From Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

SARCOMA ECOSYSTEMS: SPATIAL CHARACTERIZATION AND PROGNOSTIC SIGNIFICANCE

Yanhong Su

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Sarcoma Ecosystems: Spatial Characterization and Prognostic Significance

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Yanhong Su

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Principal Supervisor:
Monika Ehnman, PhD
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Professor Arne Östman, PhD
Karolinska Institutet
Department of Oncology-Pathology
Bergen University
Center for Cancer Biomarkers

Assoc. Prof. Felix Haglund de Flon, MD, PhD
Karolinska Institutet
Department of Oncology-Pathology
Karolinska University Hospital
Department of Molecular Cancer Diagnostics

Assoc. Prof. Nick Tobin, PhD
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Göran B Jönsson, PhD
Lund University
Department of Lund University Cancer Centre

Examination Board:
Assoc. Prof. Maria Ulvmar, PhD
Uppsala University
Department of Medical Biochemistry and Microbiology

Assoc. Prof. Isabelle Magalhaes, PhD
Karolinska Institutet
Department of Clinical Science, Intervention and Technology
Division of Therapeutic Immunology and Transfusion Medicine

Assoc. Prof. Weng-Onn Lui, PhD
Karolinska Institutet
Department of Oncology-Pathology

To my families

ABSTRACT

Sarcoma is a highly heterogeneous disease with complex biological activities and unique tumor microenvironments (TME) in distinct subtypes. The limited treatment options and inadequate responses to current therapies necessitate a deeper understanding of sarcoma biology and personalized treatment strategies. Our research comprehensively explores the sarcoma TME through advanced spatial analysis and investigates sarcoma's molecular and genetic profiles through transcriptome and genome sequencing.

In **paper I**, we focused on undifferentiated pleomorphic sarcoma (UPS) using multiplex immunofluorescence (mIF) staining for in-depth spatial analysis of B cell populations and lymphocyte aggregates (LAs). LAs in UPS were found to be associated with longer overall survival (OS) and metastasis-free survival (MFS). Moreover, we unveiled distinct maturation profiles among B cell subsets, indicative of different phenotypes that contribute to functional ecosystems in TME. LA-positive tumors displayed a more well-differentiated B cell profile throughout the entire tumor section, not limited in LA regions. We introduced the B-index, an integrated measurement tool combining B cell abundance and maturity, which demonstrated predictive power for both MFS and OS. Using the TissUUmap tool, we identified B cell desert areas characterized by extremely low B cell infiltration. LA-positive tumors displayed smaller and more fragmented B cell desert areas. In **paper II**, we performed double immunohistochemistry to study CD11c-positive antigen-presenting cells (APCs) and CD8-positive cells in 177 soft tissue sarcoma (STS) patients. We found that CD11c-CD8 interactions in the TME were associated with improved MFS and OS. Transcriptomic analysis in The Cancer Genome Atlas (TCGA) sarcoma cohort supported the prognostic significance of combining CD11c with CD8, irrespective of FOXP3 levels and in the presence of CD274 (PD-L1). In **paper III**, we conducted transcriptome and targeted DNA sequencing in 91 synovial sarcomas, identifying three distinct Synovial Sarcoma Clusters (SSCs) mirroring histological subtypes. SSC-I was characterized by high cell proliferation and immune evasion with an unfavorable prognosis. SSC-II was dominated by vascular-stromal components and correlated with better outcomes. SSC-III displayed biphasic differentiation, genomic complexity, and immune checkpoint-mediated immune suppression, leading to adverse outcomes, even after a good histological response to neoadjuvant treatment. In **paper IV**, we analyzed Ewing sarcoma (ES) transcriptome signatures in four previously published cohorts and identified 29 prognostic RNA-binding protein (RBP) genes, from which we constructed and validated an RBP-associated prognostic risk model (RPRM). The RPRM demonstrated stable predictive value for prognosis, with NSUN7 emerging as an independent and favorable prognostic marker.

In summary, our research integrates spatial analysis of the sarcoma TME to identify unique immune features and prognostic markers. Moreover, we use transcriptomic and genomic analyses to categorize specific sarcoma types for more detailed survival stratification. This work provides a deeper insight into the sarcoma TME and suggests an improved grouping strategy, aiming to shape the development of personalized immunotherapy in the future.

LIST OF SCIENTIFIC PAPERS

I. **Yanhong Su,** Haoyang Mi, Panagiotis Tsagkozis, Lennart Linke, Andri Papakonstantinou, Nicholas P. Tobin, Christina L. Stragliotto, Arne Östman, Aleksander S. Popel, Felix Haglund de Flon and Monika Ehnman.
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Manuscript

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CD11c-CD8 Spatial Cross Presentation: A Novel Approach to Link Immune Surveillance and Patient Survival in Soft Tissue Sarcoma.
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III. Yi Chen, **Yanhong Su**, Isabelle Rose Leo, Ioannis Siavelis, Jianming Zeng, Xiaofang Cao, Panagiotis Tsagkozis, Asle C Hesla, Andri Papakonstantinou, Xiao Liu, Wen-Kuan Huang, Binbin Zhao, Monika Ehnman, Henrik Johansson, Yingbo Lin, Janne Lehtiö, Yifan Zhang1, Olle Larsson, and Felix Haglund de Flon.
Integrative multi-omics analysis reveals molecular subtypes and tumor evolution of synovial sarcoma.
Manuscript

