UNIVERSIDADE Ð COIMBRA

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PERIPHERAL IMMUNE STATUS OF TRABECTEDIN VERSUS ANTHRACYCLINE-BASED CHEMOTHERAPY IN SOFT TISSUE SARCOMA

Dissertação no âmbito do Mestrado em Bioquímica orientada pelo Dr. Paulo Rodrigues Santos e pelo Professor Doutor Paulo Fernando Santos e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Outubro de 2021

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Resumo

A avaliação do *status* imunológico de doentes com cancro tem sido o foco de vários estudos. No entanto, o seu valor de prognóstico ainda não é claro em doentes com sarcomas de tecidos moles (STS). Além disso, a maioria dos estudos tem como foco a análise imunológica apenas no microambiente tumoral e não considera o efeito da terapia.

O presente estudo teve como objetivo comparar o efeito de terapias baseadas em trabectedina *versus* antraciclinas no *status* imunológico no sangue periférico de doentes com STS. Foi também objetivo avaliar os efeitos do tratamento de longa duração com trabectedina (>20 ciclos), de modo a compreender melhor o seu efeito imunomodulador. Por último, a capacidade preditiva da sobrevida global dos elementos do *status* imunológico foi avaliada.

A análise da frequência e do reportório de células imunes foi realizada por citometria de fluxo, a expressão de genes relacionados com a resposta imunitária foi quantificada por PCR em tempo real e, para a quantificação dos níveis de fatores imunes solúveis, foi utilizada a tecnologia *Multiplex Analyte Profiling* (xMAP®).

Os resultados mostraram diferenças no *status* imunológico dos doentes com STS quando as terapias e o número de ciclos de trabectedina foram comparados. Nos doentes submetidos a terapias baseadas em trabectedina, os níveis de células T duplas negativas, células T CD4 ativadas, células T reguladoras de memória, células *Natural Killer* expressando PD-1 e a expressão dos genes IL1B, FCGR3A e SELL foram encontrados aumentados quando comparados com os doentes submetidos a terapias baseadas em antraciclinas. Pelo contrário, os níveis das células T CD4 *naïve*, *early myeloid-derived supressor cells* (e-MDSC) e os níveis de expressão dos genes CCL3 e CCL4 foram encontrados diminuídos. Quando analisámos o efeito do tratamento de longa duração (>20 ciclos) com trabectedina, os níveis das células $CD56^{dim}$ *Natural Killer*, das células T duplas positivas, os níveis solúveis de *programmed death-ligand* 2 (PD-L2) e B7-H2, e os níveis de expressão de CXCL1 foram encontrados aumentados quando comparados com os pacientes submetidos a ≤20 ciclos de trabectedina. Pelo contrário, as células dendríticas plasmacitóides, as células $CD3^{brigh}$ NKT-*like* ativadas e a expressão de FGF2 foram encontradas diminuídas. Considerando o valor prognóstico dos fatores com diferenças estatisticamente significativas, encontrámos os níveis solúveis elevados de PD-L2 e B7-H2 associados a uma sobrevida global mais longa.

Em suma, estes resultados demonstraram o impacto da trabectedina no *status* imunológico dos doentes com STS. Os resultados sugerem ainda que os níveis de PD-L2 e o B7-H2 no plasma podem vir a ser usados como biomarcadores preditivos de bom prognóstico em doentes com STS submetidos a terapias de trabectedina de longa duração. Estudos posteriores com maior número de doentes serão necessários para confirmar os dados preliminares do presente trabalho.

Palavras-chave: sarcoma dos tecidos moles; trabectedina; antraciclinas; monitorização imunológica; imunofenotipagem; citocinas; quimiocinas; fatores de crescimento; *immune checkpoints*; expressão génica.

Abstract

The assessment of the immunological *status* of cancer patients has been the focus of several studies. Yet, its prognostic value in soft tissue sarcomas (STS) remains unclear. In addition, most studies focused only on the analysis of the tumor microenvironment (TME) and did not consider patient therapy.

The present study analyzed peripheral blood from patients with STS in order to compare the effects of trabectedin with anthracycline-based therapy on the immunological *status*. To better understand the immunomodulatory role of trabectedin-based therapies, we assessed the immunological *status* considering the duration of therapy (>20 cycles *versus* ≤20 cycles). In addition, the predictive role of the peripheral immune elements, considering the patient therapy, was also evaluated.

We analyzed the repertoire and frequency of immune cells using flow cytometry, the expression of immune-related genes was performed by real-time PCR, and the quantification of soluble immune-related factors by Multiplex Analyte Profiling (xMAP®) tecnology.

The results showed differences in the immunological *status* of STS patients when the therapies and the number of trabectedin cycles were compared. The levels of double-negative T cells activated CD4 T cells, memory regulatory T cells, PD-1 Natural Killer cells, and the expression of IL1B, FCGR3A, and SELL were found increased in the group of patients who had undergone trabectedin-based therapy, when compared to anthracycline-based therapy. On the contrary, the levels of *naïve* CD4 T cells, early myeloid-derived suppressor cells (e-MDSC), and the expression of CCL3 and CCL4 were found decreased. When we analyzed the patients on long-term trabectedin therapy (>20 cycles), the frequencies of $CD56^{dim}$ Natural Killer (NK) cells and double-positive T cells, the levels of soluble programmed death-ligand 2 (PD-L2) and B7-H2, and the expression of CXCL1 were found increased when compared with ≤20 cycles of trabectedin. Contrary, plasmacytoid dendritic cells, activated $CD3^{brigh}$ Natural Killer T-like cells, and the expression of FGF2 were decreased. Concerning the prognostic value of the immune-related factors statistically altered, we found that higher levels of the soluble immune checkpoints PD-L2 and B7-H2 were correlated with longer overall survival.

In conclusion, these results suggest an impact of trabectedin-based therapy on the immunological *status* of STS patients. Moreover, the levels of PD-L2 and B7-H2 in

plasma could be used as good predictive biomarkers for STS patients undergoing long-term trabectedin therapy. Further studies with a larger number of patients will be necessary to confirm the preliminary data of the present study.

Keywords: soft tissue sarcoma; trabectedin; anthracyclines; immune monitoring; immunophenotyping; cytokines; chemokines; growth factors; immune checkpoints; gene expression.

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Abbreviations

ANTH	Group of patients who had undergone anthracycline-based therapy
APC	Antigen-presenting cell
BTLA	B- and T-lymphocyte attenuator
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
cDC	Conventional dendritic cell
cDC1	Conventional dendritic cell type 1
cDC2	Conventional dendritic cell type 2
cDNA	Complementary DNA
CNRQ	Calibrated normalized relative quantification
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
DN	Double negative (T cell)
DP	Double positive (T cell)
e-MDSC	early myeloid-derived suppressor cells
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
G-CSF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor
HLA-DR	Human leucocyte antigen-DR isotype
ICOSL	Inducible co-stimulatory ligand
IDO-1	Indoleamine-pyrrole 2,3-dioxygenase 1
IFN	Interferon
IgD	Immunoglobulin D
IL	Interleukin
IRF4	Interferon regulatory factor 4

IRF8	Interferon regulatory factor 8
LAG3	Lymphocyte-activation gene 3
LIF	Leukemia inhibitory factor
M-CSF	Macrophage-colony stimulating factor
M-MDSC	Monocytic myeloid-derived suppressor cells
M1	Classically activated or inflammatory (macrophage)
M2	Alternatively activated or anti-inflammatory (macrophage)
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
NY-ESO-1	New York esophageal squamous cell carcinoma-1
OS	Overall survival
PAP-GM-CSF	Prostatic acid phosphatase GM-CSF
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD-1	Programmed death-1 receptor
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
pDC	Plasmacytoid dendritic cell
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cell
PTX3	Pentraxin 3
RGMB	Repulsive guidance molecule B
RIG	Retinoic acid-inducible gene
ROS	Reactive oxygen species
Sirpα	Signal regulatory protein α
SSC	Side scatter
STS	Soft tissue sarcomas
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
Tc	Cytotoxic T cell
TCGA	The Cancer Genome Atlas

TCR	T-cell antigen receptor
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	Tumor-infiltrating lymphocytes
Tim-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAB	Group of patients who had undergone trabectedin-based therapy
TRAB>20	Group of patients who completed >20 trabectedin cycles
TRAB≤20	Group of patients who completed ≤20 trabectedin cycles
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tregs	Regulatory T cells
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
xMAP	Multiplex Analyte Profiling
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BLC	B lymphocyte chemoattractant
ENA	Epithelial neutrophil activating peptide
I-TAC	Interferon–inducible T cell alpha chemoattractant
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma interferon
TSLP	Thymic stromal lymphopoietin
TWEAK	TNF-related weak inducer of apoptosis
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IP-10	Interferon gamma-induced protein 10
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NGF	Nerve growth factor
SDF	Stromal cell-derived factor

SCF	Stem cell factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HVEM	Herpes virus entry mediator
MIC	MHC class I polypeptide–related sequence
PVR	Poliovirus receptor
TACTILE	T Cell-activated increased late expression protein
ULBP	UL16 binding protein
TIMD	T cell immunoglobulin and mucin domain containing
VISTA	V-domain Ig suppressor of T cell activation

Chapter 1

INTRODUCTION

1 Introduction

1.1 The Immune System

It is clear that we need a defense mechanism to protect our bodies from the constant aggressions that we are exposed to. Simplistically, this defense is composed of 3 levels. The first, immediate, consists of anatomic and physical barriers, such as intact skin, vigorous mucociliary clearance mechanisms, low stomach pH, and bacteriolytic lysozyme in tears, saliva, and other secretions[1,2]. Any pathogen that has the ability to overcome this first level encounters two further ones, the innate induced and the adaptive system[1,3].

Immunologists have been trying to uncover the complex physiological processes involved in an immune response, proposing diverse theories over the decades[4]. First, Burnet proposed the clonal selection theory, where the antigen selects the appropriate cell to bind to, depending on the antibody receptor on the cell surface, promoting the cell proliferation and the specific antibody production[5]. At that time, immunology was very different from what is today, comprising mostly antibody production and specificity[6]. The view of the immune system as an immune network of interacting lymphocytes and antibodies was first described in the idiotypic network theory[7]. However, both theories only described the role of the immune system as a simple safeguard against pathogens. Its role in homeostasis and disease tolerance was then described by Medzhitov *et al.*[8]. Later, the discontinuity theory described that the immune system responds to sudden alterations in antigenic stimulation[9]. More recently, the S(c)ensory Immune System Theory was proposed by Veiga-Fernandes and Freitas[4]. They proposed that immune responses incorporate sensory immune functions adapted to the environment, ensuring systems physiology, homeostasis, and perpetuation of its replicating molecules.

The last years have witnessed an increased interest in the immune system. In the beginning, our distant predecessors protected themselves from a microbe or a parasite by releasing chemicals, producing a barrage of defensive protein molecules, or unleashing phagocytic cells. The evolution of these primitive components led to innate immunity[10,11]. The adaptive immunity evolved much later, developing in the context of a functioning innate immunity. So, the immune system takes advantage of two types of responses: innate and adaptive. They differ from each other mainly in speed and specificity. Besides

the differences, both are composed of an organization of cells and molecules specialized in defending against infection and usually work as a team[3].

1.1.1 The Innate Immunity

Innate immunity protection encompasses all tissues and is a task performed by cells of both hematopoietic and nonhematopoietic origin. With a hematopoietic origin, the innate response uses phagocytic cells, such as macrophages, neutrophils, mast cells, eosinophils, dendritic cells (DC), and Natural Killer (NK) cells[1]. Nonhematopoietic cells, such as skin and the epithelial cells lining the respiratory, gastrointestinal, and genitourinary tracts, are also involved in innate immunity protection. Furthermore, innate immunity also counts with a humoral component to enhance these cellular defenses. The humoral component includes several molecules, such as cytokines, chemokines, complement proteins, lipopolysaccharide-binding protein, C-reactive protein, and other pentraxins, collectins, and antimicrobial peptides[2].

1.1.1.1 Monocytes

The myeloid cells family comprises three major subtypes of mononuclear phagocytes: DC, macrophages, and monocytes[12]. The last subtype derives from precursors in the bone marrow and circulates in the blood, playing a critical role in supporting tissue homeostasis, initiating and propagating the response to pathogens, and resolving immune responses to avoid tissue damage[13–15]. These cells comprise a heterogeneous system of cells with diverse functions[14]. Nowadays, three subsets have been established: classical monocytes, nonclassical, and intermediate monocytes[16,17]. Common monocyte progenitors are converted into classical monocytes and then into nonclassical monocytes, being the intermediate monocytes a transition state[18]. Although most nonclassical monocytes seem to derive from classical monocytes, there is a possible existence of a progenitor able to differentiate directly into nonclassical monocytes[19].

Classical monocytes are recruited to inflamed tissues and have the ability to recruit other immune cells through secreted cytokines and antimicrobial factors[14]. Besides the monocyte’s functions mentioned early, they have emerged as important regulators of cancer development and progression[20–22]. Within the tumor microenvironmental, classical monocytes can directly kill malignant cells by phagocytosis or cytokine-mediated induction of cell death, for example, Tumor necrosis factor-related apoptosis-

inducing ligand (TRAIL) or antibody-dependent cytotoxicity. However, their cytotoxicity is limited by the tumor microenvironmental. Cancerous cells can shield themselves, for instance, through the expression of CD47, avoiding phagocytosis. Furthermore, numerous cancer cells are resistant to TRAIL-mediated apoptosis. Tumor-educated monocytes could differentiate into tumor-associated macrophages (TAM) that promote immune suppression[14,23–27].

Nonclassical monocytes primarily remain in the vasculature during homeostasis. These patrolling monocytes scavenge endothelium-derived cellular debris and flag-damaged endothelial cells through the recruitment of neutrophils[28]. Nonclassical monocytes appear to also extravasate during inflammation but at a lower rate when compared to classical monocytes. The fate of this subtype of monocytes during cancer requires further investigation[14].

1.1.1.2 Macrophages

Elie Metchnikoff introduced the term ‘macrophage’ that means ‘big eater’, due to its phagocytic nature[29,30]. For many years, it was thought that macrophages emerged from the differentiation of circulating monocytes[31,32]. However, recently this hypothesis was refuted due to the morphological and functional differences between these two cells[33]. In fact, macrophages are seeded before birth, and their maintenance is independent of monocytes[33–35].

These cells play an essential role in the innate immunity response[11]. Besides this function, macrophages are important in almost every aspect of an organism’s biology, including cancer[32].

In addition to the functional diversity, macrophages are characterized by a considerable plasticity[36]. This plasticity allows macrophage polarization, a process whereby they acquire distinct functional phenotypes depending on environmental cues. At the time of polarization, these cells may acquire a classically activated or inflammatory (M1), or an alternatively activated or anti-inflammatory (M2) phenotype. The M1 macrophages are responsible for removing pathogens through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and reactive oxygen species (ROS) generation. In addition, this phenotype is responsible for anti-tumoral activity. The response of M1 macrophages is regulated by the anti-inflammatory function of M2 macrophages. With the M2 phenotype, macrophages are responsible for the regulation of

inflammation. Furthermore, in cancer, M2 macrophages promote tumor formation and progression[37].

1.1.1.3 Dendritic Cells

DC are the most efficient antigen-presenting cells (APC). Playing a role in the innate response, these cells recognize and respond to pathogen and danger-associated signals. Moreover, they capture, process, and present antigens to T cells through major histocompatibility complex (MHC) molecules, bridging the innate and the adaptive response. Although this is their main characteristic, they are a population phenotypic heterogeneous[38,39].

DC could be found in two distinct functional states: mature and immature. Within the immature state, DC have low expression of surface stimulatory molecules, surface chemokine receptors, and do not release immunostimulatory cytokines. Moreover, these immature cells have a high expression of inhibitory molecules, such as programmed death-ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and secrete anti-inflammatory cytokines. These features condition the antigen-specific induction of *naïve* T cells but are essential to prevent responses against healthy tissues[40–42]. Although their poor ability to perform cross-presentation, immature DC have a high endocytic capacity, being important sentinels, capturing apoptotic and necrotic cells[43–47].

When exposed to extracellular factors, immature cells suffer significant alterations in surface proteins, intracellular pathways, and metabolic activity, turning to mature DC[48–51]. A long series of transcriptional adaptations are involved in DC maturation. However, the complete set of the factors responsible for this maturation is not precisely defined yet[52]. After maturation, DC migrate from peripheral tissues to secondary lymphoid organs and activate T lymphocytes through antigen presentation. Furthermore, DC, influenced by interactions with other immune cells, can secrete cytokines and other factors responsible for modifying ongoing immune responses[53,54].

It is clear that the main characteristic of these cells is the ability to present antigens to T cells. However, besides their maturation state, DC comprises a variety of subsets with different phenotypes. Rising from committed DC precursors in the bone marrow, DC could differentiate into specialized subsets, including plasmacytoid dendritic cells (pDC), conventional dendritic cells type 1 (cDC1), and conventional dendritic cells type 2 (cDC2). This differentiation is controlled by a specific repertoire of transcription

factors, notably the interferon regulatory factor 8 (IRF8) and the interferon regulatory factor 4 (IRF4). The DC subset and the type of stimulus received define the nature of downstream T cell responses[38,39,55–59]. Human cDC1 have the most potent antigen presentation abilities via MHC class I, sharing a superior capacity to induce CD8 T cell immune responses. Furthermore, through interleukin (IL)-12, cDC1 promote T helper (Th) 1 and NK responses[38,60–62]. cDC2 have a wide range of receptors, allowing cross-presentation with appropriate activation. cDC2 seem to induce Th1, Th2, and Th17 responses and regulatory T cells (Tregs), frequently having regulatory roles[38,57,63–65]. Unlike cDC, pDC express low levels of MHC class II and express a narrow range of receptors for cross-presentation. This subset is characterized by the secretion of high levels of IFN-α/β and plays a central role in viral infections[66–69].

These circulating subsets of DC appear to be affected in cancer. It has been observed a decreased total number and phenotypic and functional alterations in DC[70–78].

1.1.1.4 Granulocytes

Being a category of white blood cells, granulocytes have long been solely considered players during innate immune responses. However, recently, it became clear that this group of cells also has a vital role in the adaptive response[79,80]. The granulocyte family includes neutrophils, eosinophils, basophils, and mast cells. All of them appear to migrate to peripheral and lymphoid tissues during inflammation[81–85].

Neutrophils are the subset most abundant and are classically characterized by their ability to act as phagocytic cells and release lytic enzymes, playing a primary role in the clearance of extracellular pathogens[79,86,87]. Over the last decades, an extensive range of studies has been reporting neutrophils as a highly versatile and sophisticated group of cells with functional and phenotypic heterogeneity. Furthermore, their function goes far beyond the elimination of pathogens[80]. Besides their role in the innate response, there is recent evidence that neutrophils are also involved in activating and regulating adaptive immune cells, differentially influencing the immune response[79].

Contrary to neutrophils, eosinophils are a minor subpopulation of granulocytes[88]. Despite their lower levels, these cells are receiving growing interest due to their complexity and complex role in health and diseases like cancer[89]. Eosinophils are classically known as phagocytic cells, playing a critical role in infection and inflammation. In addition to these well-known functions, nowadays, this minor subset

appears to play a more complex role in the immune system. Eosinophils possess an array of receptors and surface components for antigen presentation and are able to produce several substances, leading to upregulation or downregulation of the ongoing immune response[90].

At last, mast cells and basophils, as the most prominent sources of histamine and other inflammatory mediators, are known for their role in allergies and other inflammatory diseases. Although this common characteristic, mast cells and basophils differ morphologically, ultrastructurally, immunologically, biochemically, and pharmacologically[91].

Basophils are released from the bone marrow to the blood, representing only a minor population of leukocytes. Once in circulation, basophils display important and nonredundant roles as effector cells. These cells can also affect the adaptive response, promoting the Th2 cell differentiation, for example[91–95].

Besides their recognized role as effectors in allergies, mast cells also have an essential role in both innate and adaptive responses. This group of cells has a widespread distribution and their maturation, phenotype, and function depend strongly on the local microenvironment. They specifically recognize several stimuli and respond with the release of active mediators. Furthermore, mast cells communicate with other cells implicated in immune responses. Contrary to basophils, mast cells can be divided into several subtypes according to their location, morphology, function, and pharmacological properties. So, diversity in responses can be expected[91,96,97]. The role of both mast cells and basophils in cancer is still poorly defined[91].

1.1.1.5 Natural Killer Cells

NK cells comprise the third largest population of lymphocytes, are potent producers of immunoregulatory cytokines and can directly kill target cells. As the name suggests, these effector functions do not require prior stimulation[98–100].

Once they recognized the target, NK cells kill it predominantly via two pathways. The first one involves the secretion and exocytosis of molecules that induce target cell apoptosis, such as perforin. In the second one, NK cells take advantage of ligands, such as TRAIL. Through their association with death receptors in target cells, NK cells induce a caspase-dependent apoptosis[101]. Besides their cytotoxic activities, these cells have

diverse biological functions, including an immunoregulatory role through the production and secretion of several cytokines, for example, the IFN-γ[101].

The activity of NK cells is tightly controlled by a balance between a wide range of activating and inhibitory receptors expressed on the cell surface. Since the expression of these receptors varies between cells, the NK cell population presents a sizeable heterogeneity[101,102]. The inhibitory receptors are responsible for the prevention of NK cell activation and, therefore, the prevention of host cell killing. When NK cells meet an infected or an abnormal cell with lack or lower levels of MHC class I molecules, the balance between activatory and inhibitory signals is affected, and activation is predominant, leading to NK cell killing of target cells[103–105].

With the arrival of monoclonal antibodies for NK-cell markers, it was possible to notice that NK cells could be divided into two subsets based on their cell-surface density of CD56: $CD56^{dim}$ and $CD56^{bright}$[106]. The majority of NK cells present a low density of CD56, being included in the $CD56^{dim}$ subset. This subset is characterized by a more naturally cytotoxic function and a low production of cytokines. By contrast, the $CD56^{bright}$ subset appears to be poorly cytotoxic but produces high levels of immunoregulatory cytokines[101,107,108]. NK cells also play an important role in cancer, mainly due to their ability to lyse tumor cells[109,110].

Historically, NK cells were considered a part of the innate immune system, being characterized as short-lived, rapid, and effectors cells of this type of response. However, the discovery of NK cells with an extended lifespan and memory-like functions demonstrated that they possess typical characteristics of the adaptive immune system[111,112].

1.1.2 The Adaptive Immunity

As mentioned before, the adaptive and innate responses differ mainly in speed and specificity. The innate response is characterized by its speed but lack of specificity. On the contrary, the adaptive response performed a specific response, but it requires more time[3]. The innate response is an older evolutionary defense. Later, adaptive immunity was developed from innate immunity, and for this reason, the often demarcation between innate and adaptive responses is overly simplistic[2,113].

The adaptive immunity counts mainly with T and B lymphocytes. When the surface receptors present in these cells bind to an antigen, the system is shaped and occurs

proliferation of antigen-specific B and T lymphocytes. Therefore, this immunity response was named 'adaptive'[2,3]. These cell receptors are randomly generated and highly diverse. The receptor repertoire diversity allows the adaptive immunity to recognize an elevated number of antigens, being an advantage compared to the limited number of pathogen receptors used by the innate response. However, this diversity comes with some disadvantages: the risk of autoimmune disease and the time delay. In the random process of receptor generation, receptors specific for self-proteins can be created, leading, consequently, to autoimmune diseases. Also, after exposure to a pathogen occurs a clonal expansion of antigen-specific lymphocytes. This process requires 3 to 5 days, which could be enough time for the pathogen to cause damage to our organism[1,2].

1.1.2.1 B Lymphocytes

The first studies concerning B lymphocytes demonstrated the primary function of B lymphocytes: antibody production[114–116]. Besides their essential role in secreting antibodies, B lymphocytes play other important functions in immune homeostasis[114].

After the differentiation of hematopoietic stem cells into common lymphoid progenitors, they can migrate to the thymus or remain in the bone marrow. The majority of progenitors remain in the bone marrow and become immature B (*b*one marrow-derived) lymphocytes, also referred to as 'transitional'. Immature cells are then released and further differentiated in the spleen[114,117]. Before further differentiation, B cells can capture antigens and present them on MHC class II molecules to T cells. Being activated by an antigen, B lymphocytes proliferate and differentiate into plasmablasts, short-lived effector cells capable of early antibody response. This T cell-B cell cooperation is also a crucial step in the formation of germinal centers, where activated B lymphocytes differentiate into plasma cells, long-lived cells capable of higher antibody production, or into memory B lymphocytes, that are programmed to rapidly differentiate into antibody-secreting cells in case of re-exposure to the same antigen[118–120].

1.1.2.2 T Lymphocytes

When discovered, T lymphocytes were found to be responsible for cell-mediated immune responses[114]. They were named 'T lymphocytes' or 'T cells' because, unlike B lymphocytes, their maturation occurs in the thymus. T cells became a diverse population

during this maturation, and they leave the thymus with a diverse receptor repertoire and consequently different functions. When they express CD8 glycoprotein in their surface, they are called CD8 T cells or cytotoxic T cells and are restricted to recognizing antigens presented by MHC class I molecules. On the other hand, when T cells express CD4 glycoprotein, they are called CD4 T cells or helper T cells and only recognize antigens presented by MHC class II molecules[121,122].

Primarily, CD4 T cells were thought to be responsible for a basic helper activity in antibody responses. However, research over the past decade demonstrated that these cells display a significant degree of plasticity and can differentiate into a surprising number of diverse subsets, among them Th1, Th2, Th17, and Tregs. Although it is not well clear the factors that determine the lineage choices of CD4 T cells, it is known that their differentiation depends on the costimulatory signals, such as cytokines, that they receive from DC. Once differentiated, CD4 T cell subsets differ from each other by the release of specific cytokines. Consequently, these subsets are also heterogeneous in terms of function, playing either pro- or anti-inflammatory responses[122,123]. For example, Tregs are characterized by the expression of inhibitory receptors and the secretion of inhibitory cytokines, leading to an immune suppression[124].

Although T cells leave the thymus in a mature state, an interaction with an APC is required to initiate the adaptive response. The activation of *naïve* CD4 or CD8 T cells requires the binding of CD4 and CD8 receptors with MHC class II and MHC class I, respectively. This activation leads to a differentiation of *naïve* cells into effector cells, short-lived cells that migrate to the site of infection and eliminate the pathogen, or into memory cells, long-lived cells responsible for a fast expansion and a more effective response during a re-exposure to the same pathogen[122].

