ESM1, [3-6] were also down-modulated. Gene profiling also revealed that in cediranib treated ASPS, CCL2 mRNA was associated to the presence of high level of CD163 gene expression. This coordinated gene up-regulation may suggest the selective recruitment of inflammatory monocytes that differentiate into fully mature macrophages at tumor site. However, CD163 is a marker associated to M2-like macrophages, endowed with pro-tumor and pro-angiogenic functions. Furthermore, the up-regulated expression of TIE2 in post treated samples can also be indicative of an increased accumulation of TIE2+ cells,

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known as vessel-associated macrophages, crucially involved in tumor-mediated neo-angiogenesis [7]. Thus, it is difficult to reconcile this complex scenario with the observation that the majority of the examined ASPS biopsies indeed derived from patients displaying a clinical response to treatment. The precise knowledge of the type, functional polarization and localization of immune tumor infiltrating cells in ASPS will possibly be of help in interpreting these data. While the Authors studied post-treatment changes on extractive tissues, we examined pre-treatment morphological and biochemical profiles of metastatic ASPS. We have investigated the pre-treatment immunophenotypic and biochemical profiles of 7 out of 15 patients treated with sunitinib since 2007 [8,9]. These 7 patients received surgery in our institute before treatment with sunitinib and their tumor tissues were available for immunohistochemistry (IHC) analysis. The other 8 patients underwent surgery elsewhere and material for the analyses was not at disposal. Moreover, ethical issues restrained the analysis of post-treated ASPS since there was no clinical indication for surgery after sunitinib. In a semi-quantitative scoring system, all the 7 samples displayed similar distribution and density for all the studied markers. An example, explicative for all the examined ASPS, is depicted in Figure 1. As showed in Figure 1, pre-treatment ASPS consisted of a sizeable population of CD163+ cells found in two distinct localizations. In fact, they were interspersed within nest tumor cells but, most importantly, they were also clearly detectable in the perivascular region where CD163+ cells (Figure 1 panels A and B, lower and higher magnification, respectively) were aligned to VEGFR2+ cells of endothelial nature (Figure 1, panel C: CD163 -NCL-CD163, Leica-Novocastra-green; VEGFR2 -55B11, Cell Signaling Technology- red). The CD163+ cells were also CD14+ (Figure 1, panel D) and therefore identifiable as tumor associated macrophages, and consequently aligned to CD31+ cells (panel E: CD14 -MS-1080-S1, Thermo Scientific- red; CD31 -JC70A, Dako-green). Of note, a similar distribution of immunoreactivity was observed for CSFR-1 (panel F: C20, sc-692, Santa Cruz Biotechnology). In addition, our previous investigation showed that CSFR-1 not only was expressed but also activated [8]. The CSF-1/CSF-1R signaling axis is the major regulator of survival, proliferation and functional differentiation of macrophages. All together, our observations established the presence of M2-like, CD163+ CD14+ macrophages in the tumor microenvironment of naive ASPS. These myeloid cells are active inflammatory components that may promote VEGF-mediated vasculogenesis and, although not physically part of the vasculature, they are thought to provide trophic support to the characteristic ASPS vascular network. The pre-treatment immunophenotypic ASPS signature we observed strongly suggest that myeloid immune component of the ASPS microenvironment may directly influence the response to anti-angiogenic