IV. Yi Chen, Huafang Su, **Yanhong Su**, Yifan Zhang, Yingbo Lin and Felix Haglund.
Identification of an RNA-Binding-Protein-Based Prognostic Model for Ewing Sarcoma.
Cancers (Basel). 2021 Jul 25;13(15):3736.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ANGS	Angiosarcomas
APC	Antigen presenting cell
BCR	B cell receptor
Breg	Regulatory B cell
BS	Bone sarcoma
CBR	Clinical benefit rate
CFU-GEMM	Colony-forming unit granulocyte/megakaryocyte/granulocyte progenitor
CGP	Comprehensive genomic profiling
ChT	Preoperative chemotherapy
CLP	Common lymphocyte progenitor
CNA	Copy number alteration
CSR	Class switch recombination
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DDLP	Dedifferentiated liposarcoma
DERBP	Differentially expressed RNA-binding protein
ELP	Early lymphoid progenitor
ES	Ewing sarcoma
ETP	Early T-lineage precursor
FFPE	Formalin-fixed paraffin-embedded
FNCLCC	Fédération nationale des centres de lutte contre le cancer
FO B cell	Follicular B cell
GC	Germinal center
GIST	Gastrointestinal stromal tumor
H&E	Hematoxylin and eosin

HEV	High endothelial venule
HSC	Hematopoietic stem cell
ICI	Immune checkpoint inhibitor
IDO1	Indoleamine 2,3-dioxygenase 1
I-E TIME	Infiltrated-excluded tumor immune microenvironment
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgH	Immunoglobulin heavy chain
IgM	Immunoglobulin M
IHC	Immunohistochemistry
I-I TIME	Infiltrated-inflamed tumor immune microenvironment
IMC	Imaging mass cytometry
In-T	Intratumoral
LA	Lymphocyte aggregate
LDH	Lactate dehydrogenase
LMS	Leiomyosarcoma
LPS	Liposarcoma
LUAD	Lung adenocarcinoma
MFS	Metastasis-free survival
mIF	Multiplex immunofluorescence
MZ	Marginal zone
NCI	National cancer institute
NK	Natural killing
OR	Overall response
ORR	Overall response rate
OS	Overall survival
PD1	Programmed cell death 1
PDAC	Pancreatic ductal adenocarcinoma

PD-L1	Programmed cell death ligand 1
PFS	Progression free survival
PPI	Protein-protein interaction
PR	Partial response
RBP	RNA-binding protein
RPRM	Prognostic risk model
RT	Radiation therapy
SCNA	Somatic copy number alteration
SD	Stable disease
SHM	Somatic hypermutation
SS	Synovial sarcoma
STS	Soft tissue sarcoma
TAM	Tumor-associated macrophage
TA-TLS	Tumor-associated tertiary lymphoid structure
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TIL-B	Tumor infiltrating B cell
TIME	Tumor immune microenvironment
TLS	Tertiary lymphoid structure
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer
Treg	Regulatory T cell
TSA	Tyramide signal amplification
ULMS	Uterine leiomyosarcoma
UPS	Undifferentiated pleomorphic sarcoma
WS	Whole-section
WTS	Whole transcriptome sequencing

1 INTRODUCTION

1.1 Sarcoma

1.1.1 Epidemiology

1.1.1.1 Soft tissue sarcoma

Soft tissue sarcoma (STS) is a group of heterogenous tumors which arise from embryonic mesoderm tissues, including fat, muscle, blood vessels, or other connective or supportive tissue (Linch, Miah et al. 2014). As a rare tumor, STS only accounts for 1% of all adult cancers with estimated new cases of 13,190 in USA in 2022. Regardless of the low incidences, the related death rate is relatively high, with estimated death cases of 5,130, accordingly (Siegel, Miller et al. 2022). STS can arise in all ages, with a higher incidence in adults (Siegel, Miller et al. 2022).

1.1.1.2 Bone sarcoma

Bone sarcomas (BSs) are an exceptionally rare subset of malignant neoplasms, comprising less than 0.2% of all malignancies across all age groups (Gatta, Capocaccia et al. 2017). The overall incidence rate typically ranges from 0.8 to 0.9 cases per 100,000 individuals annually. Individual subtypes of bone sarcomas exhibit even lower incidence rates, with none exceeding 0.3 incident cases per 100,000 individuals each year (de Pinieux, Karanian et al. 2021). Osteosarcoma and Ewing sarcoma (ES) are the most common types of BS. Given the heterogeneity of BS consisting of 15 distinct categories, the focus of this thesis will be directed specifically toward the discussion of ES within the context of BS.