1.2 Immuno-Oncology

1.2.1 Interactions Cancer-Immune System

The interactions cancer-immune system have been the focus of several studies. First, these studies were performed to understand how the immune system could repress tumor development, protecting the organism. The immune surveillance theory said that the immune system controlled the growth of transformed cells and prevented neoplasia. However, the development of cancers in immunocompetent individuals showed the

shortcomings of cancer immunosurveillance. Then, it became clear that the immune system affects tumor development, but it also promotes its growth. These results lead to the concept of cancer immunoediting (Figure 1)[125,126]

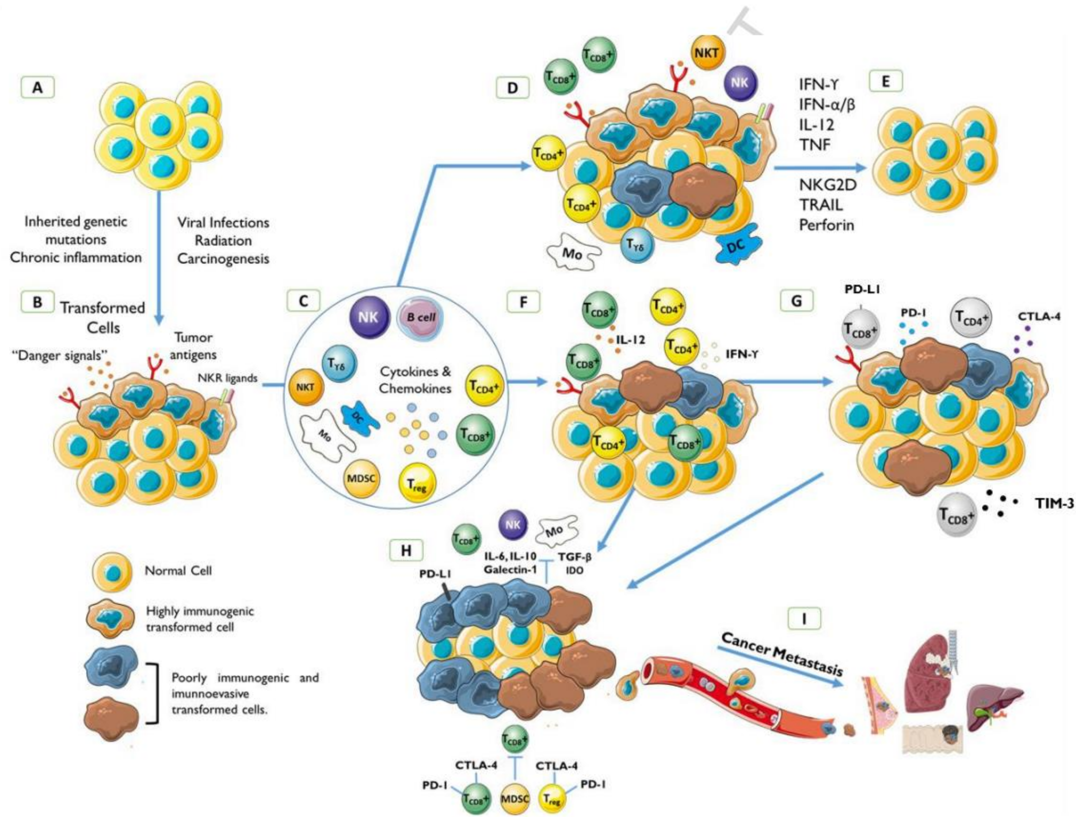

Figure 1 | Cancer and the immune system. (A) Normal tissue. (B) Transformed tissue. Transformed cells lead to the production of tumor antigens. (C) Immune system activation and response. Tumor antigens activate the immune system and occur a cross-talk between the innate and the adaptative responses. (D) Elimination. Once activated, the immune system eliminates the transformed cells through the direct cytotoxic activity of immune cells or the production of cytotoxic cytokines and chemokines. (E) Normal tissue restored. The cytotoxic ability of the immune system leads to tumor disappearance and normal tissue restoration. (F) Equilibrium. Cancer cells stay in a dormant state controlled by the adaptive immune system and gain the ability to prevent the immune system under selection pressure. (G) Immune system 'exhaustion'. The immune system is no longer able to control the tumor progression, and cancer cells rapidly proliferate. (H) The cytotoxic immune cells are inhibited, and cancer cells proliferate and overcome the immune system. (I) Metastasis. *NKR, Natural Killer cell receptor; NK, Natural Killer; DC, dendritic cells; Mo, monocytes; MDSCs, myeloid-derived suppressor cells; Treg, regulatory T cells; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; PD-L1, programmed-death ligand 1; PD-1, programmed death-1 receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; TGF, transforming growth factor; IDO, indoleamine 2, 3-dioxygenase.* (from Mendes *et al.* 2016)[127].

Cancer immunoediting is separated into three phases: elimination, equilibrium, and escape. In the first one, the cancer cells are recognized and eliminated by the innate and

adaptive immune systems before they are clinically detected (Figure 1A-E). If all cells are efficiently eliminated, this is the end of the process. On the contrary, by killing the highly immunogenic cells, the adaptive immune system shapes the immunogenicity, and the less immunogenic cells progress into the equilibrium phase (Figure 1F). This phase is thought to be the longest. Here, the cancer cells stay in a dormancy state controlled by the adaptive immune system, CD4 and CD8 T cells, as well as by cytokines, such as IFN-γ and IL-12, until eventually elimination and tumor regression, or until eventually overcoming the immune system, and progression into the last phase, escape[128,129]. Cancer cells use three main routes to overcome the immune system: acquire the ability to circumvent recognition by the immune system, acquire more resistance to cytotoxic effects or develop immunosuppression mechanisms (Figure 1H)[128]. The escape phase can also be reached when the immune system becomes 'exhausted' and loses the ability to eliminate cancer cells (Figure 1G)[127]. Once in the escape phase, cancer cells can grow and metastasize[128,129].

1.2.2 Immunotherapy

The growing knowledge about immune system regulation and immune system-cancer interactions was responsible for the emergence of immunotherapies. While chemotherapy kills cancer cells through cytotoxic features, immunotherapy tries to take advantage of the host immune system. Thus, immunotherapy can be defined as the use of agents to increase and/or reestablish the immune system ability to prevent and combat disease[130]. Resulting from the increased understanding of the immune system, several immunotherapeutic strategies have been developed, among them checkpoints inhibitors, monoclonal antibodies, adoptive cell therapies, treatment vaccines, and immune system modulators.

Jern, Milstein, and Kohler won the Nobel Prize in Physiology or Medicine for the discovery of the principal for murine monoclonal antibodies production. Later, molecular biology and protein engineering created more human-like antibodies with lower immunogenicity[131]. These findings, along with the growing understanding of how these antibodies target cancer cells, allowed the development of monoclonal antibodies therapies. Besides their direct tumor kill function, monoclonal antibodies also participate in an immune-mediated tumor cell killing[132].

Cancer vaccines comprise one of the most explored approaches of immunotherapy. They use tumor antigens to stimulate the patient immune system to fight against the tumor cells[133]. In 2010, the U.S. Food and Drug Administration (FDA) approved the first cancer vaccine Sipuleucel-T (Provenge, Dendreon, USA) for castration-resistant prostate cancer[134]. This vaccine consists of autologous peripheral-blood mononuclear cells activated with prostatic acid phosphatase granulocyte-macrophage colony-stimulating factor (PAP-GM-CSF). This encouraged further research and now a combination of vaccines with other therapeutic agents and personalized vaccines are being developed in clinical trials[133].

Another Nobel Prize in Medicine and Physiology was awarded to James P. Allison and Tasuku Honjo. They proposed a cancer immunotherapy by inhibition of negative immune regulation[135,136]. There are multiple immune checkpoints, such as CTLA-4, that is upregulated in activated T cells. When CTLA-4 binds to B7-1/CD80 on activated APC, it acts as a negative regulator of T-cell activation[137]. Another immune checkpoint pathway is the programmed death-1 receptor (PD-1). As CTLA-4, PD-1 is usually expressed on activated T cells and binds to PD-L1 or programmed death-ligand 2 (PD-L2), resulting in a signal for T cell inactivation[138]. CTLA-4 and PD-1 pathways function as a negative immune regulation[139,140]. So, agents that block these pathways became a new approach to cancer therapy and provided a foundation for additional investigation of critical pathways that modulate the immune response to cancer[135,136].

Most of the immunotherapy approaches rely on enhancing the pre-existent antitumor immune cells in the patient. However, some tumors are characterized by low immunogenicity and so this kind of therapy tends to fail. In this regard, adoptive cell therapies have emerged. These therapies consist of the administration of specific immune cells, either isolated tumor-infiltrating lymphocytes or T cells genetically engineered[141].

Another immunotherapeutic strategy consists in the use of immunomodulatory agents, for example, cytokines. It was demonstrated that cytokines, such as IL-2, IL-7, IL-15, and IL-21, can mediate and amplify the antitumor functions of cytotoxic immune cells. Therefore, their potential as therapeutic agents has been explored[142].

Despite some promising results, most of the clinical trials did not achieve a satisfactory response. Thus, the future progress in tumor immunotherapy will require combined therapies, instead of single-agent, to enhance the strength and duration of immune responses[142].

Although immunotherapeutic drugs have been approved to treat several cancers, the discipline of cancer immunotherapy started in 1891 with William B. Coley, injecting streptococcal organisms into a patient with sarcoma[143]. This procedure led to a stimulation of the immune system of the patient and the sarcoma disappeared, demonstrating the possible use of immunotherapy in this disease.

1.3 Soft Tissue Sarcoma

Soft Tissue Sarcomas (STS) are a heterogeneous and rare group of diseases with a mesenchymal origin, representing only 1% of solid tumors[144]. This group of diseases comprises over 50 different histologic subtypes and affects patients of all ages[145]. Although it can occur anywhere in the body, the most common anatomic sites are the extremities (60-70%), and the abdomen and retroperitoneum (20%)[146]. In addition to being highly heterogeneous in anatomical localization and histology, it is also heterogeneous in terms of molecular characteristics and prognosis[147].

1.3.1 Classification and Diagnosis

The World Health Organization (WHO) classification provides an organization by tumor type, considering morphology, immunohistochemical, and genetic features. This classification also stratifies STS into benign, intermediate locally aggressive, intermediate rarely metastasizing, and malignant, according to clinical behavior (Table I)[148,149].

STS diagnosis is mainly based on histological interpretations, including immunohistochemistry, cytogenetic, and molecular genetic investigations[150]. However, due to its rarity and heterogeneity, the diagnosis is difficult and requires expert analysis[151]. Furthermore, it is crucial a consensus and reproducible diagnostic criteria.

Table I | World Health Organization classification of STS.

Category	Subgroup	Grade	Entity
ADIPOCYTIC TUMORS		Benign	Lipoma
			Lipomatosis
			Lipomatosis of nerve
			Lipoblastoma/ lipoblastomatosis
			Angiolipoma
			Myolipoma of soft tissue
			Chondroid lipoma
			Extra-renal angiomyolipoma
			Extra-adrenal myelolipoma
			Spindle cell/ pleomorphic lipoma
			Hibernoma
		Intermediate (LA)	Atypical lipomatous tumour/ well differentiated liposarcoma
		Malignant	Dedifferentlated liposarcoma
			Myxoid liposarcoma
			Pleomorphic liposarcoma
			Liposarcoma, not otherwise specific
FIBROBLASTIC / MYOFIBROBLASTIC TUMORS		Benign	Nodular fasciitis
			Proliferative fasditis
			Proliferative myositis
			Myositis ossifficans
			Fibro-osseous pseudotumour of digis
			Ischemic fascitis
			Elastofibroma
			Fibrous hamartoma of infancy
			Fibromatosis colli
			Juvenile hyaline fibromatosis
			Inclusion body fibromatosis
			Fibroma os tendon sheath
			Desmoplastic fibroblastoma
			Mammary-type myofibroblastoma
			Calcifying aponeurotic fibroma
			Angiomyofibroblastoma
			Cellular angiofibroma
			Nuchal-type fibroma
			Gardner fibroma
			Calcifying fibrous tumour
		Intermediate (LA)	Palmar/ plantar fibromatosis
			Desmolds-type fibromatosis
			Lipofibromatosis
			Giant cell fibroblastoma
		Intermediate (RM)	Dermatofibrosarcoma protuberans
			Fibrosarcomatous dermatofibrosarcoma protuberans
			Pigmented dermatofibrosarcoma protuberans
			Solitary fibrous tumour
			Solitary fibrous tumour, malignant
			Inflammatory myofibroblastic tumour
			Low grade myofibroblastic tumour
			Myxoinflammatory fibrobastic sarcoma/
			Atypical myxoinflammatory fibroblastic tumour
			Infantile fibrosarcoma
		Malignant	Adult fibrosarcoma
			Myxofibrosarcoma
			Low-grade fibromyxold sarcoma
			Scleorosing epitheliod fibrosarcoma
SO-CALLED FIBROHISTIOCYTIC TUMORS		Benign	Tenosynovial giant cell tumour
			Localized type
			Diffuse type
			Malignant
			Deep benign fibrous histiocytoma
		Intermediate (RM)	Plexiform fibrohistiocytic tumour
			Giant cell tumour of soft tissue
SMOTH-MUSCLE TUMORS		Benign	Leiomyoma od deep soft tissue
		Malignant	Leiomyosarcoma
		Pericytic (perivascular)	Glomus tumour (and variants)
			Glomargiomatosis
			Malignant glomus tumour
			Myopericytoma
			Myofibroma
			Myofibromatosis
			Angioleiomyoma
SKELETAL-MUSCLE TUMOR			Rhanbdomyoma
			Embrryonal rhabdomyosarcoma
			Alveolar rhabdomyosarcoma
			Pleomorphic rhabdmyosracoma
			Spindle cell/ Sclerosing rhabdomyosarcoma
SKELETAL-MUSCLE TUMORS	Vascular tumors	Benign	Haemangioma
			Synovial
			Venous
			Arteriovenous haemangioma/ malformation
			Epithelioid haemangioma
			Angiomatosis
			Lymphangioma
		Intermediate (LA)	Kaposiform haemangioendothelioma
		Intermediate RM)	Retiform haemangioendothelioma
			Papillary intralymphatic angloendothelioma
			Composite haemangioendothelioma
			Pseudomyogenic (epithelioid sarcoma-like)
			Haemangioendothelioma
			Kapsoi sarcoma
		Malignant	Epithelioid haemangioendothelioma
			Angiosarcoma of soft tissue
	Gastrointestinal stromal tumors		
NERVE SHEATH TUMORS		Benign	Schwannoma (including variants)
			Melanotic schwannoma
			Neurofibroma (including variants)
			Plexiform neurofibroma
			Perineurioma
			Mallignant perineurioma
			Granular cell tumour
			Dermal nerve sheath myxoma
			Solitary droumscribed neuroma
			Ectopic meningioma
			Nasal glial heterotopia
			Benign Triton tumour
			Hybrid nerve sheath tumours
		Malignant	Malignant peripheral nerve sheath tumour
			Epithelioid malignant nerve sheath tumour
			Malignant triton tumour
			Malignant granular cell tumour
			Ectomesenchymoma
TUMORS OF UNCERTAIN DIFFERENTIATION		Benign	Acral fibromyxoma
			Intramuscular mycoma (including cellular variant)
			Juxta-articular myxoma
			Deep (´aggressive`) angiomyxoma
			Pleomorphic hyalinizing angiectatic tumour
			Ectopic hamartomatous thymoma
		Intermediate (LA)	Haemosiderotic fibrolipomatous tumour
		Intermediante (RM)	Atypical fibroxanthoma
			Angiomatoid fibrous histiocytoma
			Ossifying fibromyxoid tumour
			Ossifying fibromyxoid tumour, malignant
			Mixed tumour NOS
			Mixed tumour NOS, malignant
			Myoepithelioma
			Myoepithelial carcinoma
			Phosphaturic mesenchymal tumour, benign
			Phosphaturic mesenchymal tumour, malignant
		Malignant	Synovial sarcoma NOS
			Synovial sarcoma, spindle cell
			Synovial sarcoma, biphasic
			Epithelioid sarcoma
			Alveolar soft-part sarcoma
			Clear cell sarcoma os soft tissue
			Extraskeletal Ewing sarcoma
			Extraskeletal myxoid chondrosarcoma
			Desmoplastics small round cell tumour
			Extra-renal rhabdoid tumour
			Neoplasm with perivascular epithelioid cell differentiation
			PEComa NOS, benign
			PeComa NOS, malignant
			Intimal sarcoma
UNDIFFERENTIATED / UNCLASSIFIED SARCOMAS			Undifferentiated spindle cell sarcoma
			Undifferentiated pleomorphic sarcoma
			Undifferentiated round cell sarcoma
			Undifferentiatedepithoiliod sarcoma
			Undifferentiatedsarcoma NOS

LA, locally aggressive; RM, rarely metastasizing.

1.3.2 Soft Tissue Sarcoma Current Therapies

Surgical resection delivered in specialist centers has been improving and remains the standard treatment, together with or without preoperative or postoperative radiotherapy. Unfortunately, STS recurs frequently as locally inoperable or metastatic disease. For locally advanced or metastatic disease, the mainstay is chemotherapy[152]. Single-agent anthracycline is the first-line therapy and, for the second-line treatment, trabectedin has demonstrated efficacy in some subtypes of STS[147].

1.3.2.1 Anthracycline-based Therapy

Anthracyclines take place among the most effective anti-cancer drugs ever developed. Doxorubicin, belonging to the anthracycline family, changed the therapy for STS patients, whose prognostic were very poor. Doxorubicin proved to be active against this group of diseases and remained the standard treatment. Although studies using newer anthracyclines aimed to decrease the side effects and intensify the treatment, the overall survival (OS) of patients did not improve[153].

Despite its extensive clinical use, the precise mechanism of action is still unclear. Still, it is known that the primary mechanism responsible for doxorubicin anti-cancer activity is the ability to intercalate the DNA, leading ultimately to cell death. Unfortunately, these mechanisms of toxicity are not restricted to cancer cells but also occur in healthy cells, leading to notorious side effects. Concerning the side effects and drug resistance, novel therapeutic approaches that overcome these limitations are needed[154,155].

1.3.2.2 Trabectedin-based Therapy

Initially isolated from the ascidian *Ecteinascidia turbinate*, trabectedin was approved by European Medicines Agency (EMA) and FDA for the treatment of STS patients. Since this tetrahydroisoquinoline alkaloid exists in low amounts in nature, trabectedin is currently prepared synthetically[156].

Trabectedin also impacts the DNA, but its mechanism of action appears distinct from the other available DNA-damaging chemotherapeutic drugs[157]. Trabectedin structure (Figure 2) includes a monobridged pentacyclic skeleton composed of two fused tetrahydroisoquinoline rings (subunit A and B), connected to a 10-member lactone bridge

through a benzylic sulfide linkage, and linked to a tetrahydroisoquinoline through a spiro ring (subunit C). The dehydration of the carbinolamine moiety in subunit A generates an iminium intermediate that will predominantly bind to the guanine in the N2, in the minor groove of DNA. Then, van der Waals interactions and hydrogen bonds stabilized the link between the subunits A and B and neighboring nucleotides of the DNA. The formed DNA adduct leads to a DNA bending towards the major groove and perturbs the cell cycle progression. The subunit C interacts with DNA binding proteins, impacting DNA transcription and DNA repair mechanisms. In addition to this unique and complex effect on DNA, trabectedin appears to be responsible for microenvironment changes[157].

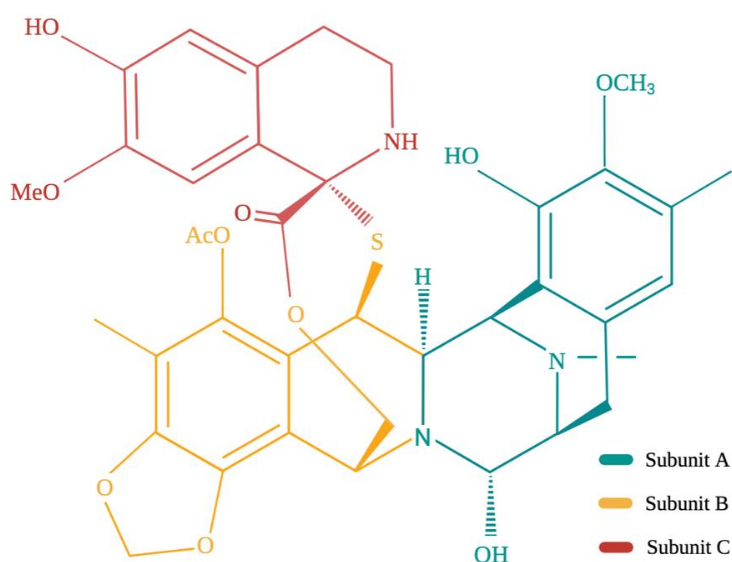

Figure 2 | Chemical structure of trabectedin. Trabectedin chemical structure consists of a monobridged pentacyclic skeleton composed of two fused tetrahydroisoquinoline rings (subunit A and B), connected to a 10-member lactone bridge through a benzylic sulfide linkage, and linked to a tetrahydroisoquinoline through a spiro ring (subunit C).

Behaving as an immunomodulatory drug, trabectedin seems to induce a subversion of the protumor microenvironment, overcoming chemo-immune resistance[157]. In ovarian cancer biopsies, trabectedin showed to inhibit the differentiation of monocytes to macrophages, and the production of the pro-inflammatory mediators chemokine (C-C motif) ligand 2 (CCL2) and IL-6 by macrophages, TAM, and monocytes[158]. In myxoid liposarcomas cell lines, an STS subtype particularly sensitive to trabectedin, this inhibition was also demonstrated[159]. Furthermore, it was showed inhibition of IL-8, IL-10, IL-1β, vascular endothelial growth factor (VEGF), chemokine (C-C motif) ligand 5 (CCL5), tumor necrosis factor (TNF)-α, and the matrix binder protein pentraxin 3 (PTX3)

in vitro. These findings were further confirmed in a patient tumor sample, proving the *in vivo* relevance of these results. Note that CCL2 expression correlates with macrophage accumulation, and TAM are strongly associated with cancer progression. IL-8 and IL-6 are potent mediators of angiogenesis and a potent tumor growth-promoting cytokine, respectively. VEGF plays an important role in angiogenesis, promoting tumor metastasis, and PTX3 has been associated with an increase in tumor mass[159]. Accordingly, the inhibition of these immune factors contributes to the antitumor activity of trabectedin.

1.4 Soft Tissue Sarcoma and the Immune System

Despite the notable improvement in cancer treatment, many STS patients do not respond to therapy. This limited efficacy of therapy is often attributable to the complexity of the disease, being STS subtypes either chemosensitive or chemoresistant. In addition to the disease complexity, the tumors can be represented as a complex ecosystem with different populations of non-tumor cells, such as immune cells, soluble plasmatic factors, and immune checkpoints[160]. These elements may be essential to identify the patients who would benefit from current treatments and also to discover novel immunotherapeutic agents or targets[150].

1.4.1 Immunotherapy in STS

As was mentioned before, the successful experiment of William B. Coley made immunotherapeutic strategies an option for treating sarcomas[143]. At the moment (July 2021), 85 ongoing clinical trials are targeting the immune system in STS patients. Phase II and phase III completed clinical trials focusing on immunotherapeutic strategies in STS are represented in Table II.

Although the expression of PD-1 and PD-L1 in STS remains controversial, some studies demonstrated the presence of these immune checkpoints in STS patients, offering a possibility for immune checkpoint blockade therapy in this group of diseases[161]. The first study concerning this type of immunotherapy in STS aimed to analyze the efficacy of targeting the immune checkpoint CTLA-4 with ipilimumab in synovial sarcoma. Unfortunately, neither an immunological activity nor clinical benefit was demonstrated[162].

Table II | Completed Clinical Trials for Immunotherapy in Soft Tissue Sarcoma.

	NCT Identifier	Phase	Enrollment	Title	Interventions
ADOPTIVE CELL THERAPY	NCT02849366	I and II	30	**Combination of Cryosurgery and NK Immunotherapy for Recurrent Sarcoma**	Cryosurgery NK cell immunotherapy
	NCT00001566	II	42	**A Pilot Study of Autologous T-Cell Transplantation With Vaccine Driven Expansion of Anti-Tumor Effectors After Cytoreductive Therapy in Metastatic Pediatric Sarcomas**	Therapeutic autologous dendritic cells Indinavir sulfate Peripheral blood stem cell transplantation
	NCT00003887	II	Not	**Lymphocyte Infusion in Treating Patients With Relapsed Cancer After Bone Marrow or Peripheral Stem Cell Transplantation**	Peripheral blood lymphocyte therapy
VACCINE THERAPY	NCT01347034	II	20	**Radiation Therapy and Intratumoral Autologous Dendritic Cells in Soft Tissue Sarcomas (STS)**	External Beam Radiation Therapy Autologous Dendritic Cells
	NCT02496520	I and II	6	**Dendritic Cell-based Immunotherapy for Advanced Solid Tumours of Children and Young Adults**	Dendritic Cells Surgery, chemotherapy, and radiation therapy as needed by the patient's tumor and stage
	NCT00365872	II	17	**External Beam Radiation With Intratumoral Injection of Dendritic Cells As Neo-Adjuvant Treatment for Sarcoma**	Dendritic Cell Injections Radiation therapy Complete Resection
	NCT00948961	I and II	70	**A Study of CDX-1401 in Patients With Malignancies Known to Express NY-ESO-1**	CDX-1401 Resiquimod (TLR7/8 agonist) Hiltonol® (Poly-ICLC, TLR3 agonist)
	NCT03357315	I and II	30	**Mix Vaccine for Metastatic Sarcoma Patients**	Mix vaccine
	NCT00005628	II	35	**Vaccine Therapy in Treating Patients With Recurrent Soft Tissue Sarcoma**	Vitespen
	NCT00001564	II	30	**A Pilot Study of Tumor-Specific Peptide Vaccination and IL-2 With or Without Autologous T Cell Transplantation in Recurrent Pediatric Sarcomas**	EF-1, EF-2, PXFK, and E7 peptides IL-2, IL-4, GM-CSF, and CD40 Ligand
	NCT00003408	II	40	**Biological Therapy Following Chemotherapy and Peripheral Stem Cell Transplantation in Treating Patients With Cancer**	Aldesleukin (synthetic IL-2) Recombinant interferon alfa Sargramostim (recombinant GM-CSF)
	NCT00923351	I and II	44	**Therapy to Treat Ewing's Sarcoma, Rhabdomyosarcoma or Neuroblastoma**	Tumor Purged/CD25 Depleted Lymphocytes Tumor Purged/CD25 Depleted Lymphocytes with Tumor Lysate/KLH Pulsed Dendritic Cell Vaccine rhIL-7 Tumor Lysate/KLH Pulsed Dendritic Cell Vaccine
	NCT02423863	II	26	**In Situ, Autologous Therapeutic Vaccination Against Solid Cancers With Intratumoral Hiltonol®**	Hiltonol® (Poly-ICLC, TLR3 agonist)

NCT, national clinical trial; NK, Natural Killer; STS, Soft tissue sarcoma; NY-ESO-1, New York esophageal squamous cell carcinoma-1; TLR, toll-like receptor; Poly-ICLC, poly-L-lysine; EF, elongation factor; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; KLH, keyhole limpet hemocyanin; rh, recombinant human;

Likewise, a phase II study targeting the immune-checkpoint PD-1 with nivolumab did not obtain any response in uterine leiomyosarcoma patients[163]. Later, the clinical trial SARC028 was the first multicenter phase II study of immune checkpoint blockade in a group of patients with bone and STS. In this trial, it was tested the anti-PD-1 therapy with pembrolizumab, and promising responses were observed in specific STS subtypes, including undifferentiated pleomorphic sarcoma and dedifferentiated liposarcoma, when the patients presented higher tumor-infiltrating lymphocytes at the baseline. Based on these promising results for specific subtypes of STS and in a specific immune microenvironment, further research and correlative studies are fundamental to improve the selection of patients who will benefit from immune checkpoint blockade therapies.