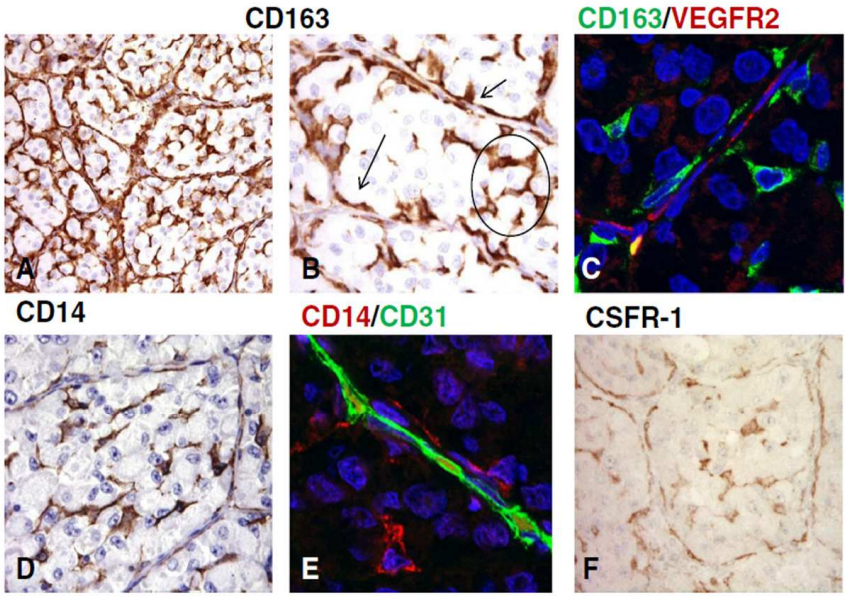

Figure 1 Macrophages are key components of the inflammatory microenvironment in metastatic alveolar soft part sarcoma. Immunohistochemical analysis of CD163+ cells infiltrating an untreated ASPS lesion **(A-B)**. As evidenced by the higher magnification image these cells are found in two distinct localizations: they are interspersed within nest tumor cells (circle) and they are also detectable in the perivascular region (arrows). **(B)** Confocal microscopy imaging of CD163+ cells (green) shows that they are aligned to endothelial VEGFR2+ cells (red) **(C)**. CD14 staining closely resembles that of CD163 **(D)** and double staining confirms that CD31+ endothelial cells (green) are lined by CD14+ macrophages (red). **(E)** A similar distribution of immunoreactivity is observed for CSFR-1 **(F)**.

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therapies and become direct target for anti-VEGF/VEGFR drugs, such as cediranib. However, Kummar's data indicated that the CCL2 and CD163 genes, known markers of inflammatory myeloid cell infiltration and associated with M2-like pro-tumor macrophages, were boosted in those ASPS of patients treated with cediranib with evidence of response. The absence of drug-induced down-regulation in the myeloid inflammatory components raises questions concerning its possible association with the profile of the tumor, with response or resistance to treatment. At first, we can argue that cediranib treatment may induce a functional shift of the infiltrating myeloid cells instead of modulating their frequency at tumor site (that would have ended up with a diminished expression of genes encoding for markers of myeloid cells). Indeed, the active role of VEGF/VEGFR signaling in the functional generation of myeloid cells with strong pro-angiogenic and immunosuppressive functions is amply documented both in animal models and in humans [10]. Thus, by blocking this pathway, cediranib might affect the type or the functional status of the inflammatory cells, eventually contributing to the transformation of the immunosuppressive, pro-angiogenic microenvironment into a more immunostimulatory, anti-tumor milieu [11]. In support of this interpretation, cediranib up-regulated the inflammatory pathway genes controlled by the nuclear factor-kB, as highlighted by the authors themselves. This strong and coordinated boost of inflammation-related genes might transform the chronic, pro-tumorigenic inflammation at the tumor site into an acute inflammation status that is perceived by the immune system as 'dangerous' and is generally correlated with an active, protective immune response [12]. In addition, as recently reported in different tumor settings [13], anti-angiogenic therapy induces a vascular normalization that alleviates tumor local hypoxia, thus removing one of the major factors responsible for the generation of an immunosuppressive environment [14]. As an alternative hypothesis, it cannot be excluded that M2-like pro-angiogenic myeloid cells present at tumor site, as shown by our immunophenotyping studies, might increase in number to counteract the massive, antiangiogenic-mediated vascular pruning. Indeed, in such a case, M2 polarized myeloid cells could be the immune-related mediators of acquired resistance. To decipher the role of this 'inflammatory' component in ASPS treated with cediranib, and, more in general, for the anti-angiogenic therapies of solid tumors, it will be crucial to assess whether or not the localization and the functional activation of the myeloid cells, resident or newly recruited, in treated ASPS overlap with those found in pre-treated tumors. This matter can only be dissected through a thorough pre/post-treatment analysis of pair-matched ASPS samples. Nonetheless, the gene expression changes induced in ASPS after cediranib treatment and the presence of a structured myeloid cell infiltration provide the rationale for further studies to investigate the feasibility of approaches targeting myeloid cells in combination with anti-angiogenic therapy. Drugs limiting the viability, function and differentiation of cells of myeloid lineage have been recently introduced in clinical setting. Among them all-trans-retinoic acid (ATRA), with differentiation potential, and synthetic triterpenoids, that reduce the intracellular reactive oxygen species, molecules mediating the suppressive function of MDSC and macrophages, have been recently used in pilot clinical studies [15,16]. Furthermore, trabectedin has been recently shown to limit the viability of monocytes and tumor-associated macrophages in sarcomas [17], and bisphosphonates, employed in the treatment of bone metastasis, may also potentially target macrophages [18]. In conclusion, the precise knowledge of the nature of tumor infiltrating cells before and after a given drug treatment may pave the way to new combined therapies aimed at overcoming drug induced resistance.

Competing interests
SS has received research funding by Pfizer for clinical study. All other authors declare they have no competing interests.

Authors' contributions
CC, MT, TN, BV, LR, SS and SP made intellectual contributions and drafted the manuscript. BV designed and performed immunohistochemical and confocal analysis. All authors read and approved the final manuscript.

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