The historical context of ES dates back to its description by James Ewing in 1921 (Koster and Weintrob 1931). ES has an approximate incidence of around 0.1 per 100,000 individuals annually (Esiashvili, Goodman et al. 2008). Notably, it is the most common type of BS in individuals under the age of 15, while cases diagnosed over the age of 30 are rare, and it is infrequent in individuals under the age of 5 (Bernstein, Kovar et al. 2006). It's also worth noting that ES is extremely rare in Afrodescendant and Asian populations and displays a slightly higher incidence among males (Pritchard 1984).

1.1.2 Clinical diagnosis

1.1.2.1 Soft tissue sarcoma

Due to the varying sources of origins and different lines of differentiation, STS has more than 100 subtypes. Common subtypes of STS include liposarcoma (LPS), undifferentiated pleomorphic sarcoma (UPS), gastrointestinal stromal tumor (GIST) and leiomyosarcoma (LMS). Heterogenous genetic and molecular variance and complex pathological behaviors compose a major challenge for clinical diagnosis. With few exceptions, children and adults share similar clinical features of STS (Ferrari, Sultan et al. 2011). STS can arise from all parts of the human body, and over 40% of cases are found in extremities (Pisters, Weiss et al.

2011). The clinical symptoms of STS lack specific manifestation. The typical symptom is characterized by a painless, gradually enlarging mass (Clark, Fisher et al. 2005). Secondary compression symptoms can occur depending on the primary tumor site and compression pressure, such as neuralgia, paresthesia, and urinary tract obstruction.

1.1.2.2 Bone sarcoma

Symptoms commonly associated with ES primarily result from the expansion of bone lesions, often manifesting as initial indicators of the condition. ES can potentially originate from any bone, the most common primary sites are the extremity bones, accounting for approximately 50% of cases, followed by the pelvis, ribs, and vertebrae. Importantly, ES can also originate in soft tissues, particularly in adult patients (Strauss, Frezza et al. 2021). Therefore, ES most frequently affects anatomical regions including the pelvis, femur, tibia, humerus, and the thoracic wall, although it has the potential to involve any bone in the body (Miser, Goldsby et al. 2007, Jedlicka 2010).

Histologically, ES presents as a homogenous assembly of undifferentiated small round cells, each featuring vesicular nuclei and a limited cytoplasm within a sparsely populated intercellular stroma. These histological attributes offer little insight into the cellular lineage responsible for the origin of ES. The definitive diagnosis of ES is made on biopsy. ES is characterized by transcription factors gene fusion event involving a member of the FET family and a member of the ETS family. Approximately 85% of ES exhibits EWSR1-FLI1 fusions, while 10% of cases involve EWSR1-ERG fusions. In about 3% of instances, fusions between EWSR1 and other members of the ETS family of transcription factors, such as FEV1, are observed. (Delattre, Zucman et al. 1992). The resultant EWS-FLI1 chimeric protein assumes the role of an aberrant transcriptional regulator, linked to the control of numerous genes governing cellular growth, signal transduction, and differentiation (Janknecht 2005).

1.1.3 Grading system

1.1.3.1 Soft tissue sarcoma

The grading of STS is one of the most important risk stratification tools based on histopathological evaluation. There are two common systems for grading sarcoma: the French Federation of Cancer Centers (Fédération nationale des centres de lutte contre le cancer; FNCLCC) and the National Cancer Institute (NCI) grading system (Costa, Wesley et al. 1984, Trojani, Contesso et al. 1984). As the most widely used grading system, FNCLCC grading is based on a combination of features including: tumor differentiation level/histology, mitotic count and tumor necrosis. NCI is a less commonly used system, and it is more difficult to objectively quantify. Moreover, for predicting distant metastasis development and tumor mortality, the FNCLCC system shows a slightly superior advantage (Guillou, Coindre et al. 1997). Notably, these two systems are applied for the grading in most subtypes of STS and are established since 1980s. Although the long-term utilities validate the reliability and validity, the dramatic changes of therapeutic solutions, especially with the advent of

immunotherapy, propose new challenges to develop a more accurate outcome-predictive tool applicable for advanced treatment measure. Unfortunately, the survival model considering tumor microenvironmental or immune features has either been in-depth studied or successfully established in STS.

1.1.3.2 Bone sarcoma

All ESs are considered as high-grade osteolytic malignant neoplasms.

1.1.4 Prognosis and treatments

1.1.4.1 Soft tissue sarcoma

The 5-year survival rate for localized STS is 81%. However, when the diseases progress to distant metastasis stage, the 5-year survival rate drops to 15% dramatically (Howlader N). Multiple clinical and histopathological features at diagnosis can affect the outcomes of STS, including age, tumor location, tumor size and depth, staging, histological type and grade and margin (Vraa, Keller et al. 1998, Trovik, Bauer et al. 2000, Maretty-Nielsen, Aggerholm-Pedersen et al. 2014). These risk factors provide valuable predictive information for local recurrence, distant metastasis and survival.

Currently, the standard treatments for STS follow the classical general principles for solid tumor therapies, including surgery, radiation therapy (RT) and chemotherapy. Depending on the disease stages, the treatment strategies vary. For stage I STS, surgical wide resection is recommended as the primary treatment while postoperative RT can be performed in selected patients. For stage II-III STS, in addition to surgery, postoperative RT or chemotherapy can be combined based on the comprehensive evaluation of age, performance status, comorbidities, tumor location, and histologic subtype. For unresectable STS, primary treatment with RT, chemotherapy, chemoradiation or regional limb therapy can be adopted.