Other immunotherapeutic approaches, such as adoptive cell therapy, have been also studied for STS treatment. Since sarcomas seem to be one of the tumors most vulnerable to the cytotoxicity of NK cells, therapies based on these cells appear to be a promising alternative treatment[164]. Based on one study where was demonstrated that rhabdomyosarcoma is sensitive to expanded NK cells[165], phase I and II clinical trials of expanded NK cells in patients with this STS subtype have begun (NCT02409576). Still concerning NK immunotherapies, another ongoing clinical trial aims to combine NK cell adoptive cell therapy with cryosurgery (NCT02849366).

Adoptive cell therapies with lymphocytes harvested from the patient or a donor, expanded, and reinfused into the patient have been also investigated in STS. One ongoing phase II clinical trial proposes a donor lymphocyte infusion in patients with relapsed malignancies, among them patients with sarcoma (NCT00003887). Similarly, another ongoing phase II clinical trial aims to eradicate minimal residual disease in sarcoma patients with autologous T cell transplantation concomitant with a tumor-specific peptides vaccine (NCT00001566). As mentioned before, adoptive cells therapies can also use T cells genetically engineered. Once studies demonstrated that some STS subtypes, especially synovial sarcomas, express the cancer testis antigen New York esophageal squamous cell carcinoma-1 (NY-ESO-1), genetically engineered T cells expressing receptors for specific recognition of this antigen might be a promising approach[166,167]. Indeed, in synovial sarcoma, a T cell receptor-based gene therapy against NY-ESO-1 demonstrated promising results[168]. These results were later confirmed in another pilot study, where an autologous T-cell expressing T-cell receptor specific for NY-ESO-1 achieved an anti-tumor response in 50% of metastatic synovial sarcoma[169]. Based on

these encouraging results, an ongoing clinical trial is trying to use a CDX-1401 cancer vaccine to create an immune response against the NY-ESO-1 antigen (NCT00948961).

Concerning cancer vaccines based on DC in STS, the reports are still limited. However, one report showed the efficacy of this type of immunotherapy, but only for some patients[170]. Currently, several ongoing clinical trials are focused on strengthening the immune system to fight sarcoma with the administration of vaccines with autologous DC (NCT01347034; NCT02496520; NCT00365872). Other types of cancer vaccines, such as peptides vaccines, are the focus of current clinical trials and could become an alternative approach to treat STS (Table II).

However, despite some positive responses, most of the clinical trials have been reporting disappointing results. This observed lack of response is often attributed to the disease complexity, which is, at least partly, supported by the complex microenvironment, including the immune cells and other immune-related factors. Therefore, further studies evaluating the immune *status* of STS patients are required to develop more efficient immunotherapies and identify biomarkers for monitoring the immunotherapy response.

1.4.2 Immune Monitoring in STS

There is a growing interest in studying the immunological *status* in STS. Besides the fact that the elements of the tumor microenvironment (TME) could represent potential therapeutical agents or targets and potential biomarkers for predicting therapy responses, they also could be used to predict patient clinical outcome. A study from The Cancer Genome Atlas (TCGA) consortium proposed an association between the elements from the TME and the prognosis of patients with different STS subtypes[171]. The TME comprises several distinct populations of non-tumor cells, including endothelial, stromal, cancer-associated fibroblasts and adipocytes, and immune cells[160]. Regarding the immune cells, in human STS, their characterization remains poorly defined. In 2020, Petitprez *et al.* suggested a new classification and stratification of STS patients considering the immune composition of the TME[172]. The clear different profiles and immune compositions found in this study led to the stratification of STS patients into five distinct sarcoma immune classes. The fact that each histological subtype was identified in each class, clarifying the hypothesis that the immune profile varies even between tumors with the same histology. Furthermore, once some of these sarcoma immune classes showed a

high expression of immune-related genes, this work confirmed that the simplistic description of STS as “non-immunogenic” tumors cannot be applied to all. Still in this study, a correlation between the immune microenvironment and the patient prognosis and response to therapy was demonstrated.

1.4.2.1 Cellular Immunity

As was mentioned before, macrophages differentiate into M1 or M2 macrophages. When this differentiation occurs in the TME, they became TAM. Several factors present in the TME, such as IL-4 and IL-13, promote an M2-like differentiation of TAM, which are responsible for an anti-inflammatory role, facilitating the tumor immune escape[173,174]. The preference for TAM polarized toward a pro-tumoral phenotype in STS was confirmed in a recent study[175]. In this study they described a presence of M2-like TAM, through immunohistochemistry, in all STS samples, supporting the possibility of targeting this macrophage phenotype for STS treatment. Contrary, M1-like TAM were only found in a few samples and in a low density. Concerning the prognostic significance of these cells, they were correlated with clinical outcome in other cancers[176,177]. However, in STS little is currently known. Still, one recent study identified TAM as a negative prognostic factor in different types of STS (Figure 3)[178]. Concerning M1 and M2-like TAM, in non-gynecologic leiomyosarcomas, the high density of both macrophages expressing CD163 and macrophages expressing CD68, markers for the M2-like and M1-like macrophages, respectively, were associated with poor clinical outcome[179]. Later, another study evaluated the correlation of both TAM phenotypes in leiomyosarcomas and confirmed the negative prognostic value of M2-like macrophages[180].

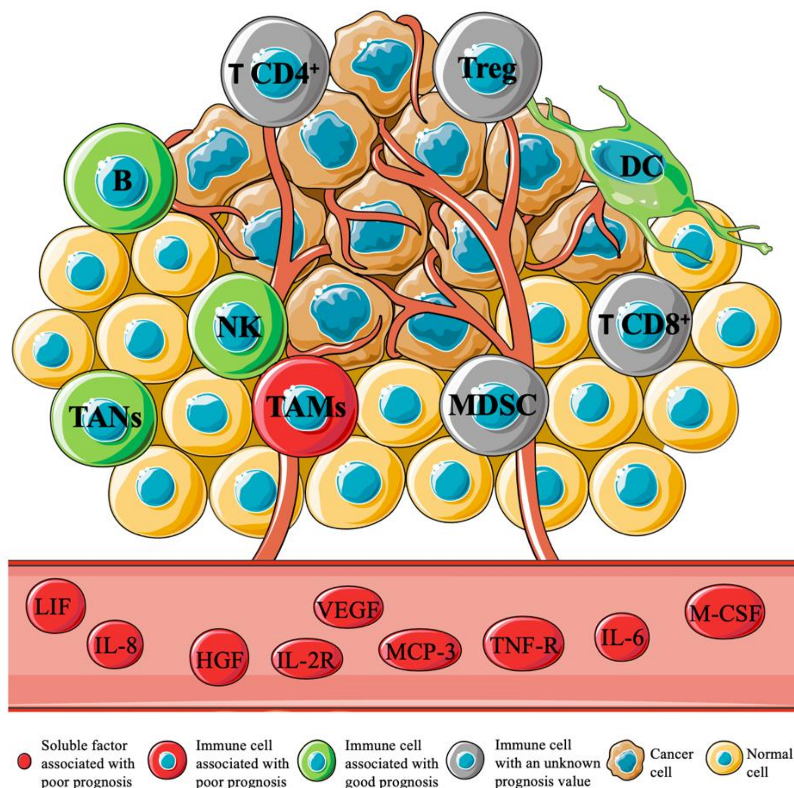

Figure 3 | Expression levels of immune cells subtypes, cytokines, chemokines, growth factors, and soluble receptors and their prognostic value in STS. The TME has been associated with the prognosis in several tumors. However, in STS this association is still underexplored. Immune cells such as B cell, DC, and NK were associated with a positive prognostic (green). Contrary, TAMs, TANs, and some soluble factors, LIF, IL-8, HGF, IL-2R, VEGF, MCP-3, TNF-R, IL-6, and M-CSF, were associated with a negative prognostic (red). The prognostic value of MDSC, Tregs, CD4 T cells, and CD8 T cells is not clear yet (grey). *TANs, tumor-infiltrating neutrophils; NK, Natural Killer; TAMs, tumor-infiltrating macrophages; Tregs, regulatory T cells; MDSC, myeloid-derived suppressor cells; DC, dendritic cells; LIF, leukemia inhibitory factor; IL, interleukin; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; MCP-3, monocyte chemotactic protein 3; TNF-R, tumor necrosis factor receptor; M-CSF, macrophage colony-stimulating factor* (from Sousa *et al*. 2021)[161].

Like TAM, tumor-associated neutrophils (TAN) in mice appear to acquire two different phenotypes: N2 phenotype, usually associated with pro-tumor activity, and N1 phenotype, usually associated with anti-tumor activity. Nevertheless, the tumor-promoting effects of the human N2 phenotype remain unclear[181]. Concerning the role of TAN in STS, Ponzetta *et al*. found an association between their high density and a favorable clinical outcome in undifferentiated pleomorphic sarcoma[182]. However, in other STS subtypes, this association was not significant.

As strong indicators of tumor immunogenicity, the role of tumor-infiltrating lymphocytes (TILs) in the progression of some tumors has been described[183]. Studies

including several sarcoma subtypes have been correlated TILs with a better patient outcome. However, most studies presented a limited sample size and considered only a few STS subtypes, so these reports could not be representative of all STS[172,184]. Indeed, two studies analyzed the expression profile of CD3E in STS to explore the level of T cell infiltration and concluded that some subtypes, such as rhabdomyosarcoma and alveolar soft part sarcoma, showed a higher T cell infiltration when compared to other subtypes[185,186]. This confirmed the idea that immune cell infiltration could be distinct between STS subtypes.

Within the T cells, studies have been studying CD4 and CD8 T cells and have been trying to correlate their frequency with the patient prognosis. Nevertheless, their prognostic value remains controversial. Although in some studies CD4 and CD8 T cells were associated with a better outcome[184,187–190], the opposite, an association with poor outcome, was also suggested[191,192]. Moreover, several studies stated that there is no statistical significance in this correlation[188,189,193]. Considering the possibility that the immune cell infiltrate depends on the STS subtype, the limited sample size and different subtypes in the study patient cohort may explain the discrepancies in these results[194,195]. Furthermore, these discrepancies may also be due to the differences in the methodology, antibody clones, and cutoff values used[191].

Recently, studies have shown that B cells play an important role in shaping the immune response against cancer cells[196]. Studies in well-differentiated and dedifferentiated retroperitoneal liposarcoma found B cells, usually, in a low density[197]. Concerning their association with disease prognosis in STS, two studies observed an association between B cells levels and a better outcome[172,175,189]. Moreover, the presence of B cells was also associated with a better response to immunotherapies[172,198].

Due to their ability to lyse tumor cells, NK cells play an important role in cancer immunosurveillance[109,110]. Their function in STS has been the focus of only a few studies. One of them found a low density of NK cells in the most well-differentiated and dedifferentiated retroperitoneal liposarcoma tissues through flow cytometry[197]. Another study stated that tumor-infiltrating NK cells were the only cells to correlate significantly with a better patient outcome in several STS subtypes[171]. Later, their positive prognostic value was corroborated by Judge *et al.*[184].

As was mentioned before, it was observed a decreased number of DC and alterations in their subsets in cancer. Is STS, there is a lack of studies concerning DC. However, the TCGA demonstrated a significant association between tumor gene

expression signature indicating the presence of tumor-infiltrating DC and favorable patient outcome in myxofibrosarcoma and undifferentiated pleomorphic sarcoma, suggesting an important role of these cells and antigen-presentation in immune responses against sarcomas[171].

Given the suppressive role of Tregs and myeloid-derived suppressor cells (MDSC), contributing to tumor escape, these cells have been studied in several cancers, where they appear to be increased[199–204]. Several studies also aimed to associate the density of tumor-infiltrating Tregs and patient outcome, but the results are controversial[205]. In STS, a study observed a high density of Tregs in most patients, corroborating the findings in other cancers[192]. Also corroborating the previous works, one study correlated the Tregs infiltration with a poor outcome in STS[206]. However, an association with a better outcome was also observed in another study[207]. Moreover, in this last study, the higher infiltration of Tregs was associated with a better response to pembrolizumab, anti-PD-1 monotherapy.

Concerning the role of MDSC, in mice bearing rhabdomyosarcoma was observed an expansion of total MDSC in the tumor site, preferentially polymorphonuclear MDSC (PMN-MDSC), and their essential role in the tumor immune escape was demonstrated[203]. Nevertheless, the role of MDSC in human STS remains underexplored.

1.4.2.2 Soluble Proteome

The network of pro- and anti-inflammatory chemokines and cytokines also contributes to the complexity of the TME and orchestrates the immune responses. Their multifaceted roles in tumor development, progression, and recurrence have been studied in a broad range of tumors. Moreover, their expression profile might be a prognostic factor for patient outcome[208–210]. Likewise, other soluble factors, like growth factors and soluble receptors, play an important role in cancer[211,212]. The prognostic value of several chemokines, cytokines, growth factors, and soluble receptors in STS is summarized in Figure 2.

In STS patients, studies described an elevated serum level of some chemokines, cytokines, growth factors, and soluble receptors, when compared to healthy donors. For instance, VEGF and fibroblast growth factor (FGF), both responsible for promoting angiogenesis, were found increased in the serum of STS patients[213–215]. Similarly, increased levels of IL-6, IL-8, IL-10, IL-2Rα, receptors for TNF (TNF-RI and TNF-RII),

and macrophage-colony stimulating factor (M-CSF) were found in STS patients[216–218]. Concerning their role as prognostic markers, Rutkowski *et al*. correlated the serum levels of IL-2Rα, TNF RI, M-CSF, and VEGF with the increased tumor size, the serum levels of IL-8 with higher tumor grade and decreased survival, and the serum levels of IL-6 with increased tumor size, higher grade, metastases, and decreased survival[216].

Concerning IL-6, few further studies confirmed its association with poor patient outcome[219]. Furthermore, Hagi *et al*. demonstrated a correlation between high serum levels of IL-6 and the presence of STS, suggesting IL-6 as a marker for the differential diagnosis[219].

The leukemia inhibitory factor (LIF) was associated with the progression and the metastatic behavior of rhabdomyosarcoma cells[220]. Also in rhabdomyosarcoma cells, IL-8 seemed to play a pivotal pro-angiogenic role and the IL-4R-dependent signaling pathway seemed to regulate the tumor cell progression, highlighting the possible critical role of IL-4 in this STS subtype[221,222]. However, these results were observed only in rhabdomyosarcoma cells, studies in rhabdomyosarcoma patients and other STS subtypes are required.

Besides its receptors, TNF was also investigated in STS, and similar to its receptors, high serum levels of TNF were found in STS patients[216]. Nevertheless, there was no significant association between its levels and tumor grade, size, metastases, or recurrence. Likewise, no associations between these features and the serum levels of IL-10 and granulocyte colony-stimulating factor (G-CSF) were found[216].

Regarding IL-2Rα, its higher serum levels were associated with tumor size and its lower serum levels were correlated with prolonged OS. In the same study, low monocyte chemotactic protein (MCP)-3 levels and low hepatocyte growth factor (HGF) levels were correlated with a better outcome[223].

1.4.2.3 Immune Checkpoints

Immune checkpoints are crucial in the regulation of the immune responses and, as was mentioned before, they can be dysregulated in cancer, facilitating the tumor immune escape[224]. Besides their role as immunotherapeutic agents or targets, they could also be used as predictive markers for patient outcome and therapy response. The prognostic value of several immune checkpoints in STS is summarized in Figure 4.

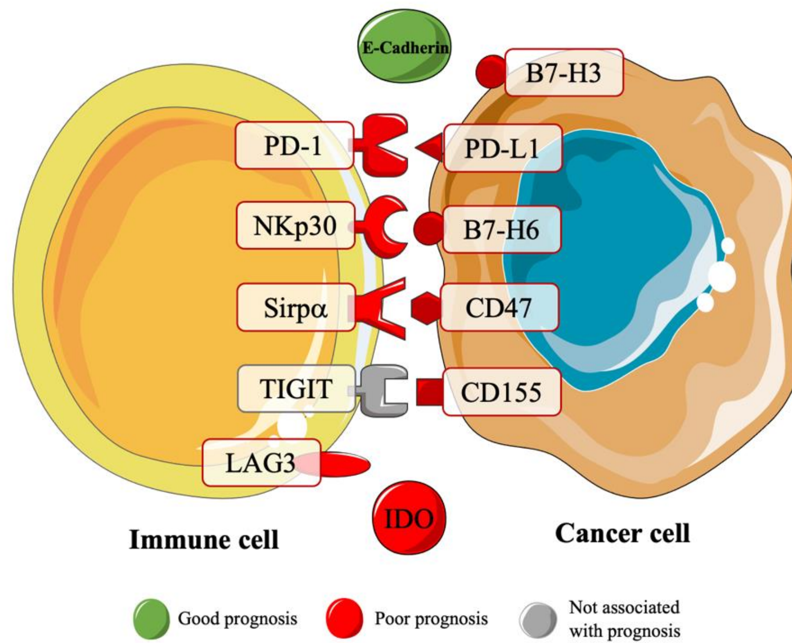

Figure 4 | Prognostic value of immune checkpoints in STS. Studies have been trying to correlate the presence of immune checkpoints with the prognosis of patients with STS. These studies have demonstrated a negative prognostic value for B7-H3, PD-1, PD-L1, NKp30, B7-H6, Sirpα, CD47, CD155, LAG3, and IDO (red). A positive prognostic value was correlated with the immune checkpoint E-Cadherin (green). *PD-1, programmed death-1 receptor; PD-L1, programmed death-ligand 1; TIGIT, T cell immunoreceptor with Ig and ITIM domains; LAG3, lymphocyte-activation gene 3* (from Sousa *et al.* 2021)[161]

Kim *et al.* evaluated the impact of PD-1 and PD-L1 in STS for the first time[137]. In this study, an intratumoral infiltration of PD-1 positive lymphocytes and the expression of PD-L1 were observed by immunohistochemistry in most STS samples. Additionally, the presence of PD-1 and PD-L1 were correlated with a negative prognostic. Later, more studies aimed to evaluate these immune checkpoints in STS and confirmed their presence and their negative prognostic value[138,225–233]. Conversely, in other studies, PD-1 and PD-L1 were found to be low or absent, and not associated with patient outcome[138,184,192,194,207,225,234]. These discrepancies might be due to the use of different methods of expression assessment, cutoff values, antibody clones, and tissue samples analyzed before and after therapeutical interventions. Moreover, one study proposed that PD-1 and PD-L1 expression depended on the STS subtype, so the use of different STS subtypes may also justify the controversial results[235]. Besides their use as prognostic markers, these immune checkpoints could be used to indicate the patients who will benefit from PD-1 therapies. Indeed, a recent study stated that STS patients who exhibited more

PD-L1-expressing macrophages responded to pembrolizumab, an anti-PD-1 monotherapy[207].

Other immune checkpoints have also been studied in other tumors. However, in STS there are only a few reports. Dancsok *et al*. found a high expression of lymphocyte-activation gene 3 (LAG3) on infiltrating CD8 T cells[225]. Another study confirmed this overexpression by immunohistochemistry and proposed a correlation between LAG3 expression and poor patient outcome[191]. In the same study, Dancsok *et al*. demonstrated that the expression of B- and T-lymphocyte attenuator (BTLA) was lower in sarcoma and mainly in infiltrating CD4 T cells[225]. Concerning the immune checkpoints indoleamine-pyrrole 2,3-dioxygenase 1 (IDO-1) and E-Cadherin, it was suggested that the expression of IDO-1 was correlated with a poor patient outcome in undifferentiated pleomorphic sarcoma, and E-cadherin expression was associated with a better patient outcome in STS[236–238]. The expression of B7-H6 and B7-H3 has also been evaluated and the expression of both molecules was correlated with a poor patient outcome in metastatic gastrointestinal stromal tumors and rhabdomyosarcoma, respectively[239,240]. Likewise, the expression of the immune checkpoints CD47 and signal regulatory protein α (Sirpα) were correlated with poor outcome in sarcomas[174]. Lastly, the expression of the exhaustion marker T cell immunoreceptor with Ig and ITIM domains (TIGIT) was evaluated in STS using the TCGA, but not associated with the patient outcome[184]. Nevertheless, in the same study, the expression of CD155, its dominant ligand, was correlated with worse patient outcome in STS.

1.4.2.4 Immune-Related Genes

Like the presence of immune cells populations, the expression of immune-related genes has been studied in cancer, and its prognostic value has been evaluated in several cancers, including lung cancer, ovarian cancer, head and neck squamous cell carcinoma, and renal cancer[241–244]. Although some studies aimed to evaluate the expression of immune-related genes in STS and correlated it with patient outcome, more studies are required to clarify its prognostic significance (Figure 5).

Figure 5 | Studies correlating the expression of immune-related genes with the patient prognosis in STS. Five main studies tried to clarify the use of immune-related genes expression as biomarkers for patient outcome in STS. Immune-related genes associated with a better prognosis in STS are represented in green. Contrary, immune-related genes correlated with a poor prognosis are represented in red. From the peripheral to the center, the circles represent the genes encoding extracellular proteins, transmembrane proteins, intracellular proteins, the methodology used, and the respective first author and publication year of the study. [1] *Prognostic value in synovial sarcomas;* [2] *Prognostic value in gastrointestinal stromal tumors;* [3] *Prognostic value in myxoid liposarcomas;* [4] *Prognostic value in sarcomas with complex genetics.*

Chen *et al*. found that high transcription levels of IL33 were correlated with the recruitment of CD8 T cells[245]. In the same study, the low expression of ST2, IL-33 receptor, was associated with the recruitment of Tregs and MDSC. Furthermore, both immune-related genes were correlated with a good prognosis. Another study aimed to evaluate 364 differentially expressed immune-related genes in STS patients and found

that 18 of these genes were associated with patient survival[246]. Similarly, Dufresne *et al*. evaluate the expression of 93 immune-related genes in 253 STS samples and demonstrated a correlation between the immune signature and each sarcoma subtype, corroborating the idea that the prognostic value may depend on the STS subtype[247].

Another study, proved the effective performance of five immune-related genes, including IFIH1, CTSG, STC2, SECTM1, and BIRC5, in risk stratification of patients, confirming the potential use of these genes to predict the patient outcome[248]. Lastly, in 2020, another study identified seven genes, including C3, CD36, DOCK9, FCER2, FOS, HLA-DRB4, and NCAM1, associated with a poor outcome and six immune-related genes, including BIRC5, DUSP4, FOXP3, HLA-DQA1, HLA-DQB1, and LAG3, associated with a better outcome in high-grade STS tissue samples[249].

1.4.2.5 Peripheral Blood Immune *Status*

Besides the studies that aimed to evaluate the serum levels of immune-related factors, the studies concerning the immune cells, the immune checkpoints, and the immune-related genes usually focus on the TME, analyzing tumor tissue samples. Thus, the studies assessing the peripheral blood immune *status* are sparse.

In 2014, the lymphocyte/monocyte ratio in the peripheral blood of STS patients was evaluated for the first time[250]. In this study, they concluded that this ratio could represent a negative prognostic marker. However, another study performed in 2019 did not find significant differences between the lymphocyte/monocyte ratio and the clinical outcome in STS[251]. The neutrophil-to-lymphocyte ratio in STS was also the focus of two meta-analyses[252,253]. Both concluded that a higher neutrophil-to-lymphocyte ratio was correlated with worse clinical outcome.

Recently, Kim *et al*. assessed the peripheral immunological *status* in STS and observed that high levels of monocytic MDSC (M-MDSC) and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) positive CD8 T cells were associated with a worse prognosis[254]. Contrary, they found a correlation between the high levels of NKG2D positive CD8 T cells and a better prognosis.

Concerning the peripheral levels of NK cell subsets, one study evaluated them in the peripheral blood of chemotherapy-*naïve* STS patients and STS patients with a progression or relapse after chemotherapeutical treatment[255]. In both groups, NK cells were found to be dysfunctional, corroborating the studies in other cancers. Furthermore,

through flow cytometry, they observed a lower frequency of $CD56^{dim}$ NK cells in the second group of patients when compared to controls. Also in the group of STS patients with a progression or relapse after therapy, the expression of NKG2D, CD3ζ, and perforin was associated with NK cells activation. However, another study aimed to also evaluate peripheral NK cells in gastrointestinal stromal tumor patients and did not find significant differences in the levels of NK cells nor in the NKG2D expression when compared to controls[256]. Still, they observed a higher expression of NKp30c, an immunosuppressive isoform of the NKp30 receptor, correlated with a poor patient outcome.

Finally, one study intended to assess the immune cells in both peripheral blood and tumor tissue[184]. They observed that T cells and NK cells were both more activated and exhausted in the tumor site when compared to the peripheral blood. Moreover, the $CD56^{bright}$, the less cytotoxic subset, was found less expressed in the tumor site when compared to the peripheral blood. As far as we are aware, this is the only study comparing the immunological *status* of peripheral blood and tumor site in STS patients. The significant differences found demonstrated that the immune *status* varies between these two locations.

Chapter 2

OBJECTIVES

2 Objectives

With the development of immunotherapy, the assessment of the immunological *status* of cancer patients is growing in importance. Indeed, studies have been shown that immune cells and other mediators of the immune response, such as soluble immune-related factors, immune checkpoints, and immune-related genes, may represent potential immunotherapeutic agents or targets, or potential biomarkers for an accurate prognosis and therapy response.