Unresectable disease can be treated primarily with RT, chemoradiation, chemotherapy, or regional limb therapy. If tumors become resectable with acceptable persevered functions after primary treatment, the treatment strategy is usually surgery followed by RT (if not used before) with or without postoperative chemotherapy. For stage IV and metastasis disease, the potential survival benefit of metastasectomy remains controversial, but primary tumor management for patients with limited metastasis who are amenable to local therapy is the same as for stage II or III tumors. In patients presenting with disseminated metastasis, asymptomatic patients can follow a “watchful waiting” observation strategy, while symptomatic patients can be treated with palliative RT, surgery, or chemotherapy (von Mehren, Randall et al. 2018). Systemic therapy is one of the most important therapeutic measurements in the advanced-disease setting, in which the major option remains chemotherapy. In addition to the classical cytotoxic drug, such as anthracycline-based chemotherapy which is recommended as first-line treatment for advanced disease, novel chemotherapy regiments, such as combined therapy with trabectedin, and small molecular kinase inhibitors such as Pazopanib, Regorafenib, etc. have been investigated (Grunwald,

Karch et al. 2020, Riedel, Ballman et al. 2020, Le Cesne, Blay et al. 2021). Unfortunately, none of these attempts show significant survival improvement. The median overall survival (OS) for advanced STS never surpasses 2 years in all clinical trial settings.

1.1.4.2 Bone sarcoma

The presence of metastatic disease upon diagnosis serves as the most crucial determinant of ES survival outcomes. Approximately 25% of ES are primarily diagnosed with pre-existing metastases (10% with lung, 10% with bones or bone marrow, and 5% with combinations or other metastatic patterns). Notably, multiple bone metastases indicate a notably graver prognosis, with a 5-year survival rate of less than 20%, compared to 5-year survival rates from 50% to 60% in patients with lung or pleural metastases (Cotterill, Ahrens et al. 2000). Other recognized adverse prognostic indicators include tumor volume, elevated levels of lactate dehydrogenase (LDH), axial localization of tumors, older age (> 15 years), inadequate histological response to preoperative chemotherapy (ChT), and incomplete or absent surgical intervention for the primary tumor site. Irrespective of the specific gene fusion involved, patients with ES demonstrate similar outcomes following standard treatment protocols (Le Deley, Delattre et al. 2010). In the absence of systemic treatments, 5-year survival rate is less than 10%. However, with contemporary multimodal approaches involving ChT, the landscape significantly improves, yielding approximately 60% to 75% 5-year survival rates for localized disease and approximately 20% to 40% for those with metastatic disease.

The primary treatment for localized ES is neoadjuvant chemotherapy, using regimens like vincristine, doxorubicin (Adriamycin), and cyclophosphamide. Following at least 9 weeks of chemotherapy, imaging tests determine if the tumor has responded, making it amenable to surgical removal. If surgery is not feasible, RT, in combination with chemotherapy, is typically the next treatment option. In cases of metastatic disease, similar principles apply, with chemotherapy being the primary approach, followed by surgical intervention, radiation therapy, and ongoing chemotherapy as indicated by the patient's response to treatment (Network).

1.2 The sarcoma tumor microenvironment

1.2.1 Composition and organization

The tumor microenvironment (TME) in sarcomas is dominated by malignant cells during tumor growth and progression. However, it consists not only of the proliferating sarcoma cells, but also stromal cells, vessels, infiltrating immune cells and a variety of associated tissue cells. Inside the TME, molecular and cellular interactions actively occur between the various cell types, to shape or even determine the fate of the tumor regarding proliferation, metastasis and eradication. Moreover, the TME exerts a key influence on treatment response and resistance.

To understand the function of the TME in terms of predicting immune checkpoint inhibitor (ICI) responsiveness, a new concept of tumor immune microenvironment (TIME) with a special focus on the immune context in the TME has attracted increasing attention. According to the maps of infiltrated immune cells, TIMEs can be clustered into several major types:

1) Immune desert

A TME where T cells are absent is identified as an immune desert, which is shown to be correlated with worse clinical outcomes. (Joyce and Fearon 2015)

2) Infiltrated–excluded (I–E) TIME

In an I–E TIME, the presence of cytotoxic lymphocytes (CTLs) is observed. However, the CTLs are only located along the tumor capsule or the invasive margin, failing to penetrate the tumor core.

3) Infiltrated–inflamed (I–I) TIME

An I-I TIME is characterized with high infiltration of CTLs in the tumor mass and is considered as an “immunologically hot” tumor, and is thereby also more likely to respond to ICI treatments.

4) TLS-TIME

A TLS-TIME is a special subtype of I-I TIME with the presence of tertiary lymphoid structures (TLSs). TLSs are aggregates of immune cells with similar composition and structure as lymph nodes, which are believed to be the locations for immune cell recruitment, activation and differentiation, with the potential to boost anti-tumor immune reactivity (Binnewies, Roberts et al. 2018). Recently, remodeling of the TME has brought unexpected therapeutic benefits and treatments targeting the TME have emerged as one of the most successful anti-tumor therapies in a broad range of tumor types.

1.2.2 Lymphocyte aggregates and tertiary lymphoid structures

Aggregation of lymphocytes in non-lymphoid tissue can be induced by long-term exposure to an inflammatory environment. The presence of TLS can be observed in chronic inflammatory

conditions including autoimmune and infectious diseases, transplantation, inflammatory disorders and tumors. In the tumor context, TLS can locate in the stroma, invasive margin and/or less frequently the core of the tumor mass depending on the tumor type (Dieu-Nosjean, Giraldo et al. 2016).

Classical tumor-associated TLSs (TA-TLSs) are composed of follicle-like structures, containing one or more germinal centers (GCs) with follicular dendritic cells and proliferating B cells. Germinal centers are surrounded by T cell-rich zones juxtaposing mature dendritic cells along with plasma cells. High endothelial venules (HEVs) appear in mature TLSs as special types of postcapillary venules. HEVs contain cuboidal blood endothelial cells expressing abundant sulfated sialomucins for binding lymphocytes, resulting in the recruitment and transmigration of lymphocytes into tumor sites from peripheral blood. TLSs are privileged sites where local tumor-associated antigens are presented to T cells by antigen presenting cells (APCs), followed by an immune activation process for T and B cells involving cell activation, proliferation, and differentiation, generating memory T and B cells, cytotoxic effectors and antibody-producing plasma cells (Figure 1).

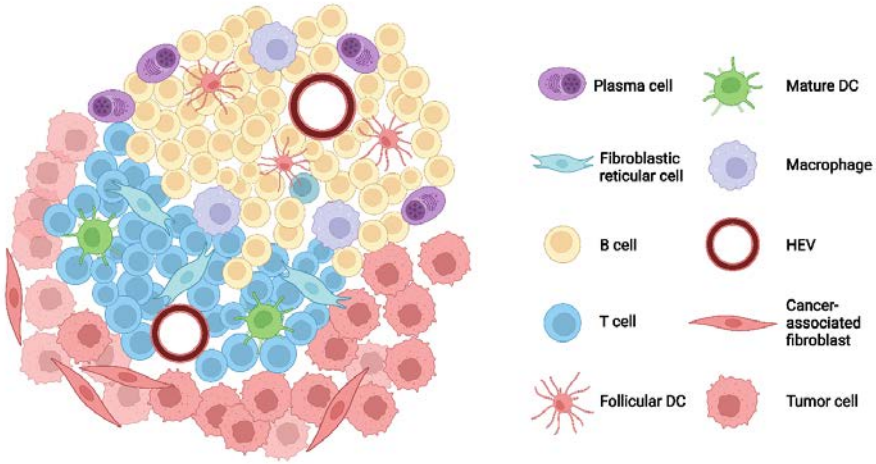

Figure 1. Cellular composition of a mature tumor-associated TLS. Created with BioRender.com.

In addition to the classical structure, a TA-TLS can also show a non-classical organization as an immature TLS, featuring small lymphocyte aggregates (LAs) containing mainly T cells and B cells without GC and HEVs. The composition and maturation of TA-TLSs also exhibit heterogeneity across tumor types. Notably, the diverse histological subtypes of sarcomas introduce further complexity to TLS characteristics, resulting in unique TLS profiles. For example, our unpublished data reveal a more mature TLS profile in liposarcoma, characterized by the presence of large LAs with well-defined GC structures. In contrast, UPS

typically exhibits smaller lymphocyte clusters with a less compact structure, and GCs are infrequently observed (Figure 2).

The structural organizations of TA-TLS are obviously intricately linked with the specific TME. Currently, it remains uncertain whether the absence of compartmentalization in non-classical TA-TLSs implies less effective tumor-antigen presentation and T-cell activation compared to classical structures. Additionally, it is unclear whether any of these variables contribute to the differentiation of effector T-cells.

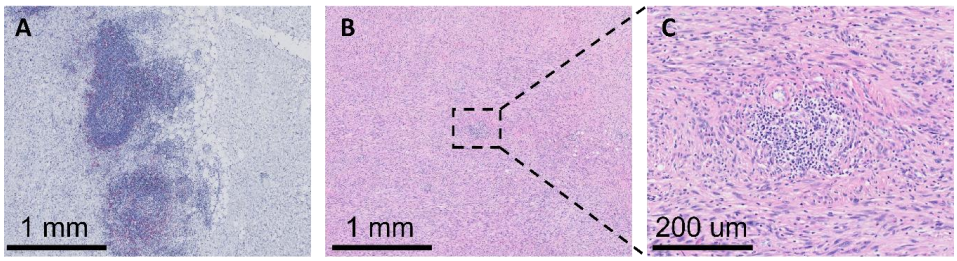

Figure 2. Histological image of representative TLSs. (A) TLSs in liposarcoma. (B) A LA in UPS. (C) A high magnification H&E image of a LA in UPS. Unpublished data from Karolinska hospital.