Although initially STS were simply classified as 'non-immunogenic' tumors, studies have proved that this characterization does not apply to all. Actually, different TME compositions have been found in STS patients, and some of them exhibited an elevated infiltration of immune cells and immune-related factors. Furthermore, the TME has also been associated with patient prognosis and patient response to therapy in STS.

However, the TME evaluation requires an invasive procedure, making sample harvest difficult, thus limiting patient cohort and follow-up. Therefore, a minimally invasive procedure, such as the analysis of peripheral blood samples, is inevitably required. Unfortunately, there is a lack of studies that seek to evaluate and correlate the peripheral immune *status* of STS patients with patient prognosis, and the scarce results reported are sometimes controversial. Moreover, different therapies are included in most studies without agent-specific effect analysis.

With this in mind, our primary and secondary objectives were as follows:

Primary objectives:

1. Evaluation of the effect of therapy on the peripheral immunological *status* of STS patients.

2. Evaluation of the prognostic value of peripheral immunological *status* in STS, considering the patient therapy.

Secondary objectives:

1. Comparison of the immunological *status* between patients undergoing trabectedin and anthracycline-based therapies, and between patients undergoing long-term trabectedin treatments (>20 cycles) and ≤20 trabectedin treatment cycles.

   1.1. Frequency analysis of circulating immune cells populations.
   1.2. Analysis of soluble levels of immune-related factors.
   1.3. Analysis of immune-related gene expression levels.

2. Correlation between the immune contexture assessed and the patient OS.

Chapter 3

MATERIAL AND METHODS

3 Material and Methods

3.1 Study Design

The study group consisted of 31 patients with STS. The demographic and clinicopathological patient characterization are present in Table III. To evaluate the peripheral immunological *status* considering the patient therapy, STS patients were divided into two therapy groups: 7 patients who had undergone anthracycline-based therapy (ANTH; doxorubicin 90 mg plus dacarbazine 900 mg, alternated with ifosfamide 10g/m^2) and 24 patients who had undergone trabectedin-based therapy (TRAB; 1.5mg/m^2). In addition, to understand whether the immunological *status* varies with long-term trabectedin therapy, the TRAB patients were divided into two separated groups: 11 patients who had completed ≤20 trabectedin cycles (TRAB≤20) and 13 patients who had completed >20 trabectedin cycles (TRAB>20).

The peripheral blood samples and clinical data were collected at Orthopedic Service from Coimbra Hospital and University Center, from November 2015 to February 2021. All the volunteers agreed and signed informed consent to participate. The present work was approved by the Ethical Committee of the Faculty of Medicine of the University of Coimbra and the Coimbra Hospital and Universitary Centre, Portugal (CHUC-021-19).

3.2 Flow Cytometry

Flow cytometry is a powerful tool with wide applications, including in the immunology field. It allows the characterization of a complex mixture of immune cell populations in highly heterogeneous body fluids, such as peripheral blood, being very effective for the study of the immune system at the single cell level[257].

Flow cytometry uses multiple lasers and detectors to analyze single cell suspensions. Each cell is analyzed by visible light scatter in two different directions. The forward scatter (FSC) indicates the relative size, while the side scatter (SSC) indicates the complexity or granularity. In addition to visible light, the cells are also analyzed according to fluorescence parameters after incubation with fluorescent conjugated antibodies or probes[257].

Table III | Demographic and clinicopathological composition of the cohort included in this study.

Clinicopathological characteristic	Value	Percentage
N	31	
Median age (range), years	54 (19-78)	
Sex		
Female	16	51.6
Male	15	48.4
Soft tissue sarcoma histology		
Leiomyosarcoma	14	45.2
Liposarcoma	4	12.9
Synovial sarcoma	3	9.7
Haemangiosarcoma	2	6.5
Undifferentiated sarcoma	1	3.2
Fibromyxosarcoma	1	3.2
Malignant fibrous histiocytoma	1	3.2
Pleomorphic rhabdomyosarcoma	1	3.2
Clear cell sarcoma	1	3.2
Malignant peripheral nerve sheath tumor	1	3.2
Alveolar soft part sarcoma	1	3.2
Phyllodes tumor of the breast	1	3.2
Localization		
Connective and soft tissue of limb	10	32.3
Retroperitoneum	5	16.1
Utero	5	16.1
Connective and soft tissue of thorax	3	9.7
Connective and soft tissue of trunk	2	6.5
Others	6	19.4
Tumor type		
Primary	10	32.3
Recurrent	8	25.8
Metastatic	6	19.4
Recurrent/Metastatic	7	22.6
Therapy		
Anthracycline-based therapy	7	22.6
Trabectedin-based therapy	24	77.4
Trabectedin cycles		
≤ 20 cycles	11	45.8
> 20 cycles	13	54.2
Prior chemotherapy		
Yes	26	83.9
No	5	16.1

Our characterization of the immune cells by flow cytometry included the evaluation of the major immune cell populations: T cells, B cells, DC, monocytes, NK cells, and MDSC, as well as their subsets. Furthermore, it was also evaluated their activation and memory, and the expression of the immune checkpoints PD-1 and PD-L1.

3.2.1 Whole blood staining and sample acquisition

To summarize the protocol, the major immune cells populations presented in the whole blood samples, collected into EDTA tubes, were counted with a hematological counter (DxH500, Beckman Coulter, Pasadena, CA, USA). Then, 100 µL of whole blood or up to 1 million cells were incubated with extracellular antibodies (Supplementary Table I), for 15 minutes in the dark at room temperature, according to a predefined combination panel (Table IV). After incubation, red blood cells were lysed with BD Lysing Solution (BD Biosciences, San Jose, CA, USA) for 10 minutes in the dark. The cell suspensions were centrifuged at 450 x *g* for 5 minutes and the supernatants were discarded. The suspensions were washed with PBS and lastly, samples were acquired in BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA) using FACSDiva™ version 6.1.3 software (BD Biosciences, San Jose, CA, USA).

Table IV | Eight-color antibody panel.

Fluorochrome	T cells	Tregs	Ths and Tcs	B cells	DC, monocytes, and NK cells	MDSC
FITC	PD-1	PD-1	PD-1	CD24	PD-L1	CD45
PE	CCR7	CD25	CXCR3	PD-1	CD56	CD33
PerCP-Cy5.5	CD4	CD4	CD4	CD19	CD123	CD3/19/56
Pe-Cy7	CD45RA	CCR4	CCR6	CD27	CD11c	CD15
APC	CD38	CD127	CD38	CD38	PD-1	CD11b
APC-H7	CD8	CD45RO	CD8	CD20	CD3/19/20	CD16
V450	CD3	CD3	CD3	CD3	CD14	CD14
V500	HLA-DR	HLA-DR	HLA-DR	IgD	HLA-DR	HLA-DR

Tregs, regulatory T cells; Ths, T helper cells; Tcs, T cytotoxic cells; DC, dendritic cells; NK, Natural Killer; MDSC, myeloid-derived suppressor cells; PD-1, programmed-death 1-receptor; CCR7, C-C chemokine receptor type 7; HLA-DR, human leukocyte antigen – DR isotype; CCR4, C-C chemokine receptor type 4; CXCR3, C-X-C motif chemokine receptor 3; CCR6, C-C chemokine receptor type 6; IgD, immunoglobulin D; PD-L1, programmed-death- ligand 1.

3.2.2 Analysis of immune cells populations

All the data were treated with FlowJo® v.10.7 (BD Life Sciences, Ashland, OR, USA), a software application with an integrated environment for viewing and analyzing flow cytometric data.

The live cells and then the lymphocyte population were isolated through size and complexity, using the information given by the FSC and SSC, respectively, and the doublet discrimination was performed with a FSC-Height (FSC-H) *vs* FSC-Area (FSC-A) dot plot (Figure 6).

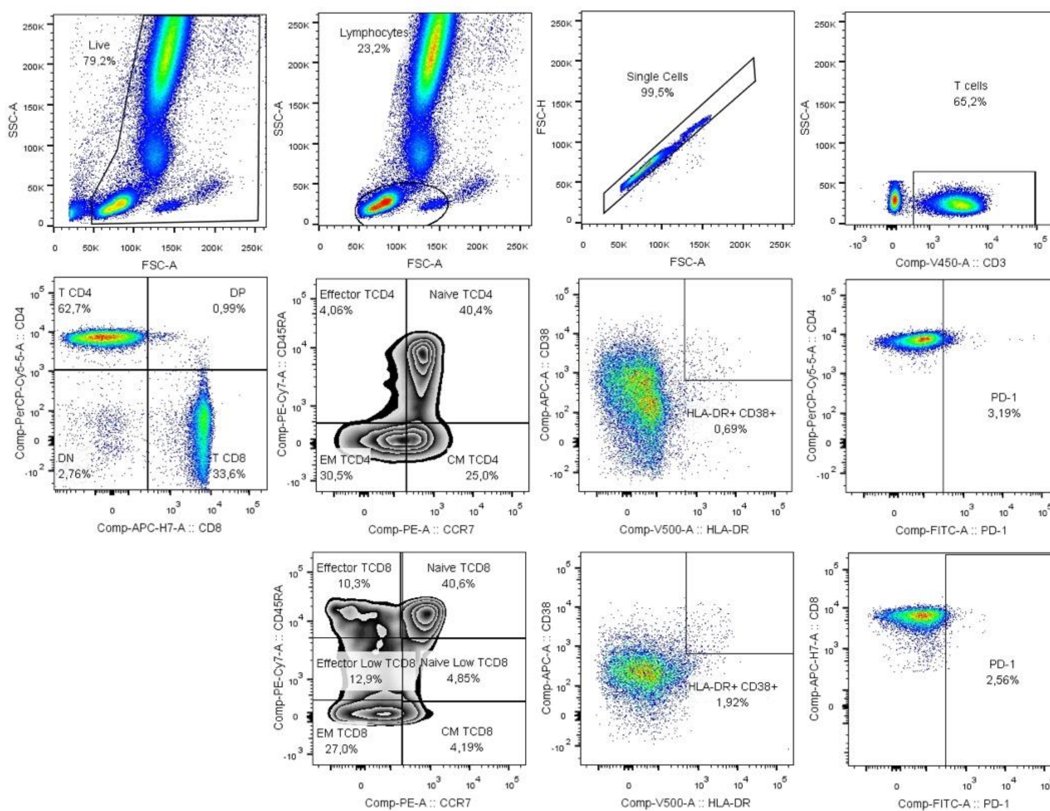

Figure 6 | T cells gate strategy. Through the analysis of the size and complexity, the events corresponding to living cells and the lymphocyte population were identified. Doublet discrimination was performed with a FSC-H *vs* FSC-A dot plot. Gated on lymphocytes, through the presence of CD3, it was possible to select the T cells. Within this population, it was selected the positive ones for CD4, corresponding to CD4 T cells, for CD8, corresponding to CD8 T cells, positive for both, and negative for both. After obtaining these different subtypes, it is possible to assess the presence of memory markers, CD45RA and CCR7 allowing the identification of *naïve* cells (CD45RA+ CCR7+), central memory cells (CD45- CCR7+), effector memory cells (CD45RA- CCR7-), and effector cells (CD45RA+ CCR7-). The activation markers, HLA-DR and CD38, and the presence of PD-1 were also assessed in both CD4 T cells and CD8 T cells. *FSC, forward scatter; SSC, side scatter; DN, double negative; DP, double positive; EM, effector memory; CM, central memory; PD-1, programmed death-1 receptor.*

To define T cells among the lymphocytes we used the positive expression of CD3. Then, the expression of CD4 and CD8 was used to distinguish the CD4 T cells, CD8 T cells, double positive (DP), and double negative (DN) T cells. Inside these two groups, it was also possible to discriminate the four subsets, using the expression of CCR7 and CD45RA: *naïve* cells (CD45RA+ CCR7+), central memory cells (CD45RA- CCR7+), effector memory cells (CD45RA- CCR7-), and effector cells (CD45RA+ CCR7-). Analyzing the expression of CD38 and human leucocyte antigen-DR isotype (HLA-DR), it was possible to define the activated *status* of either CD4 or CD8 T cells. Finally, the expression of the immune checkpoint PD-1 was also assessed.

Furthermore, within CD4 T cells, the expression of CXCR3 and CCR6 allowed the discrimination between Th1 (CXCR3+ CCR6-), Th17 (CXCR3- CCR6+), and Th2 cells (CXCR3- CCR6-) (Figure 7). Likewise, T cytotoxic (Tc)1 (CXCR3+ CCR6-), Tc17 (CXCR3- CCR6+), and Tc2 (CXCR3- CCR6-) were also identified inside CD8 T cells. The presence of activation markers, HLA-DR and CD38, and the presence of PD-1 was also assessed in Th and Tc cells.

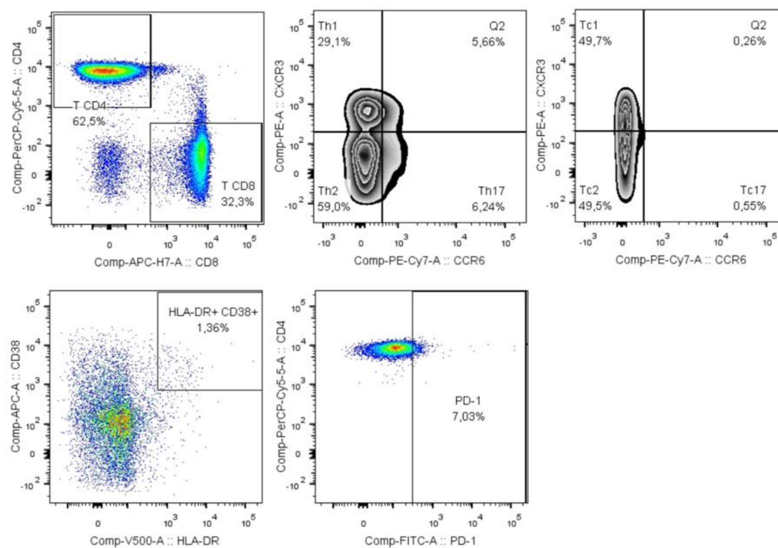

Figure 7 | Ths and Tcs gate strategy. T cells were plotted in a CD4 *vs* CD8 diagram, and the CD8 T cells and CD4 T cells were identified. Then, CD4 T cells were plotted in a CXCR3 *vs* CCR6 diagram, allowing the identification of Th1 cells (CXCR3+ CCR6-), Th17 (CXCR3- CCR6+), and Th2 cells (CXCR3- CCR6-). Similarly, CD8 T cells were plotted in a CXCR3 *vs* CCR6 diagram, and Tc1 (CXCR3+ CCR6-), Tc17 (CXCR3- CCR6+), and Tc2 (CXCR3- CCR6-) populations were discriminated. The presence of activation markers, HLA-DR and CD38, and the presence of PD-1 were also assessed. *Th, T helper cell; Tc, T cytotoxic cell; PD-1, programmed death-1 receptor.*

As was mentioned in the introduction, Tregs are also a subtype of CD4 T cells. These cells were identified by negativity for CD127 and positivity for CD25 and CCR4 antibodies (Figure 8). Further, the use of CD45RO allowed the discrimination of *naïve* Tregs (CD45RO-) and memory Tregs (CD45RO+). The activation *status* of Tregs was assessed by the presence of the activation marker HLA-DR. In addition, the presence of PD-1 was also evaluated.

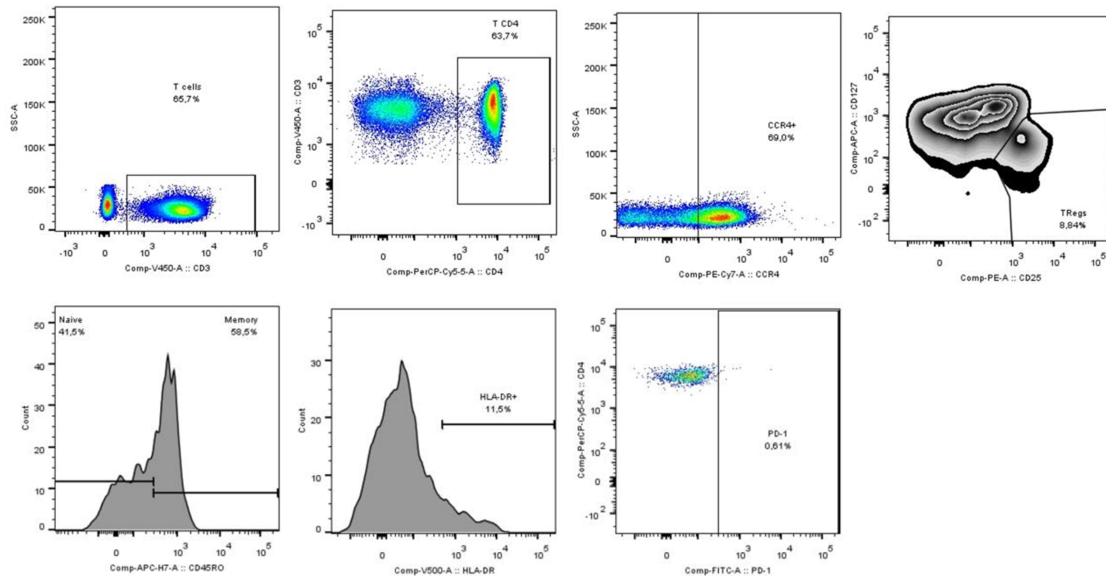

Figure 8 | Tregs gate strategy. The presence of CD3 was used to select the T cells, and the presence of CD4 was used to select the CD4 T cells. Gated on CD4 T cells, Tregs were identified by negativity for CD127 and positivity for CD25 and CCR4 antibodies. The memory marker CD45RO allowed the discrimination of *naïve* Tregs (CD45RO-) and memory Tregs (CD45RO+). The presence of PD-1 was also assessed in Tregs. *SSC, side scatter; Tregs, regulatory T cells; PD-1, programmed death-1 receptor.*

Being a part of lymphocytes, B cells were identified by the positivity for CD19 (Figure 9). Through the expression of CD27 and immunoglobulin D (IgD), *naïve* B (IgD+ CD27-), pre-switch memory cells (IgD+ CD27+), switch memory cells (IgD- CD27+), and exhausted cells (IgD- CD27-) were discriminated. The presence of CD38 and CD24 gated on *naïve* cells and switch memory B cells identified the transitional B cells ($CD38^{high}$ $CD24^{high}$) and plasmablasts/plasma cells ($CD38^{high}$ $CD24^{-}$). PD-1 positivity was also assessed for B cells.

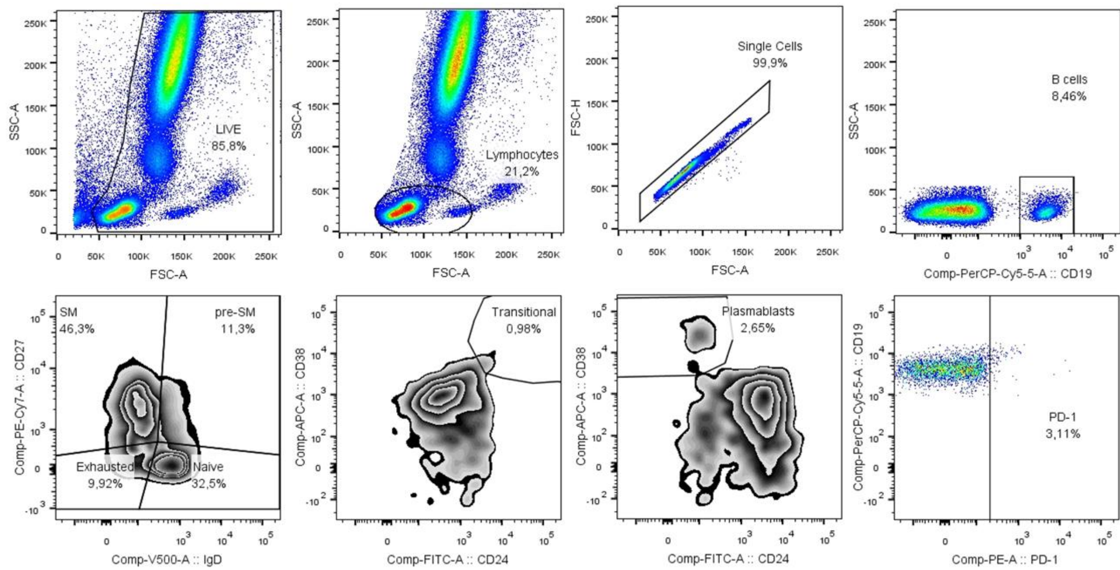

Figure 9 | B cells gate strategy. Through the analysis of the size and complexity, the events corresponding to living cells and the lymphocyte population were identified. Doublet discrimination was performed with a FSC-H *vs* FSC-A dot plot. Gated on lymphocytes, B cells were identified by CD19 positivity. B cells were plotted in a CD27 *vs* IgD diagram to allow the identification of *naïve* cells (IgD+ CD27-), pre-switch memory cells (IgD+ CD27+), switch memory cells (IgD- CD27+) and exhausted cells (IgD- CD27-). Gated on *naïve* B cells, the transitional B cells ($CD38^{high}$ $CD24^{high}$) were identified. Similarly, gated on switch memory B cells, plasmablasts/plasma cells ($CD38^{high}$ $CD24^{-}$) were discriminated. PD-1 positivity was also assessed for B cells. *FSC, forward scatter; SSC, side scatter; SM, switch memory cells; pre-SM, pre-switch memory cells; PD-1, programmed death-1 receptor.*

Within lymphocytes, NK cells (CD3- CD56+) and NKT-like cells (CD3+ CD56+) were identified (Figure 10). Further, the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, and $CD3^{dim}$ and $CD3^{bright}$ NKT-like cell subsets were discriminated. The activation *status* of NK cells and NKT-like cells were assessed by the presence of the activation marker HLA-DR. Furthermore, the presence of the immune checkpoints PD-1 and PD-L1 was also evaluated in both subsets.

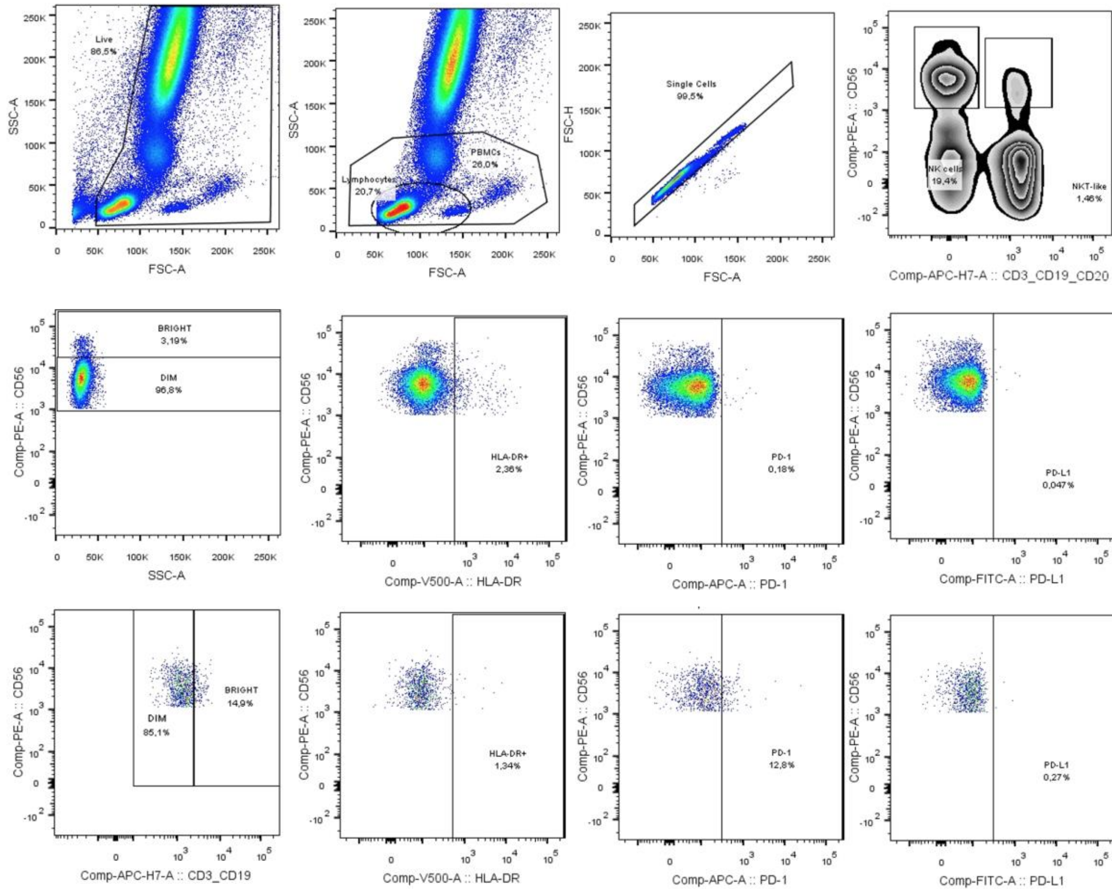

Figure 10 | NK cells and NKT-like cells gate strategy. Through the analysis of the size and complexity, the events corresponding to living cells and the lymphocyte population were identified. Doublet discrimination was performed with a FSC-H *vs* FSC-A dot plot. Gated on lymphocytes, NK cells (CD3- CD56+) and NKT-like cells (CD3+ CD56+) were discriminated. Then CD56^{dim} and CD56^{bright} NK cells, and CD3^{dim} and CD3^{bright} NKT-like cells were discriminated. The activation marker HLA-DR and the expression of PD-1 and PD-L1 were assessed in NK cells and NKT-like cells. *FSC, forward scatter; SSC, side scatter; PBMCs, peripheral blood mononuclear cells; NK, Natural Killer; PD-1, programmed death-1 receptor; PD-L1, programmed death-ligand 1.*

To identify monocytes and DC, the peripheral blood mononuclear cells (PBMCs) were discriminated through size and complexity, using the FSC and SSC (Figure 11). Then, monocytes were identified by the negativity for CD3, CD19, CD20, and CD56 and the positivity for CD14 antibody. DC were discriminated by the negativity for CD3, CD14, CD19, CD20, and CD56, and the positivity for HLA-DR antibody. Furthermore, cDC (CD11c+ CD123+/-) and pDC (CD11c- CD123+) were discriminated. The expression of PD-1 and PD-L1 was evaluated in monocytes and DC.

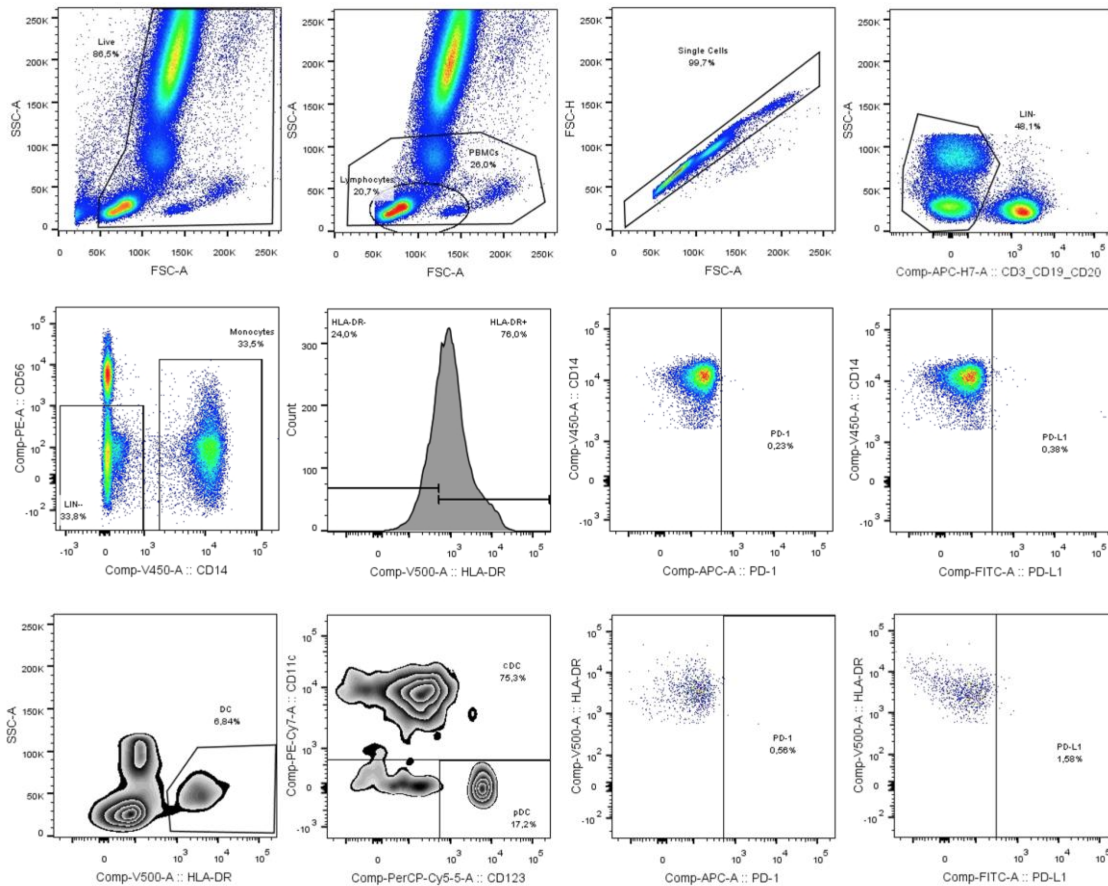

Figure 11 | Monocytes and DC gate strategy. Through the analysis of the size and complexity, the events corresponding to living cells and then the PBMCs were isolated. Doublet discrimination was performed with a FSC-H *vs* FSC-A dot plot. Gated on PBMCs, the cells with an absence of CD3, CD19, CD20, and CD56 were excluded, and the positivity for CD14 antibody identified the monocytes population. To identify DC, the lineage negative (LIN-), cells with an absence of CD3, CD19, CD20, CD56, and CD14 were selected. Then, the positivity for HLA-DR, inside the LIN- gate, discriminated DC. Gated on DCs, CD11c and CD123 antibodies were used to discriminate cDC (CD11c+ CD123+/-) and pDC (CD11c- CD123+). PD-1 and PD-L1 positivity was assessed for monocytes and DC. *FSC, forward scatter; SSC, side scatter; PBMCs, peripheral blood mononuclear cells; LIN-, negative linage; DC, dendritic cells; cDC, myeloid/conventional DC; pDC, plasmacytoid DC; PD-1, programmed-death 1 receptor; PD-L1, programmed-death ligand 1.*

To identify the MDSC we excluded the CD3, CD16, CD19, CD56, and HLA-DR positive cells (Figure 12). In the remaining population, MDSC were discriminated through their positivity for CD33 and CD11b antibodies. Gated on MDSC, the expression of CD15 and CD14 were used to discriminate the early myeloid-derived suppressor cells (e-MDSC) (CD14-CD15-), M-MDSC (CD14+CD15±), and PMN-MDSC (CD14-CD15+).

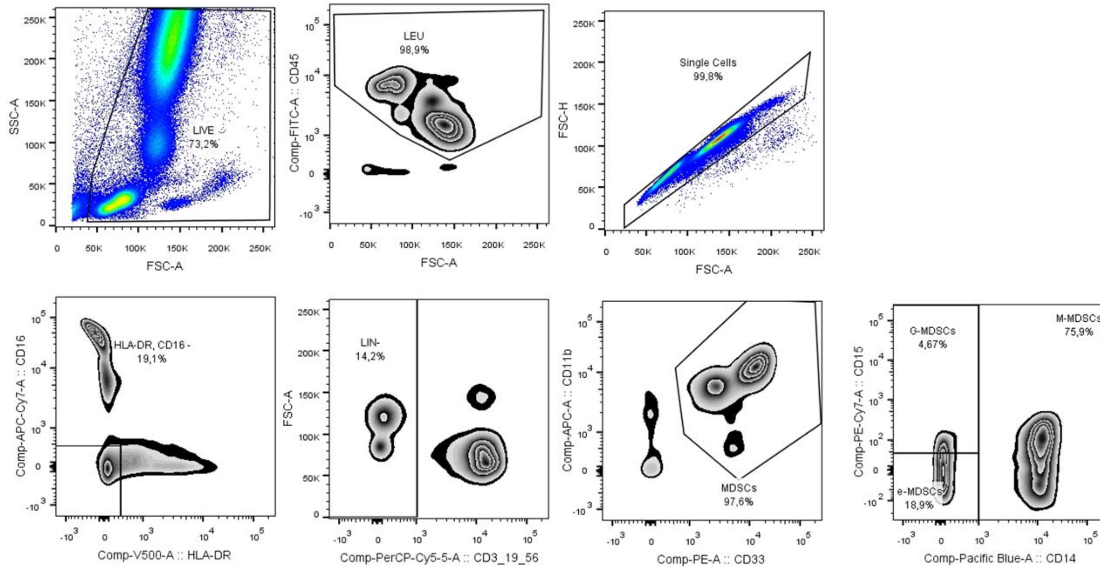

Figure 12 | MDSCs gate strategy. The events corresponding to the live cells were isolated through the analysis of the size and complexity. Doublet discrimination was performed with a FSC-H *vs* FSC-A dot plot. Then, MDSC were identified, gated on leucocytes, through their negativity for CD16, HLA-DR, CD3, CD19, and CD56, and their positivity for CD33 and CD11b antibodies. Gated on MDSC, the expression of CD15 and CD14 were used to discriminate the e-MDSC (CD14-CD15-), M-MDSC (CD14+CD15±), and PMN-MDSC (CD14-CD15+). *FSC, forward scatter; SSC, side scatter; LEU, leucocytes; LIN-, negative linage; MDSCs, myeloid-derived suppressor cells; e-MDSCs, early MDSCs; M-MDSCs, monocytic MDSCs; G-MDSCs, granulocytic MDSCs.*

3.3 Quantification of immune-related gene expression

3.3.1 Sample preparation

To perform the analysis of immune-related genes the whole blood, collected into PAXgene® Blood RNA tubes (Qiagen, Germany), was used. After collection, the tubes were incubated for 2 hours at room temperature, allowing the complete lysis of blood cells, and then stored at -80^{0}C. Before starting the RNA extraction procedure, the tubes were equilibrated at room temperature and incubated at this temperature for 2 hours.

3.3.2 RNA isolation, quantification, and purity assessment

After 2 hours of incubation, we used the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, to perform the RNA

extraction. Succinctly, the tubes were centrifugated to pellet nucleic acids. This pellet was then washed, resuspended, and incubated at $55^{o}C$ with optimized buffers and proteinase K, allowing protein digestion. The lysate was submitted to a centrifugation through the PAXgene Shredder spin column to homogenize the solution and remove cell debris. Ethanol was added to the supernatant resulting from this centrifugation, to adjust the binding conditions, and then the solution was applied to a PAXgene RNA spin column, where the RNA was selectively bound. Several washing steps were performed to eliminate remaining contaminants and the bound DNA was removed with DNase I treatment. At last, elution buffer was added to the column and the eluted RNA was heat-denatured. Following the extraction, the purity of the RNA was assessed through the ratio of the absorbance at 260 nm to the absorbance at 280 nm, using a NanoDrop 2000 (ThermoFisher Scientific, Wilmington, DE, USA).

3.3.3 cDNA synthesis

To synthesize the complementary DNA (cDNA), the iScript^{TM} Reverse Transcription Supermix for RT-qPCR (BIO-RAD, Hercules, CA, USA) was used. This cDNA synthesis supermix contains all the necessary components for reverse transcription except the RNA template, including RNase H+ Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, dNTPs, oligo(dT), random primers, buffer, $MgCl_2$, and stabilizers. This supermix was mixed with the RNA templates and incubated in a thermal cycler (Mastercycler Pro S, Eppendorf AG, Germany). The protocol consisted of an incubation of 5 minutes at $25^{o}C$ for priming, an incubation of 20 minutes at $46^{o}C$ for the reverse transcription, and lastly an incubation of 1 minute at $95^{o}C$ for reverse transcription inactivation. The resulted cDNA was stored at $-20^{o}C$ until the gene expression analysis.

3.3.4 Gene expression analysis

All cDNA samples were added to a 96 wells master plate. Taking advantage of a semi-automated pipetting system (epMotion® 96, Eppendorf AG, USA), the master plate was replicated, obtaining a 96 well plate for the quantification of each gene. Besides the cDNA samples of STS patients, non-template control reactions were performed in each plate to guarantee no unspecific amplifications.

The real-time PCR was performed using the kit iTaq™ Universal SYBR® Green Supermix (BIO-RAD, Hercules, CA, USA), containing a ready-to-use reaction master mix optimized, according to manufacturer´s instructions. Briefly, at room temperature, the supermix was mixed with RNase-free water and with the forward and reverse primers for the genes of interest or the reference genes (Supplementary Table II). The reference genes were selected according to the work of Vandesompele *et al*.[258]. After ensuring the solution homogeneity, equal aliquots were dispensed into each well of the 96 well plates containing the cDNA samples and the non-template controls, varying only the primers between the plates. The plates containing the reaction mix were incubated in the thermal cycler (Roche LightCycler II 480, Basel, Switzerland) previously programed for one pre-incubation cycle of 2 minutes at 95^{0}C, 50 amplification cycles of 5 seconds ate 95^{0}C and one minute at 60^{0}C, and a melt curve analysis (65-95ºC).

3.3.5 Analysis and quantification

Calibrated normalized relative quantification (CNRQ) of gene expression was obtained using qBase+ v3.2 software (Biogazelle, Gent, Belgium).

3.4 Multiplex Analyte Profiling (xMAP) of plasma

Cytokines, chemokines, growth factors, soluble receptors, and immune checkpoints were analyzed using Luminex's xMAP® technology. This technology combines advanced fluidics, optics, and digital processing with proprietary microsphere technology to deliver multiplex assay capabilities. The technique involves microsphere beads that are color-coded into up to 500 distinct sets. Each bead is then coated with a reagent specific to an analyte from the sample. Inside the Luminex® analyzer, it is possible to identify each bead and also any reporter dye captured during the assay, through a light source that excites the internal and the reporter dyes. Compared to other traditional methods, such as enzyme-linked immunosorbent assay (ELISA), western blotting, and PCR, this technique offers several advantages, including speed and high throughput, versatility, flexibility, accuracy, and reproducibility[259].

In this study, a panel of 65 cytokines, chemokines, growth factors, and soluble receptors, the Immune Monitoring 65-Plex Human ProcartaPlex™ Panel, was analyzed. Targets include proliferation-inducing ligand (APRIL), B-cell activating factor (BAFF),

B lymphocyte chemoattractant (BLC/CXCL13), CD30, epithelial neutrophil activating peptide (ENA)-78 (CXCL5), Eotaxin-2 (CCL24), Eotaxin-3 (CCL26), FGF-2, Fractalkine (CX3CL1), IL-16, IL-2R (CD25), IL-20, interferon–inducible T cell alpha chemoattractant (I-TAC/CXCL11), MCP-2 (CCL8), MCP-3 (CCL7), macrophage-derived chemokine (MDC/CCL22), macrophage migration inhibitory factor (MIF), monokine induced by gamma interferon (MIG/CXCL9), TNF-RII, TRAIL (CD253), thymic stromal lymphopoietin (TSLP), TNF-related weak inducer of apoptosis (TWEAK), CD40L (CD154), Eotaxin (CCL11), Gro-alpha (CXCL1), G-CSF (CSF-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), HGF, IFN alpha, interferon gamma-induced protein 10 (IP-10/CXCL10), IFN gamma, IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A (CTLA-8), IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, LIF, M-CSF, MCP-1 (CCL2), macrophage inflammatory protein (MIP)-1 alpha (CCL3), MIP-1 beta (CCL4), MIP-3 alpha (CCL20), matrix metalloproteinase (MMP)-1, nerve growth factor (NGF) beta, stromal cell-derived factor (SDF)-1 alpha (CXCL12), stem cell factor (SCF), TNF alpha, TNF beta, VEGF-A. (Supplementary Table III). Furthermore, 3 panels of immune checkpoints, the Immuno-Oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1 and 2, and Immuno-Oncology Checkpoint 10-Plex Human ProcartaPlex™ Panel 3, were also used. These 38 immune checkpoints include BTLA, CD137/4-1BB, CD152/CTLA-4, CD27, CD28, CD80, glucocorticoid-induced tumor necrosis factor receptor (GITR), herpes virus entry mediator (HVEM), IDO-1, LAG3, PD-1, PD-L1, PD-L2, Tim-3, E-Cadherin, MHC class I polypeptide–related sequence (MIC)A, MICB, NT5E (CD73), Nectin-2 (CD112), poliovirus receptor (PVR/CD155), Perforin, Siglec-7, Siglec-9, T Cell-activated increased late expression protein (TACTILE/CD96), UL16 binding protein (ULBP)-1, ULBP-3 and ULBP-4, B7-H6, CD134 (OX40), CD276 (B7-H3), CD47, CD48, Galectin-9, ICOSL (B7-H2), S100A8/A9, T cell immunoglobulin and mucin domain containing (TIMD)-4 and V-domain Ig suppressor of T cell activation (VISTA) (B7-H5). (Supplementary Table IV).

3.4.1 Sample preparation

To perform this analysis, the plasma was isolated from whole blood, collected into an EDTA tube, through a 1250 x *g* centrifugation for 10 minutes, and then stored at -20ºC until the analysis. Before starting the protocol, the frozen samples were thawed at 4ºC,

mixed well by vortexing, and centrifugated at 10,000 x *g* for 10 minutes in order to remove particulates. After, the supernatant of each plasma sample was added to a 96 wells plate.

3.4.2 Assay protocol

After the plate map definition, the magnetic beads were vortexed and added to each well. Then, the magnetic beads were washed, and the Universal Assay Buffer was added to each well. For wells dedicated to plasma samples, the plasma samples were added. For the standards, controls, and blanks, antigen standards prepared by sequential dilution, controls, and Universal Assay Buffer were added, respectively. After an incubation for 120 minutes with shanking at 500 rpm at room temperature, the plate was washed twice, and the detection antibody mixture was added to each well. Following this, the plate was incubated for 30 minutes with shanking at 500 rpm at room temperature, and then it was washed twice again. The Streptavidin-PE solution was added, the plate was again incubated for 30 minutes with shanking at 500 rpm at room temperature and washed twice. Lastly, the beads were resuspended in reading buffer, incubated for 5 minutes at 500 rpm and at room temperature, and acquired on Luminex® xMAP® 100/200™ system.

3.4.3 Analysis and quantification

Data were analyzed according to the operation manual in the ProcartaPlex™ Analysis App (https://apps.thermofisher.com/apps/procartaplex). The analytes with concentrations outside the limits of quantification were excluded from the analysis.

3.5 Statistical analysis

All statistical analyses and the graphs were performed and generated using GraphPad Prism 9.2.0 for Windows (GraphPad Sofware, San Diego, CA, USA). The Mann-Whitney test was used to compare the means between two groups. The data is presented as mean ± standard deviation and a value of $p < 0.05$ was considered statistically significant.

For the visualization of clusters of multivariate data, we use the Principal Component Analysis (PCA) and heatmaps, accessed online in ClustVis

(https://biit.cs.ut.ee/clustvis). In the PCA analysis, original values were ln(x+1)-transformed, unit variance scaling was applied to rows, and singular value decomposition with imputation was used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain the indicated percentages of the total variance. Prediction ellipses are such that with a probability of 0.95, a new observation from the same group will fall inside the ellipse. For the heatmaps, original values were also ln(x + 1)-transformed, rows were centered, unit variance scaling was applied to rows, imputation was used for missing value estimation, and both rows and columns were clustered using Manhattan distance and Ward (unsquared distances) linkage. Clustering distances were obtained using Pearson correlation subtracted from 1. Ward linkage method was calculated using the sum of squared differences from points to centroids as the distance.

For the OS analysis, we used the Kaplan-Meier survival analysis. OS time was defined as the time, in months, from the sample harvest to the date of death or the date of the last follow-up (censored patients). The Kaplan–Meier curves were performed in IBM SPSS statistics version 26.0 (IBM Corp., Armonk, NY, USA). The levels of the immune-related factors in patient blood were considered high if their percentage were above the median and low if their percentage were under the median. Then, a log-rank test was used to assess the potential associations between the immune-factors significantly altered and the patient survival.

Chapter 4
RESULTS

4 Results

In the present study, an extensive characterization of the immune cells, immune-related gene expression, and immune-related soluble factors, present in the peripheral blood of STS patients, was performed to identify immunological parameters dependent on the treatment. Our data revealed differences in the frequencies and repertoire of immune cells, and in the expression of immune-related genes between therapies. Regarding the soluble levels of several cytokines, chemokines, immune-checkpoints, and other immune-related factors, no differences were found. Moreover, we analyzed the influence of long-term trabectedin therapy in the immunological *status*. Here, we found alterations in the frequency of immune cell populations, in the expression of immune-related genes, and in the immune-related soluble factors analyzed between TRAB≤20 and TRAB>20 groups. Lastly, we performed survival analyses based on the parameters with a significantly different expression between the groups.

4.1 Immunophenotyping of STS patients according to treatment

Concerning the absolute and relative frequency of the major populations of leucocytes (granulocytes, monocytes, DC, and lymphocytes) and, particularly, the subpopulations of lymphocytes (T, B, NK, and NKT-like cells), there were no significant differences between ANTH and TRAB groups (Figure 13). To clarify the immunomodulatory role of trabectedin, we compared the same peripheral immune cell subsets between TRAB≤20 and TRAB>20. Similar to the results obtained between therapy groups, the absolute and relative frequency of the major populations did not vary with a long-term trabectedin therapy.

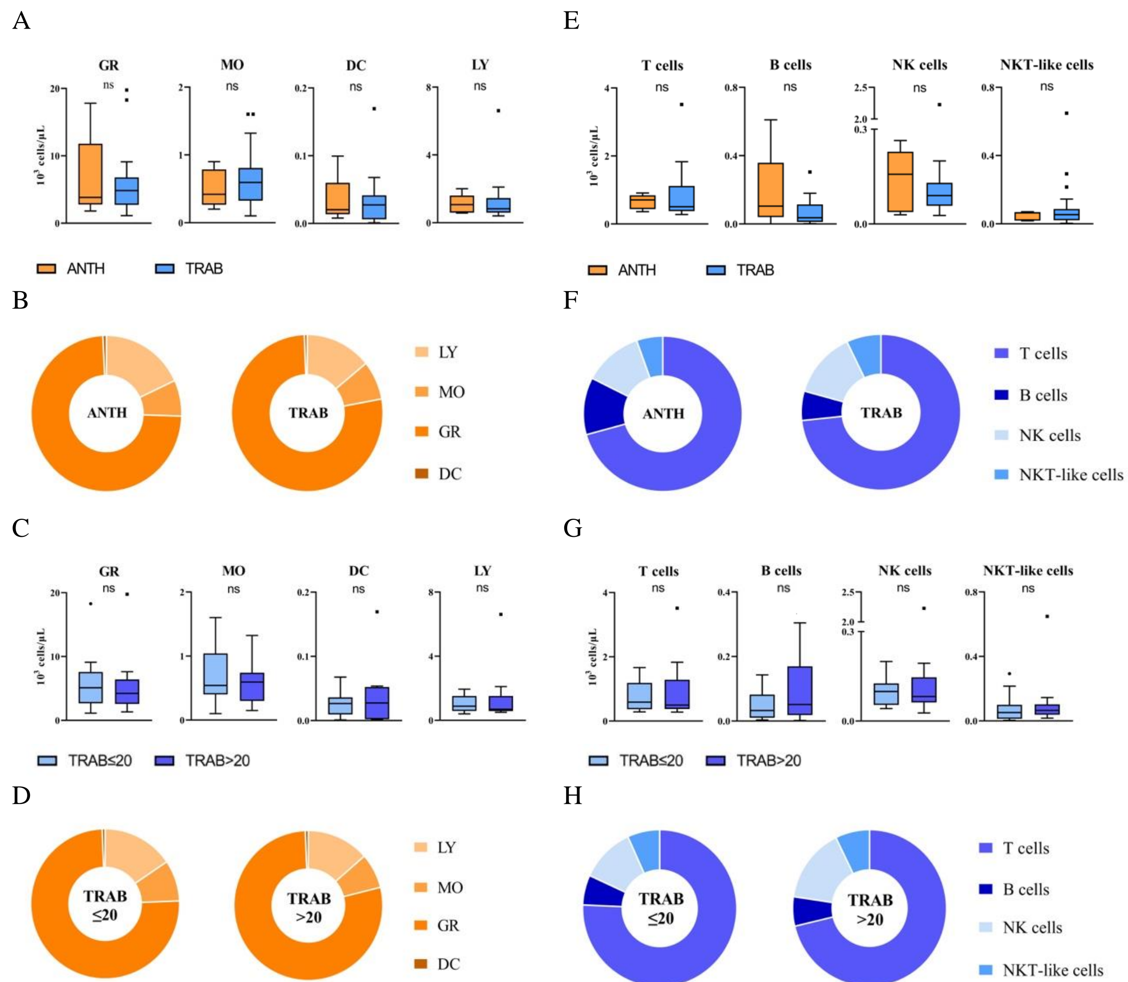

Figure 13 | Major populations of leucocytes and lymphocytes in the ANTH *vs* TRAB and TRAB≤20 *vs* TRAB>20 group of patients. Fresh whole blood from STS patients was stained with extracellular antibodies and analyzed by flow cytometry. **(A-B)** Absolute and relative frequency, respectively, of leucocyte subsets in ANTH (N= 7) and TRAB patients (N=24). **(C-D)** Absolute and relative frequency, respectively, of leucocyte subsets in TRAB≤20 (N= 11) and TRAB>20 patients (N=13). **(E-F)** Absolute and relative frequency, respectively, of lymphocyte subsets in ANTH (N= 7) and TRAB patients (N=24). **(G-H)** Absolute and relative frequency, respectively, of lymphocyte subsets in TRAB≤20 (N= 11) and TRAB>20 patients (N=13). Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; GR, granulocytes; MO, monocytes; DC, dendritic cells; LY, lymphocytes; NK, Natural Killer; ns, not statistically significant.*

4.1.1 CD4/CD8, activated, and memory T cell subsets

Although the absolute frequency of T cell subsets remained similar between groups (Figure 14A and 14C), we found alterations in the relative frequency of these subsets in both analyses (Figure 14B and 14D). TRAB patients exhibited a higher

frequency of DN T cells (3 ± 3% *vs* 6 ± 4%; p = 0.03), and TRAB>20 patients exhibited a higher frequency of DP T cells (0.9 ± 0.3% *vs* 1.4 ± 0.7%; p = 0.04).