The presence of TLSs is considered as evidence for intense intra-tumoral lymphocyte proliferation and in situ immune hyperreactivity. For example, in human lung cancer tissue, a high density of TLS-associated mature dendritic cells is correlated with increased infiltration of activated CD38+ or CD69+ CD8+ T cells, along with a higher level of effector-memory CD8+ T cells (Goc, Germain et al. 2014). Regardless of detection method, TLSs are usually associated with a favorable prognosis in most tumor types, not only in lung cancer, but also in invasive breast cancer, pancreatic cancer, and colorectal cancer, indicating a long-lasting anti-tumor guard role of TA-TLSs (Dieu-Nosjean, Antoine et al. 2008, Di Caro, Bergomas et al. 2014, Hiraoka, Ino et al. 2015, Savas, Salgado et al. 2016).

In metastatic melanoma and sarcoma, the presence of TLSs is more prevalent in tumors with a higher response rate to ICI treatment than those with poor response. Thus, TLSs could be considered a predictor for ICI response before drug administration, or could be regarded as an evaluating tool for ICI response (Cabrita, Lauss et al. 2020, Helmink, Reddy et al. 2020, Petitprez, de Reynies et al. 2020). However, the ambiguous role of TLSs should not be neglected, that is, instead of boosting immune hyperreactivity, TLSs can also establish an immunosuppressive microenvironment. For example, regulatory T cells (Tregs) located in TLSs can dampen endogenous anti-tumor immune responses (Joshi, Akama-Garren et al. 2015).

On the other hand, the precise location of TLSs, either peri-tumoral, at the tumor invasive margin, or intra-tumoral, indicates different biofunctions. For example, an intra-tumoral TLS

in pancreatic cancer patients functions as a favorable factor for outcome prediction, but not a peri-tumoral TLS (Hiraoka, Ino et al. 2015). In breast cancer, patients with high densities of both adjacent TLSs (peritumoral TLS within 5 mm from the tumor invasive margin border) and distal TLS (peritumoral TLS further than 5 mm from the tumor invasive margin border) exhibit the worst outcome (Sofopoulos, Fortis et al. 2019). The location of TLSs likely emerges as a critical factor in determining their efficacy in outcome prediction, but such investigations have not been extended to sarcomas within the existing literature.

1.2.3 Molecular subtyping with immune microenvironment signatures

In clinical practice, the diagnosis and treatment decisions in sarcomas rely heavily on conventional histological assessments and the expertise of pathologists. However, in light of the rapid advancements in multi-omics research, innovative sarcoma subtyping models are established to enhance diagnostic precision, identify prognostic biomarkers, and suggest potential treatment targets. In a comprehensive genetic analysis encompassing 2,138 sarcoma cases representing 45 different pathological entities, unsupervised clustering based on genetic alterations reveals 17 distinct sarcoma subtypes, with several subtypes exhibiting significant heterogeneity in histological cluster association. Uterine leiomyosarcoma (ULMS), UPS, and osteosarcoma have high entropy scores of clustering assignments. These findings suggest that sarcomas, which may share similar histological characteristics, can possess diverse genetic variants. Additionally, the identification of cluster-defining genetic events associated with specific vulnerabilities supports the consideration of basket trials, where treatments are based on genotype rather than histology, offering a personalized and potentially more successful approach to sarcoma treatment (Nacev, Sanchez-Vega et al. 2022).

Importantly, subtyping sarcomas based on TIME signatures provides valuable insights into the intricate immune landscape. It serves as a valuable tool for risk-stratifying sarcoma patients and identifying those who may derive greater benefit from immunotherapies. In a comprehensive transcriptome analysis encompassing 608 tumors across various STS subtypes, Petitprez et al. introduced an immune-based classification regarding TME composition, unveiling five distinct phenotypes: immune-low (A and B), immune-high (D and E), and highly vascularized (C) groups. This study highlights B cell infiltration as a pivotal discriminative feature associated with improved patient survival. Notably, the class-E group, distinguished by the presence of TLSs rich in T cells, follicular dendritic cells and B cells, demonstrates better survival and exhibits a high response rate to pembrolizumab in a phase 2 clinical trial (Petitprez, de Reynies et al. 2020). Therefore, TIME molecular subtyping in sarcomas holds the potential to inform clinical decision-making and facilitate the development of innovative immune based therapies.

1.3 B cells in physiology and tumor pathology

1.3.1 B cell development

The development and maturation of B cells is a very long journey. This journey starts from the hematopoietic cells (HSCs) in the bone marrow. The pluripotent HSCs differentiate into two major progenitors: common granulocyte/megakaryocyte/granulocyte progenitor (CFU-GEMM) and early lymphoid progenitor (ELP). ELP gives rise to two main precursors of common lymphocyte progenitor (CLP) and early T-lineage precursor (ETP), which will eventually differentiate into NK cells/B cells and T cells, respectively. Received stimulus signals from bone marrow stromal cells, functional rearrangement of heavy chain gene locus (IgH) occurs in lymphoid progenitor cells to become early pro-B cells (Adler 2008).