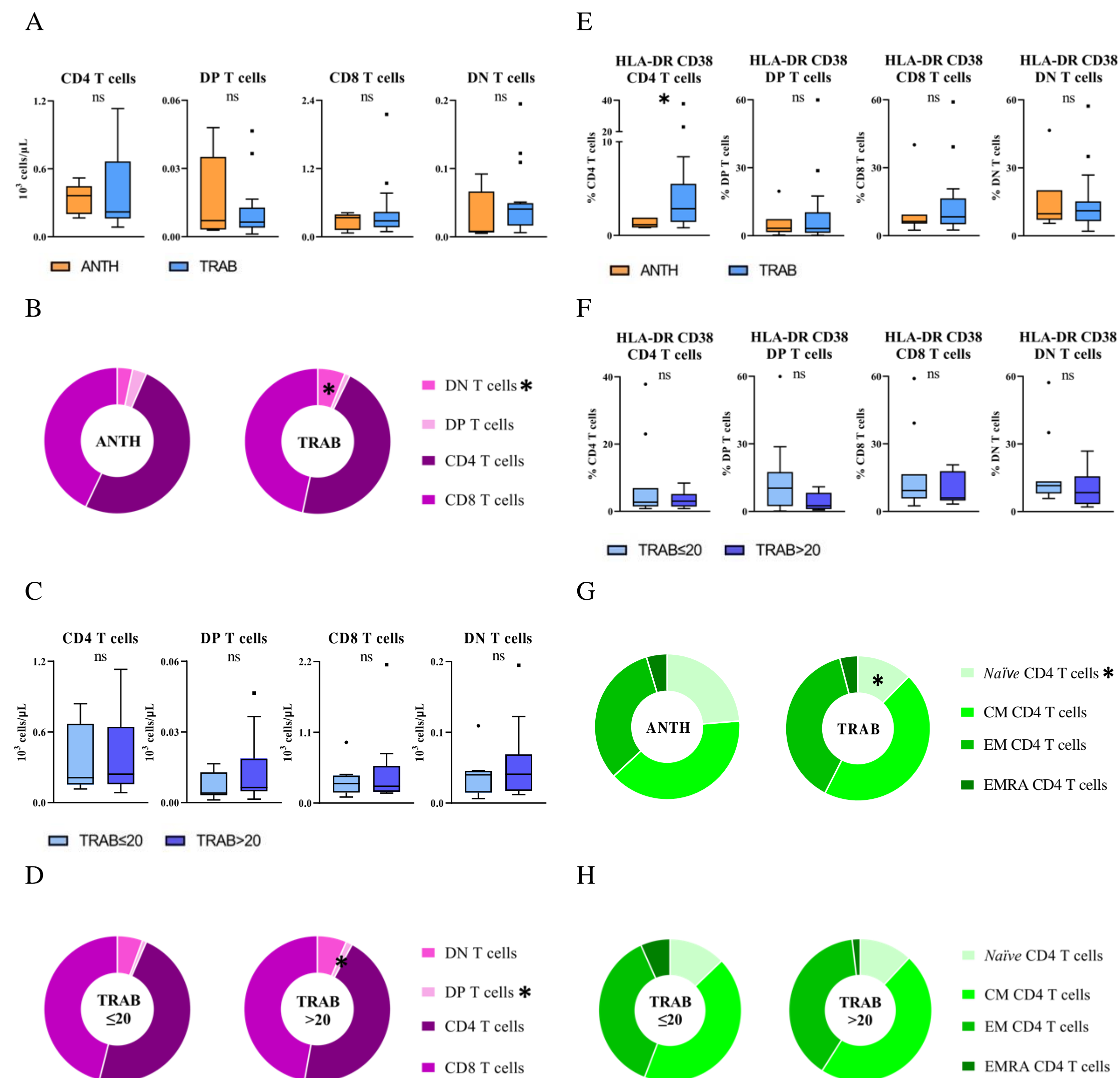

Figure 14 | T cells in the ANTH *vs* TRAB and TRAB≤20 *vs* TRAB>20 group of patients. Fresh whole blood from STS patients was stained with extracellular antibodies and analyzed by flow cytometry. **(A-B)** Absolute and relative frequency, respectively, of T cell subsets in ANTH (N= 7) *vs* TRAB patients (N=24). **(C-D)** Absolute and relative frequency, respectively, of T cell subsets in TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13). **(E-F)** Relative frequency of activated T cell subsets in ANTH (N= 7) *vs* TRAB patients (N=24), and TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13), respectively. **(G-H)** Relative frequency of memory T cell subsets in ANTH (N= 7) *vs* TRAB patients (N=24), and TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13), respectively. Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; DN, double negative; DP, double positive; EMRA, effector memory cells re-expressing CD45RA; ns, not statistically significant; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

To perform a more comprehensive analysis of the peripheral immune content of STS patients, we assessed the memory and activation *status* of several immune cell populations. Concerning the T cell subsets, a higher relative frequency of activated CD4 T cells (HLA-DR+ CD38+ CD4 T cells) (1 ± 1% *vs* 5 ± 8%; *p* = 0.01) (Figure 14E) and a lower frequency of *naïve* CD4 T cells (24 ± 12% *vs* 12 ± 9%; *p* = 0.02) (Figure 14G) were found in the TRAB patients). In addition, we evaluated the impact of DN T cells, DP T cells, activated CD4 T cells, and *naïve* CD4 T cells on patient outcome, but no significant differences in patient OS were found.

4.1.2 Natural Killer and Natural Killer T-like cells

While the total NK cell levels did not alter between therapies and number of trabectedin cycles, $CD56^{bright}$ NK cells were significantly decreased (21 ± 12% *vs* 12 ± 10%; *p* = 0.03), and consequently, $CD56^{dim}$ NK cells were increased in the TRAB>20 group (79 ± 12% *vs* 88 ± 10%; *p* = 0.03) (Figure 15). We also assessed the surface expression of PD-1 and found increased PD-1 NK cells in the peripheral blood of TRAB patients (0.2 ± 0.2% *vs* 2.6 ± 6.9%; *p* = 0.03). Although the expression of the immune checkpoint PD-1 was also assessed in other immune cell subsets, it was found constantly low and no significant differences between groups were observed.

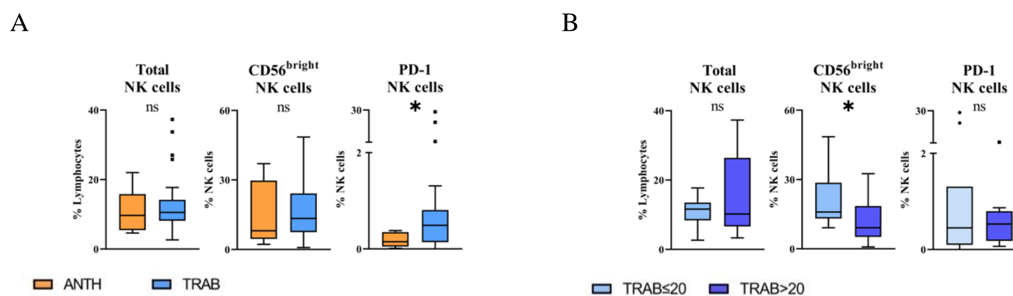

Figure 15 | NK cells in the ANTH *vs* TRAB and TRAB≤20 *vs* TRAB>20 group of patients. Fresh whole blood from STS patients was stained with extracellular antibodies and analyzed by flow cytometry. **(A-B)** Relative frequency of total NK cells, $CD56^{bright}$ NK cells, and PD-1 positive NK cells in ANTH (N= 7) *vs* TRAB patients (N=24), and TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13), respectively. Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; NK, Natural Killer; ns, not statistically significant; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

Concerning NKT-like cells, two subsets of NKT-like cells were identified through the surface expression of the CD3, the CD3^{dim} NKT-like cells, and the CD3^{bright} NKT-like cells. Additionally, we also assessed the activation *status* of these cells. We did not observe significant differences in the frequency of both subsets between patient therapies and number of trabectedin cycles (Figure 16). However, the activated subset of CD3^{bright} NKT-like cells, identified by their positivity for the activation marker HLA-DR, were found decreased in the TRAB>20 group of patients (28 ± 19% *vs* 13 ± 10%; p = 0.02).

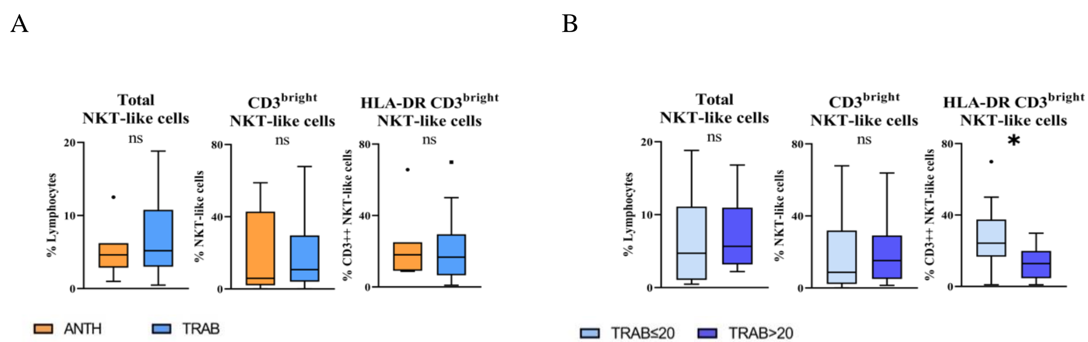

Figure 16 | NKT-like cells in the ANTH *vs* TRAB and TRAB≤20 *vs* TRAB>20 group of patients. Fresh whole blood from STS patients was stained with extracellular antibodies and analyzed by flow cytometry. **(A-B)** Relative frequency of total NKT-like cells, CD3^{bright} NKT-like cells, and activated CD3^{bright} NKT-like cells in ANTH (N= 7) *vs* TRAB patients (N=24), and TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13), respectively. Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; NK, Natural Killer; ns, not statistically significant; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

Furthermore, we tried to correlate the high levels of CD56^{bright} NK cells, CD56^{dim} NK cells, PD-1 NK cells and activated CD3^{bright} NKT-like cells with patient OS, but no significant differences were observed.

4.1.3 Dendritic cells

Once more, no alterations were found in the absolute and relative levels of total DC in both analyses. However, the analysis of their major subsets showed a decreased level of pDC in the TRAB>20 group of patients (20 ± 14% *vs* 11 ± 9%; p = 0.04) (Figure 17).

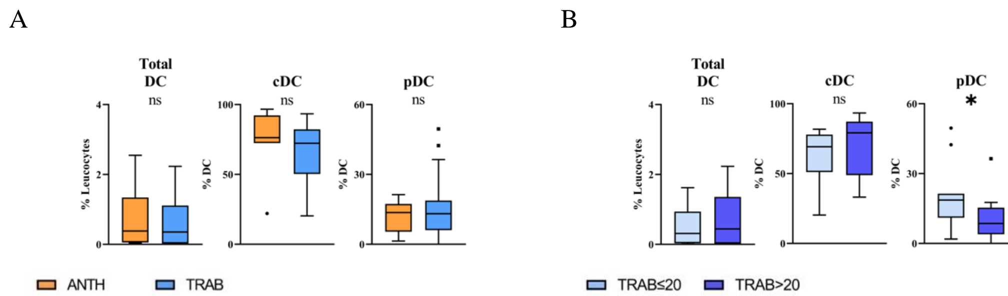

Figure 17 | DC in the ANTH *vs* TRAB and TRAB≤20 *vs* TRAB>20 group of patients. Fresh whole blood from STS patients was stained with extracellular antibodies and analyzed by flow cytometry. **(A-B)** Relative frequency of total DC, cDC and pDC in ANTH (N= 7) *vs* TRAB patients (N=24), and TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13), respectively. Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; DC, dendritic cells; cDC, conventional dendritic cells; pDC, plasmacytoid dendritic cells; ns, not statistically significant; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

4.1.4 Regulatory T cells and myeloid-derived suppressor cells

Given the suppressor role of Tregs and MDSC, we compared the frequency of these cells between therapies and number of trabectedin cycles (Figure 18). Total Tregs were not altered in both analyses. Nevertheless, when we analyzed the expression of the memory marker CD45RO on Tregs, we found higher levels of memory Tregs in the TRAB group (87 ± 3% *vs* 91 ± 7%; $p = 0.04$). Similarly, no significant differences in the total MDSC were found, but in the TRAB group, a significant decrease in the e-MDSC (Lin-CD11b+CD33+CD14-CD15-) (36 ± 23% *vs* 15 ± 16%; $p = 0.02$), with a consequent expansion of the M-MDSC (Lin-CD11b+CD33+CD14+CD15±), was observed. In the survival analysis, neither the high levels of memory Tregs nor e-MDSC were significantly correlated with the patient OS.

Figure 18 | Suppressive cells in the ANTH *vs* TRAB and TRAB≤20 *vs* TRAB>20 group of patients. Fresh whole blood from STS patients was stained with extracellular antibodies and analyzed by flow cytometry. **(A-B)** Absolute and relative frequency, respectively, of MDSC subsets in ANTH (N= 7) *vs* TRAB patients (N=24). **(C-D)** Absolute and relative frequency, respectively, of MDSC subsets in TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13). **(E-F)** Relative frequency of total Tregs, memory Tregs, and activated Tregs (HLA-DR Tregs) in ANTH (N= 7) *vs* TRAB patients (N=24), and TRAB≤20 (N= 11) *vs* TRAB>20 patients (N=13), respectively. Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; MDSC, myeloid-derived suppressor cells; e-MDSC, early-stage MDSC; M-MDSC, mononuclear MDSC; PMN-MDSC, polymorphonuclear MDSC; Tregs, regulatory T cells; ns, not statistically significant; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

4.2 Immune-related gene expression profiling

We performed a PCA and a cluster analysis in order to identify expression patterns associated with the two types of therapy or associated with the number of trabectedin cycles (Figure 19).

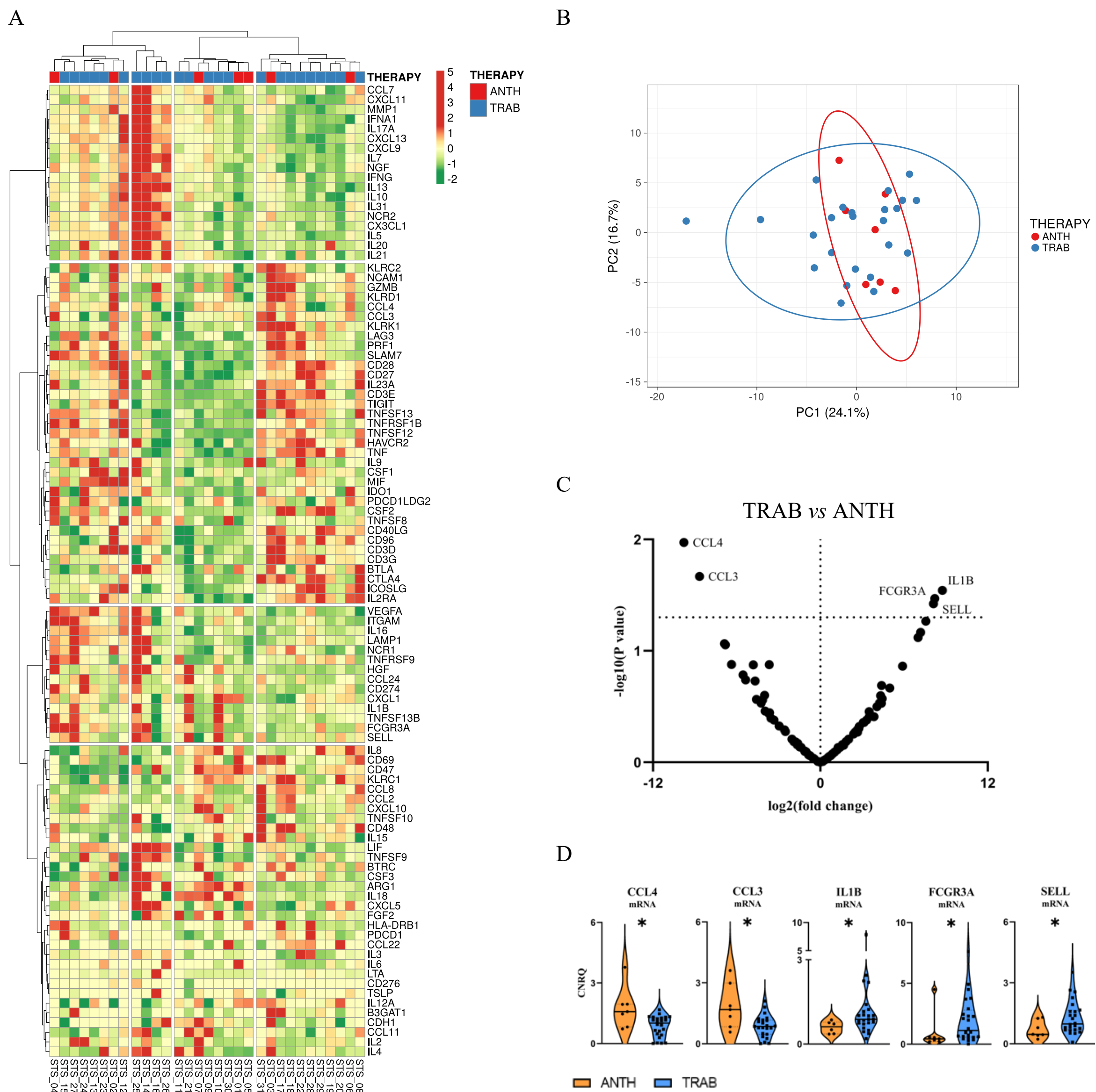

Figure 19 | Expression of immune-related genes in ANTH *vs* TRAB patients. RNA was extracted from the whole blood of STS patients, cDNA was synthesized, and the expression of immune-related genes was assessed by real-time PCR. **(A)** Heatmap for the serum expression levels of the immune-related genes analyzed in ANTH (N=7) and TRAB patients (N=24). **(B)** PCA for the expression of the immune-related genes analyzed in ANTH (N=7) and TRAB patients (N=24). **(C)** Volcano plot for differential immune-related genes expression in ANTH *vs* TRAB patients. Scattered points represent genes, the x-axis is the log2 fold change for the ratio ANTH *vs* TRAB, whereas the y-axis is the negative log (P value), and where the P value is the probability that a gene has statistical significance in its differential expression. The dots identified on the right side correspond to the genes over-expressed, and the dots identified on the left side correspond to the genes under-expressed in TRAB patients. **(D)** Calibrated normalized relative quantity of mRNA expressing the genes CCL4, CCL3, IL-1β, FCGR3A, and SELL in ANTH (N=7) and TRAB patients (N=24). Mann-Whitney test was used for the statistical analysis. *ANTH, patients who had undergone anthracycline-based therapy; TRAB, patients who had undergone trabectedin-based therapy; PC1, principal component 1; PC2, principal component 2; CNRQ, calibrated normalized relative quantity; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

Concerning the two types of therapy, the PCA analysis showed two overlapping groups (Figure 19B). The cluster analysis showed some evident clusters (Figure 19A). However, there are patients from both therapy groups in most clusters formed, in other words, the therapy groups do not form separate clusters based on the expression of immune-related genes. Further, a Mann-Whitney test comparing the expression of each immune-related gene between groups was performed. In these analyses, the genes encoding IL-1β (IL1B), CD16A (FCGR3A), and L-selectin (SELL) were found more expressed in TRAB patients (0.6 ± 0.2% *vs* 1.3 ± 1.5%; $p = 0.03$, 1.0 ± 1.6% *vs* 1.9 ± 1.9%; $p = 0.03$, 0.7 ± 0.4% *vs* 1.3 ± 0.8%; $p = 0.04$, respectively) (Figure 19C and 19D). On the contrary, the genes encoding CCL4 and CCL3 were found less expressed in this group of patients (1.8 ± 1.0% *vs* 0.9 ± 0.5%; $p = 0.01$, 1.8 ± 1.1% *vs* 0.9 ± 0.5%; $p = 0.02$, respectively). Moreover, we evaluated the correlation between these genes and the patient OS and no significant associations were observed.

We repeated the immune-related gene expression analysis, now comparing TRAB≤20 and TRAB>20 group of patients. Once more, the cluster analysis showed some evident clusters (Figure 20A). However, the two groups are distributed through the clusters. Similarly, the PCA analysis showed two overlapping groups (Figure 20B). Nevertheless, a Mann-Whitney test comparing the expression of each immune-related gene between groups was performed, and the gene FGF2 was found less expressed (14 ± 14% *vs* 4 ± 3%; $p = 0.04$) while the gene CXCL1 were more expressed in TRAB>20 patients (1.0 ± 0.9% *vs* 1.6 ± 0.8%; $p = 0.02$) (Figure 20C and 20D). Again, no significant correlation between the high expression of these immune-related factors and the patient OS was found.

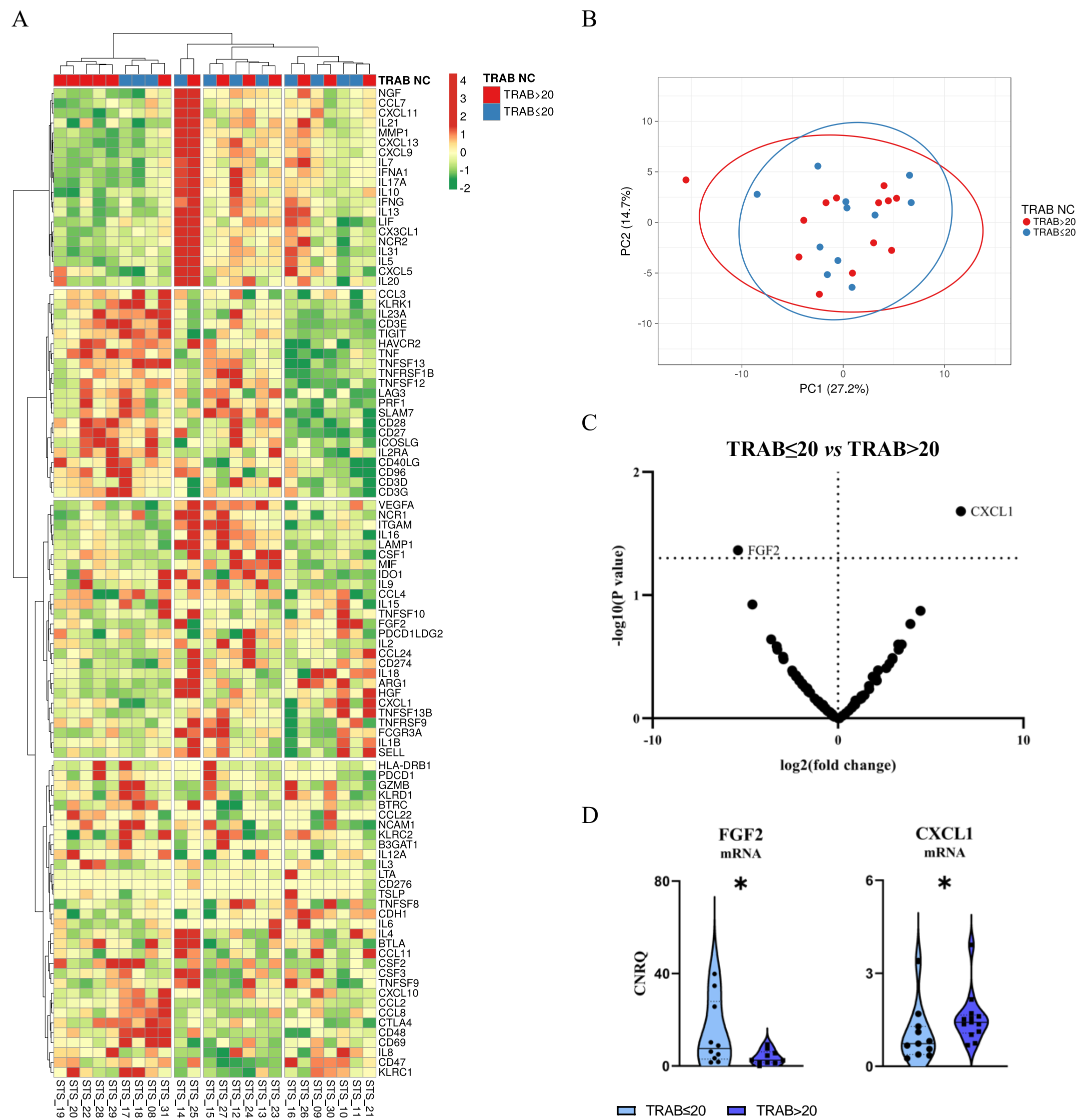

Figure 20 | The effect of the number of trabectedin cycles in the expression of immune-related genes. RNA was extracted from the whole blood of STS patients, cDNA was synthesized, and the expression of immune-related genes was assessed by real-time PCR. **(A)** Heatmap for the expression levels of the immune-related genes analyzed in TRAB≤20 (N=11) and TRAB>20 patients (N=13). **(B)** PCA for the expression of the immune-related genes analyzed in TRAB≤20 (N=11) and TRAB>20 patients (N=13). **(C)** Volcano plot for differential immune-related genes expression in TRAB≤20 *vs* TRAB>20 patients. Scattered points represent genes, the x-axis is the log2 fold change for the ratio between the two groups, whereas the y-axis is the negative log (P value), and where the P value is the probability that a gene has statistical significance in its differential expression. The dots identified on the right side correspond to the genes over-expressed, and the dots identified on the left side correspond to the genes under-expressed in TRAB>20 patients. **(D)** Calibrated normalized relative quantity of mRNA expressing the genes FGF2 and CXCL1 in TRAB≤20 (N=11) and TRAB>20 patients (N=13). Mann-Whitney test was used for the statistical analysis. *TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; PC1, principal component 1; PC2, principal component 2; CNRQ, calibrated normalized relative quantity; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

4.3 Plasmatic levels of soluble immune-related factors

The network of pro- and anti-inflammatory cytokines and chemokines, as well as other immune-related factors, are essential during an immune response. Also, their role in tumor development, progression, and recurrence has been suggested in a large diversity of cancers, including STS[161].

Since studies have shown an impact of trabectedin on the production of some immune-related factors in the TME[159], we evaluated the presence of immune-related factors in the plasma. In this analysis, we found no significant differences in the soluble level of any immune-related factors analyzed between the TRAB and ANTH groups. Considering the TRAB≤20 and the TRAB>20 group of patients, a PCA analysis showed, once more, two overlapping groups (Figure 21B). The cluster analysis showed two distinct patterns, but patients from the two groups were found in both clusters (Figure 21A). Further, we performed a Mann-Whitney test comparing the levels of each soluble immune-related factor between the two groups of patients (Figure 21C). Here, we found significant higher levels of the soluble immune-checkpoints PD-L2 and B7-H2 (3395 ± 428 pg/mL *vs* 4764 ± 1517 pg/mL; $p = 0.03$, 408 ± 307 pg/mL *vs* 1165 ± 491 pg/mL; $p = 0.004$) (Figure 21D). Furthermore, higher levels of soluble PD-L2 were significantly correlated with longer OS in patients who had undergone long-term trabectedin therapy [high PD-L2: time = 19.7 ± 4.0 months (11.8 – 27.6) *vs* low PD-L2: time = 7.8 ± 3.3 months (1.2 – 14.4); $p = 0.04$] (Figure 21E). Similarly, higher B7-H2 soluble levels were also correlated with a better outcome [high B7-H2: time = 20.0 ± 2.7 months (14.7 – 25.3) *vs* low B7-H2: time = 0.8 ± 0.5 months (0.0 – 1.8); $p = 0.002$].