D-JH recombination is completed during Pro-B cell stage and by late pro-B cell stage, VH to DJH immunoglobulin (Ig) gene segment recombination is initiated to enter Pre-B cell stage in most cells. In this stage, Ig heavy chain genes complete V-D-J recombination and light chain rearrangement is also initiated. Once light chain rearrangement has been successfully completed and u chain on the cell membrane is synthesized, the intact immunoglobulin M (IgM) receptor can be expressed, forming the immature B cell. Immature B cells have functional IgM, but no other Ig expression (Table 1) (Adler 2008). At this stage, immature B cells leave the bone marrow to enter the secondary lymphoid organs where they will encounter antigens and participate in antigen-dependent immune reactions.

Table 1. Events in early B cell development.

Early B cell development						
Phase	**Lymphoid progenitors**	**Early pro-B**	**Late pro-B**	**Large pre-B**	**Small pre-B**	**Immature B**
H chain genes	**Germline**	**D-J joining**	**V-DJ joining**	**VDJ rearranged**	**VDJ rearranged**	**VDJ rearranged**
L chain genes	**Germline**	**Germline**	**Germline**	**Germline**	**V-J joining**	**VJ rearranged**
Surface Ig	**None**	**None**	**None**	**u chain in pre-B receptor**	**m chain in cytoplasm/surface**	**Membrane IgM**

Table adapted from Immunobiology. 7ed. (Garland Science 2008)

When newly produced immature B cells emerge from bone marrow into the periphery, they enter follicles in peripheral lymphoid structures, which provide signals necessary for B cell survival. In the spleen, immature B cells differentiate through transitional B cells named T1 and T2, followed by differentiating into long-lived mature follicular (FO) or marginal zone (MZ) B cells (Thomas, Srivastava et al. 2006). Mature FO B cells recirculate among secondary lymphoid organs searching for antigens. After encountering their antigens, with the activation from helper T cells, B cells can enter several developmental possibilities:

In one way, B cells with bound antigens meet T cells at the borders of follicles in the secondary lymphoid tissue, and the T-cell-B-cell interaction produces an initial proliferation

of B cells, followed by differentiation into plasmablasts and eventually into plasma cells (Thomas, Srivastava et al. 2006). However, without experiencing somatic mutation and Ig class switch recombination (CSR), these extrafollicular plasma cells are short-lived cells secreting IgM antibodies as a rapid initial response to antigen.

In another way, the B cell immune response occurs at a later phase when activated B cells together with associated T cells migrate into follicles and proliferate to form a GC. A GC is a specialized microenvironment composed of proliferating B cells, antigen-specific T cells and follicular dendritic cells. In the GC, B cells undergo rounds of intense proliferation along with important modifications to produce a more effective antibody response (Edwards and Cambridge 2006). Antibodies play a remarkable role in immune reactions, not only for the diversity of antibody binding sites, but also for the versatile functions, which are determined by the isotype of the antibody. GC B cells undergo somatic hypermutation (SHM) in the variable region to generate abundant clones with radically differing B cell receptors (BCRs).

SHMs with improved affinity for antigens are selected, resulting in a B cell pool with high affinity to specific antigens. On the other hand, CSR initiates following B cells activation. During CSR, the μ constant regions in Ig genes are replaced by other constant regions, resulting in different classes of antibodies (Adler 2008).

To conclude, SHM and clonal selection enable affinity maturation, while CSR allows the selected B cell clones to exert various effector functions. In the end, low affinity and autoreactive B cells die while memory B cells and plasma cells with high affinity BCRs of switched isotypes leave the GC.

Table 2. Marker expressions during B cell development

	HSC	Pro-B	Pre-B	Immature	Transitional	Mature	Activated /GC B cell	Memory B	Plasmablast	Plasma cell
Location	BM	BM	BM	BM	Entry to spleen	Periphery	Periphery	Periphery	Periphery	Periphery
Function					Precursor to mature naive B cell	With functional BCR	Activated by antigen/T cell	Recall responses/ humoral immunity	Antibody secretion/ proliferation	Antibody secretion
CD19										
CD20										
CD38				hi	hi					hi
CD138										hi
CD27									hi	
IgM					hi	low				
Pax 5										

Negative
Positive
Low expression
High expression
Negative/positive

HSC, hematopoietic cell. BM, bone marrow.

1.3.2 B cells in solid tumors

Although the biology and function of the T cell in tumors have been well established, another major type of lymphocyte, the B cell, is poorly investigated in the setting of non-B cell solid tumors. The importance of tumor-infiltrating B cells (TIL-Bs) has been underestimated for a long time, until this decade. Unlike macrophages and T cells, B cells are relatively few, or even absent, in the TME. B cells usually concentrate at tumor borders or invasive margins, sometimes forming aggregates in the form of immature immune cell clusters or mature TLSs. The low frequency of TIL-Bs hinders capturing the whole B cell repertoire inside a tumor. In addition, the diversity and complexity of B cell phenotypes and functions remind us that TIL-Bs may play both pro-tumor and anti-tumor roles in the TME, depending on the context.