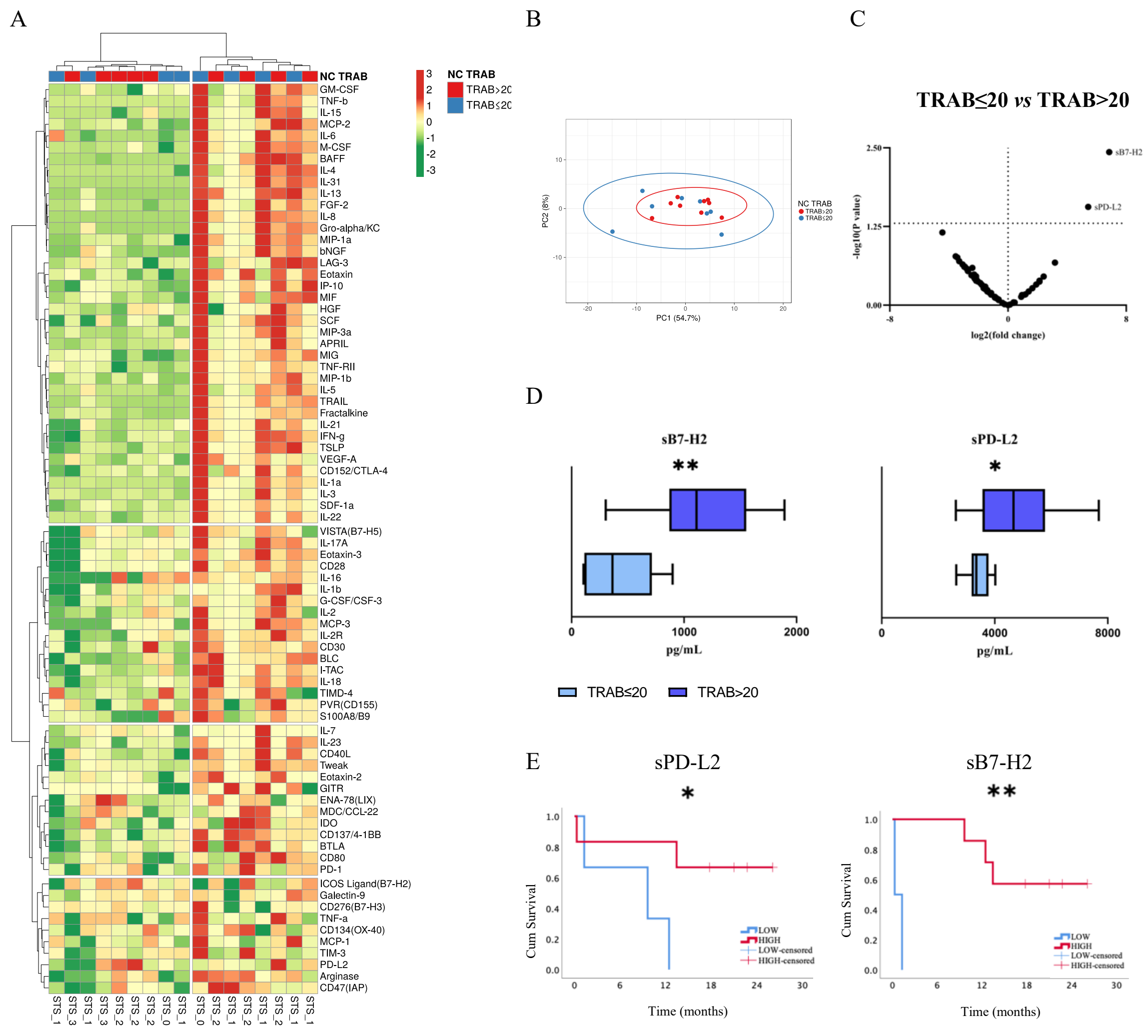

Figure 21 | The effect of the number of trabectedin cycles in the levels of soluble immune-related factors. Plasma was isolated from the whole blood of STS patients, and the levels of several cytokines, chemokines, growth factors, and immune checkpoints were measured using ProcartaPlex Human Immune Monitoring 65-Plex Panel, ProcartaPlex Human Immuno-Oncology Checkpoint Panel 1 14-Plex, ProcartaPlex Human Immuno-Oncology Checkpoint Panel 2 14-Plex, and ProcartaPlex Human Immuno-Oncology Checkpoint Panel 3 10-Plex. **(A)** Heatmap for the levels of the soluble immune factors analyzed in the plasma of TRAB≤20 (N=8) and TRAB>20 patients (N=9). **(B)** PCA for the levels of the soluble immune factors analyzed in the plasma of TRAB≤20 (N=8) and TRAB>20 patients (N=9). **(C)** Volcano plot for differential soluble levels of immune-related factors in TRAB≤20 *vs* TRAB>20 patients. Scattered points represent soluble factors, the x-axis is the log2 fold change for the ratio between the groups, whereas the y-axis is the negative log (P value), and where the P value is the probability that a soluble factor has statistical significance in its differential levels. The dots identified on the right side correspond to the soluble factors with increased levels, and the dots identified on the left side correspond to the soluble factors with decreased levels in TRAB>20 patients. **(D)** Concentration in pg/mL of sPD-L2 and sB7-H2 in TRAB≤20 (N=8) and TRAB>20 patients (N=9). Mann-Whitney test was used for the statistical analysis. **(E)** Overall survival in months of TRAB>20 patients according to the levels sPD-L2 and sB7-H2. Analysis was performed with the Kaplan-Meier method and a log-rank test. The levels of both soluble immune checkpoints in patient blood were considered high if their percentage were above the median and low if their percentage were under the median. *TRAB≤20, patients who completed ≤20 trabectedin cycles; TRAB>20, patients who completed >20 trabectedin cycles; sPD-L2, soluble programmed death-ligand 2; PC1, principal component 1; PC2, principal component 2; p-value < 0.05*, <0.01**, <0.001***, or < 0.0001****.*

Chapter 5
DISCUSSION

5 Discussion

Aiming to evaluate the peripheral immunological *status*, we assessed the presence of immune cells, immune-related soluble factors, and the expression of immune-related genes in the blood of STS patients. To understand if the peripheral immunological *status* varies between therapies, we divided the patients into two groups: patients who had undergone anthracycline-based therapy and patient who had undergone trabectedin-based therapy. Further, to better understand the immunomodulatory role of trabectedin, we divided the trabectedin group into: patients who had undergone less than 20 cycles and patients who had undergone more than 20 cycles. Then, we compared the immunological *status* between these groups and assessed the prognostic value of the differentially expressed factors.

Our results showed no significant differences in the major immune cell populations neither between therapies nor between number of trabectedin cycles. Similarly, the cluster and PCA analyses showed that the patterns of expression and the soluble levels of the immune-related factors analyzed varies largely between the patients. However, this variance is not due to the therapy used or the number of trabectedin cycles. Even so, when we analyzed the presence of more specific immune cell subsets or when we compared each immune-related factor individually, we did find significant differences between the groups (Figure 22). These results suggest the need for deeper immunological analyses, since the therapy caused alterations in the levels of several specific immune cell subsets, even when the major immune cells did not appear to be altered.

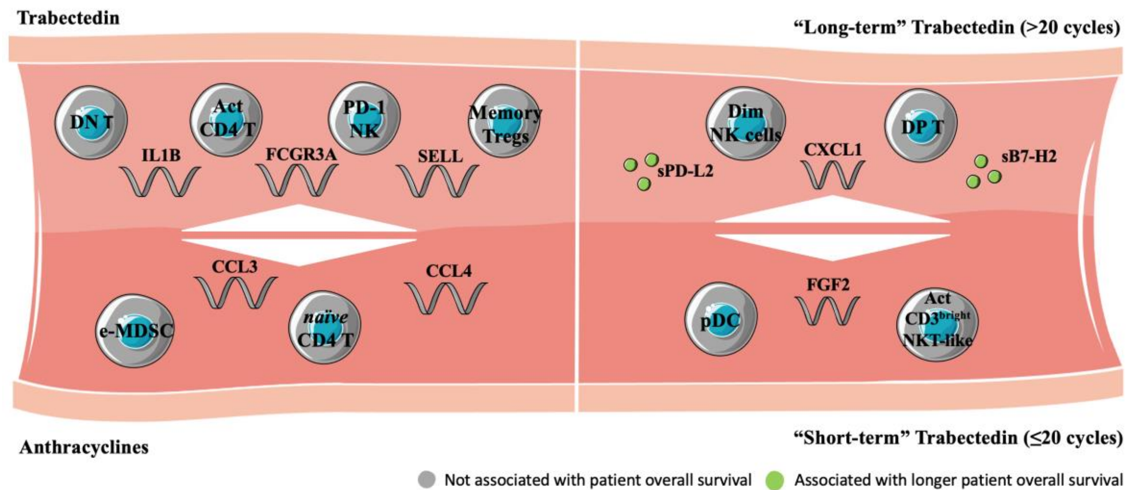

Figure 22 | The effect of therapy and number of trabectedin cycles in the peripheral immunological *status* of STS patients. The frequency of immune cells was assessed by flow cytometry, the expression of immune-related genes was performed by real-time PCR, and the quantification of soluble immune-related factors by Multiplex Analyte Profiling (xMAP®) technology, in blood samples of STS patients. The levels of DN T cells, activated CD4 T cells, PD-1 NK cells, memory Tregs, and the expression of IL1B, FCGR3A, and SELL were found increased in the group of patients who had undergone trabectedin-based therapy, when compared to anthracycline-based therapy. The levels of *naïve* CD4 T cells, e-MDSC, and the expression of CCL3 and CCL4 were found decreased in the TRAB group. When we analyzed the patients on long-term trabectedin therapy (>20 cycles), the frequencies of CD56^{dim} NK cells and DP T cells, the levels of sPD-L2 and sB7-H2, and the expression of CXCL1 were found increased when compared with ≤20 cycles of trabectedin. Contrary, pDC, activated CD3^{brigh} NKT-like cells, and the expression of FGF2 were decreased in the patients submitted to long-term trabectedin therapy. Higher levels of sPD-L2 and sB7-H2 were correlated with longer overall survival (green). *DN, double negative; Act, activated; PD-1, programmed death-1 receptor; Tregs, regulatory T cells; e-MDSC, early myeloid-derived suppressor cells; NK, Natural Killer, DP, double positive; pDC, plasmacytoid dendritic cells; sPD-L2, soluble programmed death-ligand 2.*

5.1 Immunophenotyping of STS patients according to therapy

5.1.1 CD4/CD8, activated and memory T cell subsets

The DN T cells, characterized by the expression of CD3 but lack of CD4 and CD8, comprise a small but essential fraction of T cells, with ~90% representing gamma-delta T cells[260,261]. During an immune response, DN T cells can play a dual role, presenting a cytotoxic or an immunosuppressive phenotype[262]. In human pancreatic cancer, this cell population was able to inhibit the proliferation and invasion of tumor cells[260]. Later, an expansion protocol was developed, and the expanded human DN T cells demonstrated an effective antitumor activity against leukemia cells *in vitro*[263]. Considering the promising results in leukemia, *ex vivo* expanded DN T cells were also tested in non-small-cell lung cancer cell lines and xenograft models, leading to an effective cytotoxic activity and

inhibition of tumor growth, respectively[264]. Besides their promising use in adoptive T cell therapy, the function of DN T cells remains unclear and occasionally controversial. In addition, the role of peripheral DN T cells, in particular in STS, remains unknown. Here, we observed an increase in DN T cells in the TRAB group. Taking into account their apparent antitumor effect, the increased levels of DN T cells in the TRAB group may contribute to the trabectedin efficacy.

Contrary to DN T cells, the DP T cells are characterized by the expression of CD3 and both CD4 and CD8. Initially, they were seen only as a development stage within the thymus. Nevertheless, the presence of this subset in the blood of normal individuals as well as in several pathological conditions, including cancer, motivated the investigation on DN T cell function[265]. So far, their function is controversial and remains to be elucidated. While some studies reported their cytotoxic activity in the TME, an association with poor outcome was also suggested[265–267]. In addition to their controversial role in the TME, their role in the peripheral blood was poorly explored. In the blood of patients with urological cancers, DP T cells were found to be responsible to favor the Th2 polarization of *naïve* CD4 T cells, promoting the tumor escape. Concerning STS patients, the function of circulating DP T cells remains to be explored. We found an increase of this subset in the TRAB>20 group, but no significant correlation with patient OS was found. The fact that both cytotoxic and immunosuppressive roles were attributed to DP T cells may indicate heterogeneity or pleiotropic functions in this cell subset. These distinct functions could be due to the influence of different microenvironments, so these cells need to be investigated in each particular disease context.

CD4 T cells, also known as T helper cells, help the priming, the migratory potential, and also the cytotoxic activity of CD8 T cells, being essential systemically for an effective antitumor immune response[268,269]. Although no significant alterations were observed in their total frequency, the activated CD4 T cells, identified by the expression of CD38 in combination with HLA-DR, were increased in the TRAB group. In STS the clinical relevance of this subtype in the peripheral blood remains unknown, and in the TME, CD4 T cells role is controversial. While some studies claim an association between their presence and a favorable outcome[189,190], the opposite was also observed in other studies[191,192]. Besides CD4 T cells activation, we also assessed their memory *status* and found a decrease in the frequency of *naïve* CD4 T cells in the peripheral blood of TRAB group. The function of this specific subtype in cancer, and particularly in STS, is still unknown. However, Su *et al*. reported that the circulating *naïve* CD4 T cells could

differentiate into Tregs *in situ*, enhancing the Tregs infiltration and suppressing the immune response against the breast tumor[270]. They also concluded that when the recruitment of *naïve* CD4 T cells is blocked, the tumor progression is inhibited.

5.1.2 Natural Killer and Natural Killer T-like cells

Named by their ability to kill tumor cells without prior sensitization, NK cells could be distinguished based on their expression of CD56 into: $CD56^{bright}$ and $CD56^{dim}$ NK cells. While $CD56^{bright}$ NK cells are more responsible for an immunoregulatory role, $CD56^{dim}$ NK cells have a higher capacity to destroy target cells[108]. Their influence on clinical outcome has been evaluated in several cancers[271–273]. In STS, studies found generally a low density of NK cells infiltration, and demonstrated a correlation between higher infiltration and better patient outcome[171,184,197]. Regarding the peripheral NK cells levels, studies found both $CD56^{bright}$ and $CD56^{dim}$ subsets in the blood of STS patients. In our study, we also found both subsets, being the $CD56^{bright}$ subset decreased, and consequently, the $CD56^{dim}$ increased in the TRAB>20 group. Considering their distinct functions, long-term trabectedin therapy appear to favor the subset with higher cytotoxic abilities, which may explain its efficacy against STS. Nevertheless, we did not observe any significant correlation with patient OS.

Originally identified in T cells, PD-1 and their ligands have been the focus of research. The impact of this immune checkpoint in STS was evaluated for the first time by Kim *et al.*[137]. They observed an intratumoral infiltration of PD-1 positive lymphocytes and PD-L1 expression in most STS samples, both correlated with poor prognosis. Although more studies in STS had confirmed this correlation, the opposite was also described[161]. In the peripheral blood, the expression of these markers has been neglected. In melanoma, only a few PD-1 expressing circulating T cells were found[274]. We evaluated the expression of PD-1 and PD-L1 in several circulating immune cell populations and found it low or absent in most STS blood samples. Understandably, the research has focused on PD-1 expression on T cells. However, the expression of PD-1 in NK cells is also relevant, especially in tumors that have lost or down-regulated MHC class I molecules, escaping T cell antitumor activity[275]. Despite the lower percentage of circulating PD-1 NK cells in both groups, they were increased in the patients undergoing trabectedin-based chemotherapy. This suggests that PD-1 NK cells could be also an

important factor to study in STS, especially when the patients had undergone trabectedin-based therapies.

For instance, NKT-like cells belong to the subset of T cells and express both the T-cell antigen receptor (TCR) and NK-cell markers[276,277]. This group of cells lies at the interface between innate and adaptive response and plays a role in anti-infection and anti-tumoral function[277]. However, their nature, function, and clinical relevance in cancer, and particularly in STS, remain largely unexplored. In other cancers, such as multiple myeloma and gastric cancer, $CD3^{dim}$ NKT-like cell function was found to be impaired, and this impairment was correlated with worse outcome[278,279]. Moreover, in colorectal and lung cancers, high levels of $CD3^{dim}$ NKT-like cells have been associated with a better outcome[280,281]. TRAB>20 patients exhibited a low frequency of activated $CD3^{bright}$ NKT-like cells. However, no association was found with patient survival. Further studies are required to understand the role of this specific subset and to make

5.1.3 Dendritic cells

As professional APC and robust producers of IFN-α, DC promote both innate and adaptive immune responses. Usually, these cells are classified into two distinct subsets: cDC and pDC[38]. Despite their ability to produce large amounts of IFN-α and promote immune responses, pDC are capable to perform either immunogenic or tolerogenic functions depending on the environment[282]. In cancer, it was demonstrated an impairment of IFN-α production, enhancing the tolerogenic capacity and establishing an immunosuppressive TME[283]. Furthermore, higher levels of tumor-associated pDC were associated with an increase of Tregs and shorter OS[284,285]. Although the role of pDC in the periphery remains underexplored, Hartmann *et al.* observed a higher production of IFN-α and higher activation of CD4 and CD8 T cells in the lymph nodes when compared to the tumor tissue of head and neck squamous cell carcinoma. These results showed for the first time that the impairment function of pDC may occur mainly in the TME, meaning that the tumor does not appear to influence the systemic pDC[286]. In STS, few studies noted the relevance of DC and their influence on patient outcome. In undifferentiated pleomorphic sarcoma and myxofibrosarcoma, tumor-infiltrating DC were correlated with improved disease-specific survival. So far, to the best of our knowledge, there are no studies assessing the levels of DC subsets and their correlation with STS outcome. In our study, pDC were found to decreased with the number of trabectedin cycles. Believing that

circulating pDC are less dysfunctional and more able to promote the anti-tumor function of T cells, this decreased might prejudicated the immune response against cancer. Nevertheless, no association with OS was observed. Further research is warranted to clarify the role of circulating DC and their subsets in cancer and a larger cohort is necessary to confirm their prognostic role in STS.

5.1.4 Regulatory T cells and myeloid-derived suppressor cells

Tregs were discovered in the periphery in the early 2000s and have since become a focus of research in cancer immunology[199,287]. In cancer patients, they appear to be increased and possess a potent immunosuppressive activity, so strategies to selective deplete circulating Tregs have emerged[200,201,288]. However, in STS the role of circulating Tregs remains to be explored. Here, we did not find a significant difference between the percentages of total Tregs between the TRAB and ANTH groups. We did, however, observed an increase in the percentage of memory Tregs. Our findings corroborate previous studies suggesting that the decrease of *naïve* CD4 T cells was due to an expansion of Tregs.

MDSC are mainly responsible for the suppression of T cells and are frequently divided into: PMN-MDSC, M-MDSC, and e-MDSC. Their presence in the peripheral blood has been associated with a poor outcome in several other cancers, including malignant colon cancer, myeloma, and pancreatic cancer[289–292]. In STS, studies concerning the role of MDSC in both the TME and the peripheral blood are still sparse[161]. Still, Kim *et al*. observed an association between high levels of M-MDSC and shorter disease-free survival and progression-free survival[254]. Concerning e-MDSC, this subset comprises a group of immature progenitor cells. Although they have been identified in the circulation and TME of several cancers, their role still needs to be defined[293–296]. In our group of patients, we did not find differences in the frequency of total MDSC between the therapies evaluated. Still, we observed a significant decrease in the relative frequency of e-MDSC in the TRAB group, mainly due to the expansion of the M-MDSC. Here, we found no significant correlation between this subset and OS in both therapy groups. However, based on the relevant role of MDSC and their evident expansion in cancer, as well as in patients undergoing trabectedin treatment, our results suggest that MDSC could be of interest to study in this group of patients.

5.2 Immune-related gene expression profiling

The expression of immune-related genes has been studied in other cancers[241–244]. In STS there are only a few studies concerning their expression, so its clinical significance remains unclear[245–249]. In myxoid liposarcoma cell lines, it was demonstrated a reduced mRNA level of IL8 and CCL2 after the trabectedin treatment. However, in our study, we did not observe significant differences in the mRNA levels of these genes neither between the TRAB and ANTH patients nor between the trabectedin cycles[159].

The pro-tumorigenic properties of IL-1β, encoded by the IL1B gene, include the promotion of tumor angiogenesis and the recruitment of immunosuppressive cells[297]. Furthermore, its role as a negative prognostic factor has been demonstrated in other tumors like human renal cell carcinoma[298,299]. These pro-tumorigenic activities led to the development of IL-1β blockade therapies. IL-1β blockade combined with anti-PD-1 therapy resulted in tumor abrogation in preclinical models of breast cancer[300]. As far as we are concerned, no studies aimed to evaluate the prognostic role of IL-1β in STS. In our study, no significant associations were found between the expression of IL-1β and the patient OS. Although Germano *et al*. observed decreased mRNA levels of IL-1β in myxoid liposarcoma cell lines after trabectedin treatment[159], here, we found higher expression levels of IL-1β in the blood of the patients undergoing trabectedin therapy when compared to the anthracycline group.

FCG3A, also known as CD16a, encoded by the FCGR3A gene, is the functional form of CD16 and plays an essential role during an immune response. Being the specific receptor of IgD, FCG3A establish a bridge between humoral and cellular immunity and became the key to antibody-dependent cellular cytotoxicity[301,302]. Furthermore, it also plays an important role in the maturation of DC and, more recently, their positive impact on NK cells cytotoxicity was demonstrated[303]. Since we found increased FCGR3A expression in the patients undergoing trabectedin-based chemotherapy, more studies are required to understand if these increased serum expression levels are correlated with the enhanced anti-tumoral activity of NK cells in STS, contributing to the immunomodulatory features of trabectedin therapy.

L-selectin, encoded by the SELL gene, belongs to the selectin family, a versatile group of carbohydrate-binding proteins which have essential functions in the recruitment of leukocytes from the circulation to the diseased tissue[304]. Though their role appears advantageous, the use of selectins by cancer cells to facilitate metastasis had been

hypothesized[305,306]. Later, studies with SELL-deficient mice demonstrated a decrease in metastasis, confirming this hypothesis[307]. Moreover, in the serum of bladder cancer patients, higher concentrations of L-selectin were correlated with metastatic cancer[308]. In our study, the expression levels of SELL were superior in the patients treated with trabectedin. Furthermore, higher SELL levels tended to be associated with worse OS in both therapy groups, although this correlation did not reach significance in the trabectedin group. More studies are required to understand the dual role of selectins in STS and a larger cohort is needed to confirm these results.

Chemokines CCL3 and CCL4, encoded by CCL3 and CCL4 genes, respectively, seem to be responsible for the migration of DC and tend to promote the invasion of active cytotoxic T cells to the tumor site in the early stage of tumor development, thus they have been suggested to be potential agents for treating cancer[309]. Concerning CCL4, higher serum levels were associated with improved disease-free survival in colorectal cancer[310]. However, it was also reported that CCL4 could promote tumor development and progression through the recruitment of Tregs and macrophages with a pro-tumoral activity[311]. Moreover, higher serum levels were also associated with poor prognostic features in oral squamous cell carcinoma and diffuse large B-cell lymphoma[312,313]. Similarly, serum levels of CCL3 had been associated with a poor prognosis in other cancers, such as multiple myeloma, chronic lymphocytic leukemia, melanoma, squamous cell carcinoma, and diffuse large B-cell lymphoma[313–317]. In this study, we observed a decreased expression of CCL4 and CCL3 in the serum of patients treated with trabectedin, when compared to the patients treated with anthracycline-based therapies. Since their apparent negative role in immunomodulation in other cancers, this decrease may lead to a better outcome, contributing to the efficacy of trabectedin chemotherapy. Still, we found no association between OS and the CCL4 and CCL3 expression in STS patients.

As a member of the FGF family, the FGF2, encoded by the FGF2 gene, plays an important role in the body development and growth, wound healing, fibrosis, and inflammatory reactions. This tyrosine kinase is also closely related to tumor angiogenesis and metastasis, contributing to the pathogenesis of tumors[318–320]. In several cancers, such as lung cancer, prostate cancer, and breast cancer, the high expression of FGF2 has been associated with tumor invasion, tumor metastasis, and then, a poor prognosis[321–324]. In STS, the FGF2 gene is commonly up-regulated, and a study demonstrated that high serum levels of FGF were significantly correlated with tumor mass and histological grading, suggesting its possible role as a biomarker for tumor follow-up[217,325]. In our study, we

found a decrease in the levels of FGF2 mRNA when the STS patients were undergoing more than 20 cycles of trabectedin chemotherapy. This decrease might be due to the immunomodulatory role of trabectedin and may contribute to its demonstrated efficacy in STS patients.

Chemokine CXCL1, encoded by the CXCL1 gene, has been reported to promote tumor progression and metastasis in other cancers, as well as angiogenesis when binding to the CXCR2 receptor[326–328]. Consistently, high CXCL1 expression has been correlated with poor prognosis in these cancers, including colorectal and pancreatic cancer[329]. Although studies concerning the role of immune-related chemokines in STS patients are still sparse, one study showed that CXCL1 was also involved in the traffic of MDSC to the tumor site in rhabdomyosarcoma bearing mice. Furthermore, in the serum of patients with metastatic pediatric sarcomas, the same study observed elevated expression of this chemokine[330]. In our study, we found an increased expression of CXCL1 in the peripheral blood of TRAB>20 patients. However, we did not find any correlation with patient OS.

5.3 Plasmatic levels of soluble immune-related factors

Immune checkpoints play an essential role in regulating the immune response. There has been a growing interest in the effect of these molecules in cancer, where they are often deregulated, usually functioning as an immune resistance mechanism[224]. As was mentioned before, most studies correlated the expression of PD-1 in the TME with a worse prognosis in STS[161]. There is also a growing interest in their ligands, especially in PD-L1 that has been also associated with poor outcome[137]. However, other studies observed low or absent expression of PD-L1 and claimed no association with prognosis in STS[192]. Their serum levels, peripheral expression, and their clinical significance in the peripheral blood remain underexplored. Here we found consistently low serum levels of soluble PD-1 and PD-L1, and a low expression of these factors in peripheral blood. Further, we found no significant association between the presence of these immune checkpoints and clinical outcome in any group of patients.

Besides the PD-L1, PD-L2 also competes for binding to PD-1. Indeed, the interaction between PD-L2 and PD-1 showed higher affinity compared to the interaction between PD-L1 and PD-1. Still, PD-L2 is generally expressed at a lower level, favoring PD-L1 as the primary binding ligand of PD-1[331]. Identical to PD-L1, the binding of PD-L2 to PD-1 results in an inhibition of cytotoxic T cell response against tumor cells. But,

unlike PD-L1, the PD-L2 can also bind to the repulsive guidance molecule B (RGMB) receptor, promoting CD4 T cells responses and Th1 polarization[332]. Although PD-L2 expression in tumors had been also associated with impaired survival, the studies are much less when compared to PD-L1. This lack of interest is usually explained by its more restricted expression. We found a significant increase in soluble PD-L2 serum levels in the TRAB>20 group of patients. Furthermore, in this group, higher expression of PD-L2 was associated with longer OS. These results lead to the necessity of more studies concerning the role of this immune checkpoint in the peripheral blood as well as confirming its possible positive prognostic value in trabectedin patients.

Two different signals activate T cells. The first signal is the binding of the MHC-antigen and the antigen receptor complex with the TCR, and the second signal is the binding of the B7-CD28 and other co-stimulatory molecules[333]. The B7 family includes several co-stimulatory molecules, among them the B7-H2, also known as inducible co-stimulatory ligand (ICOSL). B7-H2 binds to the receptor ICOS on T cells and is responsible for an immunomodulatory function[334]. In the TME, high expression of B7-H2 has been associated with tumor growth and progression in glioblastoma, gastric cancer, and hematological tumors[335–338]. Contrary, a study evaluated the B7-H2 transcription levels in peripheral blood of colon cancer patients and found its expression negatively associated with pathological features[339]. In our study, we found significantly higher serum levels of soluble B7-H2 in patients who had undergone more than 20 cycles of trabectedin. Moreover, in these patients, elevated levels were correlated with longer OS. These results corroborate the previous findings in colon cancer, demonstrating a different prognostic value in peripheral blood and the TME.

Some limitations of our study must be noted. First, in addition to the small total cohort, mainly due to the STS rarity, the ANTH group had an evident smaller cohort when compared to the TRAB group. This difference has to do mainly with the clear efficacy of trabectedin-based therapy, being chosen most of the time in advanced disease. Second, although the use of different histological subtypes is a well-established procedure in studies concerning STS patients, in our study a large percentage of the samples were from patients with leiomyosarcoma and therefore our results may not be representative of the whole. So, to confirm or validate our results, further research in a large sample and on specific subtypes is needed. Furthermore, there were patients with primary disease while others had metastatic or relapsed disease, and the samples were collected at various time

points in their treatment. Since the immune *status* varies with disease progression, the assessment of peripheral blood from patients with similar clinical features might provide a more accurate prognostic prediction. Lastly, the role of the most immune-related factors in STS remains unclear. Therefore, we were unable to draw firm conclusions about the observed differences between the groups and associations with the patient outcome.

Chapter ***6***

CONCLUSION

6 Conclusion

In this work we aimed to assess the peripheral immunological *status* of STS patients, including immune cells, immune-related soluble factors, immune checkpoints, and immune-related genes, considering the patient therapy, trabectedin or anthracycline-based therapy. Aiming to better understand the influence of trabectedin-based therapy on the immunological *status*, we also assessed the immunological *status* of patients who had undergone long-term trabectedin and compared it with the others.

Our results demonstrated, once more, the highly heterogeneous immune contexture of STS patients, even between the patients undergoing similar treatments. When we compared the patients who had undergone anthracycline-based therapy with the patients who had undergone trabectedin-based therapy we did not find significant differences in the major immune cell populations. However, when we deepened our research to more immune cell subtypes, memory and activation *status*, we observed some significant differences between the two therapies. The same occurred when we evaluated the influence of long-term trabectedin therapy. This highlighted the importance of studies evaluating a larger range of immune cell subsets as well as their memory and activation markers.

Although studies had shown a selective effect of trabectedin for tumor-infiltrating macrophages and monocytes, we did not observe differences in the frequency of these cells. This could indicate that this effect of trabectedin may not be significant in the peripheral blood. Nevertheless, we found significant alterations mostly in the T cells, NK cells, and immune suppressive cells. On one hand, trabectedin seems to increase the levels of DN T cells, activated CD4 T cells, and $CD56^{dim}$ NK cells. Since these cells are predominantly responsible for a potent effector activity, their increased levels might contribute to the trabectedin efficacy in STS. On the other hand, trabectedin appears to increase the levels of memory Tregs and M-MDSC, both known for their immunosuppressive role and, in some studies, correlated with patient poor outcome.

In the immune-related gene expression analysis, trabectedin appears to promote the immune response against cancer cells, through the increased expression of FCGR3A, and through the decreased expression of CCL3 and CCL4, both responsible for an apparent negative role in the immunomodulation in other cancers. Moreover, trabectedin therapy

also seems to decrease the levels of FGF2, which promotes tumor angiogenesis and metastasis. On the contrary, in the trabectedin group, we observed an increased expression of IL-1β, SELL, and CXCL1, which are associated with tumor progression and metastasis.

The other objective of our study consisted in the evaluation of the impact of the immunological *status* in STS patient outcome. So, we compared the OS of STS patients with the levels of the immune factors significantly altered. This analysis demonstrated a significant correlation between the high serum levels of the soluble immune-checkpoints PD-L2 and B7-H2 and prolonged OS in the group of patients who had undergone more than 20 trabectedin cycles. These results suggest a potential role of the soluble PD-L2 and B7-H2 as biomarkers for good prognosis in long-term trabectedin therapy.

Nevertheless, concerning the study limitations, future in-depth studies should be performed to validate this work, better understand the precise effect of trabectedin in the peripheral immunological *status*, and clarify the role of the peripheral immune components in predicting the outcome of STS patients.

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Supplementary Material

Supplementary Table I | Monoclonal antibodies used for flow cytometry studies.

Antibody	Conjugate	Clone	Brand	Cat#
CD3	V450	UCHT1	BD Horizon™	561416
CD3	APC-H7	SK7	BD Pharmingen™	560176
CD3	PerCp-Cy5.5	HIT3a	BioLegend®	300328
CD4	PerCp-Cy5.5	OKT4	BioLegend®	317428
CD8	APC-H7	HIT8a	BD™	641400
CD11b	APC	ICRF44	BioLegend®	301310
CD11c	PE/Cy7	B-ly6	BD Pharmingen™	561356
CD14	V450	MOP9	BD Horizon™	560349
CD15	PE/Cy7	HI98	BD Pharmingen™	560827
CD16	APC-Cy7	3G8	BioLegend®	302018
CD19	APC-H7	SJ2501	BD Pharmingen™	560177
CD19	PerCp-Cy5.5	HIB19	BioLegend®	302230
CD20	APC-H7	2H7	BD Pharmingen™	560734
CD24	FITC	ML5	BD Pharmingen™	555427
CD25	PE	M-A251	BD Pharmingen™	555432
CD27	PE/Cy7	M-T271	BD Pharmingen™	560609
CD33	PE	WM53	BD Pharmingen™	555450
CD38	APC	HIT2	BD Pharmingen™	555462
CD45	FITC	HI30	BD Pharmingen™	555482
CD45RA	PE/Cy7	5H9	BD Pharmingen™	561216
CD45RO	APC-H7	UCHL1	BD Pharmingen™	561137
CD56	PerCp-Cy5.5	HCD56	BD Pharmingen™	560842
CD56	PE	B159	BioLegend®	318306
CD123	PerCp-Cy5.5	6H6	BioLegend®	306016
CD127	AF647	HIL-7R-M21	BD Pharmingen™	558598
CD183 (CXCR3)	PE	IC6/CXCR3	BD Pharmingen™	550633
CD194 (CCR4)	PE/Cy7	IG1	BD™	577864
CD196 (CCR6)	PE/Cy7	11A9	BD Pharmingen™	560620
CD197 (CCR7)	PE	150503	BD Pharmingen™	560765
CD274 (PD-L1)	FITC	MIH1	BD Pharmingen™	558065
CD279 (PD-1)	FITC	MIH4	BD Pharmingen™	557860
CD279 (PD-1)	APC	MIH4	BD Pharmingen™	558694
CD279 (PD-1)	PE	MIH4	BD Pharmingen™	557946
HLA-DR	V500	G46-6	BD Horizon™	561224
IgD	V500	IA6-2	BD Horizon™	561490

Supplementary Table II | Primers used for the immune-related genes expression analysis.

Gene name	NCBI Gene ID	GenBank Accession number	Primer forward	Primer reverse	PrimerBank ID or Ref.
Genes of interest					
ARG1	383	NM_000045	GTGGAAACTTGCATGGACAAC	AATCCTGGCACATCGGGAATC	346986433c1
B3GAT1	27087	NM_018644	CCTGGCGTGGTCTACTTCG	GCAGGTTGACGGCAAATCC	77695913c1
BTLA	151888	NM_001085357	CATCTTAGCAGGAGATCCCTTTG	GACCCATTGTCATTAGGAAGCA	145580618c1
BTRC	8945	NM_003939	CCAGACTCTGCTTAAACCAAGAA	GGGCACAATCATACTGGAAGTG	379030597c1
CCL11	6356	NM_002986	CCCCTTCAGCGACTAGAGAG	TCTTGGGGTCGGCACAGAT	22538399c1
CCL2	6347	NM_002982	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT	4506841a1
CCL22	6367	NM_002990	ATCGCCTACAGACTGCACTC	GACGGTAACGGACGTAATCAC	300360575c1
CCL24	6369	NM_002991	ACATCATCCCTACGGGCTCT	CTTGGGGTCGCCACAGAAC	22165426c1
CCL3	6348	NM_002983	AGTTCTCTGCATCACTTGCTG	CGGCTTCGCTTGGTTAGGAA	4506843a1
CCL4	6351	NM_002984	CTGTGCTGATCCCAGTGAATC	TCAGTTCAGTTCCAGGTCATACA	4506845a1
CCL7	6354	NM_006273	GACAAGAAAACCCAAACTCCAAAG	TCAAAACCCACCAAAATCCA	340
CCL8	6355	NM_005623	TGGAGAGCTACACAAGAATCACC	TGGTCCAGATGCTTCATGGAA	22538815c1
CD28	940	NM_001243078	CTATTTCCCGGACCTTCTAAGCC	GCGGGGAGTCATGTTCATGTA	340545509c1
CD27	939	NM_001242	CAGAGAGGCACTACTGGGCT	CGGTATGCAAGGATCACACTG	117422442c1
CD274	29126	NM_014143	TGGCATTTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT	292658763c1
CD276	80381	NM_001024736	CTTGTTCGATGTTCACAGCG	GCCGTAGAGCTGTCTTGGATC	341
CD3D	915	NM_001040651	ACTGGCTACCCTTCTCTCG	CCGTTCCCTCTACCCATGTGA	98985800c1
CD3E	916	NM_000733	CCTCTTATCAGTTGGCGTTTGG	TTCAGTGACAGGTGATCCTCA	166362733c1
CD3G	917	NM_000073	TGGCCCAGTCAATCAAAGGAA	CAAGTCAGAAGTACCGAACCATC	166362738c1
CD40LG	959	NM_000074	ACATACAACCAAACTTCTCCCCG	GCAAAAAGTGCTGACCCAATCA	58331233c1
CD47	961	NM_001777	AGAAGGTGAAACGATCATCGAGC	CTCATCCATACCACCGGATCT	68223312c1
CD48	962	NM_001778	AGGTTGGGATTCGTGTCTGG	AGTTGTTTGTAGTTCTCAGGCAG	365733591c1
CD69	969	NM_001781	ATTGTCCAGGCCAATACACATT	CCTCTCTACCTGCGTATCGTTTT	221554485c1
CD96	10225	NM_198196	CAAACACAGACAGTAGGCTTCTT	GGGGATGATAGACAGCAATCAG	93141044c1
CDH1	999	NM_004360	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG	169790842c1
CSF1	1435	NM_172210	TGGCGAGCAGGAGTATCAC	AGGTCTCCATCTGACTGTCAAT	166235149c1
CSF2	1437	NM_000758	TCCTGAACCTGAGTAGAGACAC	TGCTGCTTGTAGTGGCTGG	371502128c1
CSF3	1440	NM_001178147	GCTGCTTGAGCCAACTCCATA	GAACGCGGTACGACACCTC	296011056c1
CTLA-4	1493	NM_005214	GCCCTGCACTCTCCTGTTTTT	GGTTGCCGCACAGACTTCA	339276048c1
CX3CL1	6376	NM_002996	ACCACGGTGTGACGAAATG	TGTTGATAGTGGATGAGCAAAGC	54111253c1
CXCL1	2919	NM_001511	GCGCCCAAACCGAAGTCATA	ATGGGGGATGCAGGATTGAG	342
CXCL10	3627	NM_001565	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT	323422857c1
CXCL11	6373	NM_005409	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT	307611978c1
CXCL13	10563	NM_006419	GCTTGAGGTGTAGATGTGTCC	CCCACGGGGCAAGATTTGAA	194733765c1
CXCL5	6374	NM_002994	AGCTGCGTTGCGTTTGTTTAC	TGGCGAACACTTGCAGATTAC	41872613c1
CXCL9	4283	NM_002416	CCAGTAGTGAGAAAGGGTCGC	AGGGCTTGGGGCAAATTGTT	4505186c1
FCGR3A	2214	NM_000569	CCTCCTGTCTAGTCGGTTTGG	TCGAGCACCCTGTACCATTGA	24429586a1
FGF2	2247	NM_002006	AGAAGAGCGACCCTCACATCA	CGGTTAGCACACACTCCTTTG	153285460c1
GZMB	3002	NM_004131	CCCTGGGAAAACACTCACACA	GCACAACTCAATGGTACTGTCG	221625527c1
HAVCR2	84868	NM_032782	CTGCTGCTACTACTTACAAGGTC	GCAGGGCAGATAGGCATTCT	354681988c1
HGF	3082	NM_001010931	GCTATCGGGGTAAAGACCTACA	CGTAGCGTACCTCTGGATTGC	58533162c1

HLA-DRB1	3123	AJ297586	GAGCAGGTTAAACATGAGTGTCA	CTCTCCACAACCCCGTAGT	15387629a1
ICOSLG	23308	NM_015259	GCAGCCTTCGAGCTGATACTC	GTTTTCGACTCACTGGTTTGC	58331247c1
IDO1	3620	NM_002164	GCCAGCTTCGAGAAAGAGTTG	ATCCCAGAACTAGACGTGCAA	323668304c1
IFNA1	3439	NM_024013	GCCTCGCCCTTTGCTTTACT	CTGTGGGTCTCAGGGAGATCA	13128950a1
IFNG	3458	NM_000619	TCGGTAACTGACTTGAATGTCCA	TCGCTTCCCTGTTTTAGCTGC	56786137c1
IL10	3586	NM_000572	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG	24430216c1
IL12A	3592	NM_000882	CCTTGCACTTCTGAAGAGATTGA	ACAGGGCCATCATAAAAGAGGT	325974478c1
IL13	3596	NM_002188	CCTCATGGCGCTTTTGTTGAC	TCTGGTTCTGGGTGATGTTGA	26787977c1
IL15	3600	NM_172175	TTGGGAACCATAGATTTGTGCAG	GGGTGAACATCACTTTCCGTAT	26787986a1
IL16	3603	NM_172217	GCCGAAGACCCTTGGGTTAG	GCTGGCATTGGGCTGTAGA	289063450c1
IL17A	3605	NM_002190	TCCCACGAAATCCAGGATGC	GGATGTTCAGGTTGACCATCAC	27477085c1
IL18	3606	NM_001562	TCTTCATTGACCAAGGAAATCGG	TCCGGGGTGCATTATCTCTAC	342349317c1
IL1B	3553	NM_000576	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA	27894305c1
IL2	3558	M22005	TACAAGAACCCGAAACTGACTCG	ACATGAAGGTAGTCTCACTGCC	386818a1
IL20	50604	NM_018724	ATGAAAGCCTCTAGTCTTGCCT	GCCCCGTATCTCAGAAAATCC	50845426c1
IL21	59067	NM_021803	TAGAGACAAACTGTGAGTGGTCA	GGGCATGTTAGTCTGTGTTTCTG	365733583c1
IL23A	51561	NM_016584	CTCAGGGACAACAGTCAGTTC	ACAGGGCTATCAGGGAGCA	28144902c1
IL2RA	3559	NM_000417	GTGGGGACTGCTCACGTTC	CCCGCTTTTTATTCTGCGGAA	269973860c1
IL3	3562	NM_000588	CAGACAACGCCCTTGAAGACA	GCCCTGTTGAATGCCTCCA	28416914c1
IL31	386653	NM_001014336	CACGTTGCCCGTCCGTTTA	TCTTCGAGAGGGACTGTAATTCC	62122910c1
IL4	3565	NM_000589	CCAACTGCTTCCCCCTCTG	TCTGTTACGGTCAACTCGGTG	4504669a1
IL5	3567	NM_000879	TGGAGCTGCCTACGTGTATG	TTCGATGAGTAGAAAGCAGTGC	28559032c1
IL6	3569	NM_000600	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	224831235c1
IL7	3574	NM_000880	TTGGACTTCCTCCCCTGATCC	TCGATGCTGACCATTAGAACAC	4504677a1
IL8	3576	NM_000584	TTTTGCCAAGGAGTGCTAAAGA	AACCCTCTGCACCCAGTTTTC	10834978a1
IL9	3578	NM_000590	CTCTGTTTGGGCATTCCCTCT	GGGTATCTTGTTTGCATGGTGG	10834980a1
ITGAM	3684	NM_001145808	GCCTTGACCTTATGTCATGGG	CCTGTGCTGTAGTCGCACT	224831238c1
KLRC1	3821	NM_007328	AGCTCCATTTTAGCAACTGAACA	CAACTATCGTTACCACAGAGGC	283046824c1
KLRC2	3822	NM_002260	GCCAGCATTTTACCTTCCTCA	ACTGCACAGTTAAGTTCAGCAT	4504883a1
KLRD1	3824	NM_007334	CAGGACCCAACATAGAACTCCA	GGAAATGAAGTAACAGTTGCACC	167614494c1
KLRF1	51348	NM_016523	TACTGGGAATATCTGGAACCGT	TTGAGCCATTCTGATTGGCAT	7705573c1
LAG3	3902	NM_002286	GCGGGGACTTCTCGCTATG	GGCTCTGAGAGATCCTGGGG	167614499c1
LAMP1	3916	NM_005561	TCTCAGTGAACTACGACACCA	AGTGTATGTCCTCTTCCAAAAGC	112380627c1
LIF	3976	NM_002309	CCAACGTGACGGACTTCCC	TACACGACTATGCGGTACAGC	380418322c1
LTA	4049	NM_000595	ATGACACCACCTGAACGTCTC	CTCTCCAGAGCAGTGAGTTCT	6806892c1
MIF	4282	NM_002415	GAACAACTCCACCTTCGCCT	CCGTTTATTTCTCCCCACCA	343
MMP1	4312	NM_002421	AAAATTACACGCCAGATTTGCC	GGTGTGACATTACTCCAGAGTTG	225543092c1
NCAM1	4684	NM_001076682	GGCATTTACAAGTGTGTGGTTAC	TTGGCGCATTCTTGAACATGA	336285437c1
NCR1	9437	NM_001242357	TGGACCCGAAGTGATCTCG	TCCTTGAGCAGTAAGAACATGC	334358898c1
NCR2	9436	NM_004828	GGCTCTCAGGCACAATCCAAG	GCTGAAGCCTCCTTACACCA	153945781c1
NCR3	259197	NM_001145467	CCCCTGAGATTCGTACCCTG	CTCCACTCTGCACACGTAGAT	224586864c1
PDCD1	5133	NM_005018	CCAGGATGGTTCTTAGACTCCC	TTTAGCACGAAGCTCTCCGAT	167857791c1
PDCD1LDG2	80380	NM_025239	ATTGCAGCTTCACCAGATAGC	AAAGTTGCATTCCAGGGTCAC	190014604c1
PRF1	5551	NM_005041	GGCTGGACGTGACTCCTAAG	CTGGGTGGAGGCGTTGAAG	133908619c1
SELL	6402	NM_000655	ACCCAGAGGGACTTATGGAAC	GCAGAATCTTCTAGCCCTTTGC	262206314c1
SLAMF7	57823	NM_021181	ACCCTCATCTATATCCTTTGGCA	CACCAACGGAACCGACCAG	19923571c1

TIGIT	201633	NM_173799	TCTGCATCTATCACACCTACCC	CCACCACGATGACTGCTGT	256600227c1
TNF	7124	NM_000594	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG	25952110c1
TNFRSF1B	7133	NM_001066	CGGGCCAACATGCAAAAGTC	CAGATGCGGTTCTGTTCCC	23312365c1
TNFRSF9	3604	NM_001561	AGCTGTTACAACATAGTAGCCAC	GGACAGGGACTGCAAATCTGAT	315259099c1
TNFSF10	8743	NM_001190942	TGCGTGCTGATCGTGATCTTC	GCTCGTTGGTAAAGTACACGTA	300193031c1
TNFSF12	8742	NM_003809	GAGGGGAAGGCTGTCTACCT	GAACCTGGAAGAGTCCGAAGTA	23510442c1
TNFSF13	8741	NM_172088	CTCTGCTGACCCAACAAACAG	GGAGGTGGCGTTAATGGGAAC	26051248a1
TNFSF13B	10673	NM_001145645	GGGAGCAGTCACGCCTTAC	GATCGGACAGAGGGGCTTT	325053721c1
TNFRSF8	943	NM_001243	TCCACGGAGCACACCAATAAC	ACTGAGAGCATGACATCGCTG	68348710c1
TNFSF9	8744	NM_003811	GGCTGGAGTCTACTATGTCTTCT	ACCTCGGTGAAGGGAGTCC	209954675c1
TSLP	85480	NM_138551	ATGTTCGCCATGAAAACTAAGGC	GCGACGCCACAATCCTTGTA	372466598c1
VEGFA	7422	NM_001171627	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA	284172466c1
Reference genes					
ACTB	60	NM_001101	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	258
B2M	567	NM_004048	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	258
GAPD	2597	NM_002046	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	258
HMBS	3145	NM_000190	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC	258
HPRT1	3251	NM_000194	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	258
RPL13A	23521	NM_012423	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA	258
SDHA	6389	NM_004168	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	258
UBC	7316	M26880	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT	258
YWHAZ	7534	NM_003406	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	258

Supplementary Table III | Analytes analyzed in ProcartaPlex Human Immune Monitoring 65-Plex Panel.

ProcartaPlex Human Immune Monitoring 65-Plex Panel

Analyte	Bead Number	[Std1] (pg/mL)	LLOQ (pg/ml)	ULOQ (pg/ml)	Analyte	Bead Number	[Std1] (pg/mL)	LLOQ (pg/ml)	ULOQ (pg/ml)
APRIL	88	452300	110	452300	IL-2R	9	397600	97	397600
BAFF	86	13000	3.17	13000	IL-3	73	112400	27	112400
BLC	29	46400	11	46400	IL-31	37	78900	19	78900
bNGF	55	22200	5.42	22200	IL-4	20	44600	11	44600
CD30	84	38600	9.42	38600	IL-5	21	29900	7.3	29900
CD40-Ligand	74	42400	10	42400	IL-6	25	58100	14	58100
ENA-78 (LIX)	82	37500	9.16	37500	IL-7	26	3100	0.76	3100
Eotaxin	33	6150	1.5	6150	IL-8	27	11700	2.86	11700
Eotaxin-2	30	15000	15	15000	IL-9	52	32600	7.96	32600
Eotaxin-3	49	6850	1.67	6850	IP-10	22	8200	2	8200
FGF-2	75	45000	11	45000	I-TAC	57	42900	10	42900
Fractalkine	59	9250	2.26	9250	LIF	15	16400	4	16400
G-CSF/CSF-3	42	52500	13	52500	MCP-1	51	10700	2.61	10700
GM-CSF	44	59700	15	59700	MCP-2	8	4150	1.01	1038
Gro-alpha/KC	61	16700	4.08	16700	MCP-3	68	16200	16	16200
HGF	46	21600	5.27	21600	M-CSF	67	57700	14	57700
IFN-alpha	48	29500	7.2	29500	MDC/CCL 22	87	56500	14	56500
IFN-gamma	43	54000	13	54000	MIF	53	3800	0.93	3800
IL-10	28	7350	1.79	7350	MIG	69	30200	7.37	30200
IL-12p70	34	26900	6.57	26900	MIP-1alpha	12	14500	3.54	3625
IL-13	35	20000	4.88	20000	MIP-1beta	47	34300	8.37	8575
IL-15	65	12000	2.93	12000	MIP-3alpha	56	39600	9.67	39600
IL-16	70	63300	15	63300	MMP-1	64	17200	4.2	17200
IL-17A	36	15800	3.86	15800	SCF	39	18900	4.61	18900
IL-18	66	39500	9.64	39500	SDF-1alpha	13	185400	45	185400
IL-1alpha	62	7100	1.73	7100	TNF-alpha	45	51600	13	51600
IL-1beta	18	25600	6.25	25600	TNF-beta	54	23600	5.76	23600
IL-2	19	30700	7.5	30700	TNF-RII	85	10700	2.61	10700
IL-20	81	58900	14	14725	TRAIL	58	13700	3.34	13700
IL-21	72	38700	9.45	38700	TSLP	80	19500	4.76	19500
IL-22	76	67700	17	67700	Tweak	97	393900	96	393900
IL-23	63	54400	13	54400	VEGF-A	78	27400	6.69	27400
IL-27	14	51700	13	51700					

Supplementary Table IV | Analytes analyzed in ProcartaPlex Human Immuno-Oncology Checkpoint Panel 1, 2 and 3.

ProcartaPlex Human Immuno-Oncology Checkpoint Panel 1 14-Plex

Analyte	Bead Number	[Std1] (pg/mL)	LLOQ (pg/ml)	ULOQ (pg/ml)
BTLA	52	492500	120	492500
GITR	57	85500	21	85500
HVEM	36	59700	15	59700
IDO	46	13200	3.22	13200
LAG-3	47	43700	11	43700
PD-1	65	30000	7.32	30000
PD-L1	66	14500	3.54	14500
PD-L2	67	189300	46	189300
TIM-3	14	303700	74	303700
CD28	15	132800	32	132800
CD80	61	150700	37	150700
CD137/4-1BB	26	47400	12	47400
CD27	27	23900	5.83	23900
CD152/CTLA4	33	34700	8.47	34700

ProcartaPlex Human Immuno-Oncology Checkpoint Panel 2 14-Plex

Analyte	Bead Number	[Std1] (pg/mL)	LLOQ (pg/ml)	ULOQ (pg/ml)
MICA	18	53100	13	53100
MICB	21	30000	7.32	30000
Nectin-2 (CD112)	29	152800	37	152800
NT5E (CD73)	30	184800	45	184800
PVR (CD155)	56	222500	54	222500
Siglec-7	12	63000	62	63000
Siglec-9	13	7600	1.86	7600
ULBP-1	73	584700	143	584700
ULBP-3	77	81000	79	81000
Tactile (CD96)	35	291000	71	291000
E-Cadherin	44	148600	36	148600
Arginase	51	50000	12	50000
ULBP-4	78	337100	82	337100
Perforin	53	258700	63	258700

ProcartaPlex Human Immuno-Oncology Checkpoint Panel 3 10-Plex

Analyte	Bead Number	[Std1] (pg/mL)	LLOQ (pg/ml)	ULOQ (pg/ml)
B7-H6	42	485400	119	485400
CD134 (OX40)	55	30800	7.52	30800
CD276 (B7-H3)	72	584700	571	584700
CD47 (IAP)	74	25100	6.13	25100
CD48 (BLAST-1)	19	116400	28	116400
Galectin-9	38	9100	2.22	9100
ICOS Ligand (B7-H2)	34	37000	9.03	37000
S100A8/A9	76	249200	61	249200
TIMD-4	39	248600	61	248600
VISTA (B7-H5)	64	35400	8.64	35400