Long-lived B cells can function as APCs, directly presenting tumor-associated antigens via BCRs to T cells and activating T cells through CD27–CD70 interactions (Deola, Panelli et al. 2008). The B cell-T cell cooperation not only initiates T cell activation, but also maintains T cell clonal expansion (Bruno, Ebner et al. 2017). In an experimental mouse model of breast cancer, single injection of ex vivo-activated B cells induced antitumoral immunity in host T cells. The study further showed that co-injection of activated B cells and T cells resulted in tumor regression with greater effect than transferring either cell type alone (Li, Lao et al. 2011).

Other studies have demonstrated that the presence of TIL-Bs with close distance to CD8+ T cells and the colocalization of B cells and T cells indicate an improved prognosis in breast cancer, melanoma and ovarian cancer (Ladanyi, Kiss et al. 2011, Nielsen, Sahota et al. 2012, Iglesia, Vincent et al. 2014). In hepatocellular carcinoma, a subset of atypical memory B cells (IgD-IgG+CD27-CD38-) expresses surface markers with APC characteristics and cooperates with CD8+ T cells. Moreover, these B cells also produce various cytokines including IFN-g, interleukin 12p40 (IL-12p40), granzyme B, and TRAIL to promote tumor-killing immune activity (Shi, Gao et al. 2013).

As the sole antibody producer in the human body, plasma cells can actively produce abundant amounts of antibodies and cytokines even with a low cell count (Dang, Hilgenberg et al. 2014). Neoantigens generated by tumor cells or tumor associated antigens can be presented to B cells by APCs or directly recognized by B cells. Either peripheral plasma cells residing in bone marrow or spleen secret free tumor-specific antibodies circulating in serum, or tumor infiltrated plasma cells produce high levels of tumor-specific antibodies in situ. These tumor-specific antibodies can support the uptake of tumor antigens by tumor-associated macrophages (TAMs) and dendritic cells. Once tumor cells are labeled with these antibodies, an antibody-dependent cellular cytotoxicity (ADCC) process executed by tumor killing cells, such as CD8+ cytotoxic T cells and natural killing (NK) cells, can be triggered (Kurai, Chikumi et al. 2007).

In triple-negative breast cancer (TNBC), tumors with a higher abundance of TIL-B showed an expansion of immunoglobulin G (IgG) isotypes, which were associated with improved

patient outcomes. The TIL-B exhibits clonal expansion, primarily biased toward IgG, with specific variable region gene combinations and narrow repertoires. This finding indicates that these TIL-Bs are likely responding to specific antigens in the TME (Harris, Cheung et al. 2021). Similarly, the phagocytosis process by M1-like TAMs is also facilitated by tumor-specific antibodies. Moreover, upon tumor cell binding, tumor-specific antibodies can activate complement system, leading to a tumor killing effect by the innate immune system.

According to the RNA sequencing data from The Cancer Genome Atlas (TCGA) database, a high expression of B cell and plasma cell markers is associated with increased OS in several tumors, such as melanoma, lung cancer, pancreatic cancer, and head and neck squamous cell carcinoma. In a meta-analysis of other transcriptome data from the TCGA database involving 18,000 tumors, the relative abundance of tumor infiltrating plasma cells is found to be one of the most significant beneficial factors for improved survival in a broad range of solid tumors (Gentles, Newman et al. 2015).

B cells can also play an immunosuppressive role in promoting tumor growth through several mechanisms. Recent research has reported the presence of regulatory B cells (Breg) inside tumors (He, Qian et al. 2014). Bregs are characterized by the expression of inhibitory ligands and immunosuppressive cytokines, among which IL-10 and transforming growth factor- β (TGF- β) are the two most established effectors. Bregs can inhibit CD4+ and cytotoxic T cell responses by antigen-independent mechanisms (Shah, Divekar et al. 2005). In addition, Bregs can induce naive or effector CD4+ T cells to differentiate into Tregs, and promote Treg proliferation (Olkhanud, Damdinsuren et al. 2011). Apart from dampening anti-tumoral T cell activity, Bregs also modulate the activity of TAMs and myeloid-derived immunosuppressive cells. Moreover, they can directly promote tumorigenesis and angiogenesis (Schwartz, Zhang et al. 2016).

Although tumor specific antibodies produced by plasma cells exert diverse anti-tumor effects, not all antibodies are effective. A faulty antibody class fails to facilitate the antigen presentation process, and cannot mediate ADCC or phagocytosis of tumor cells. In another case, even with a proper antibody class, IgG antibody specificities may not elicit efficient T cell response or other effectors for killing tumor cells. Moreover, a mass mixture of tumor and non-tumor specific antigen-antibody immune complexes in the TME may induce chronic inflammation, leading to remodeling and eventually protecting the tumor against immune-mediated elimination (Sharonov, Serebrovskaya et al. 2020).

Despite the trendy fashion of studying immunology in various tumor types, the study of TIME in STS remains very limited, especially with a focus on B cells in STS. Up till now, the understanding of B cell compositions and functions in STS is still shallow, without a deep study nor a high-resolution inspection. In our previous study, an immunohistochemistry (IHC) staining of CD20 has been performed in 33 STS patients. The result showed that the presence of CD20+ B cells correlates with improved metastasis-free survival (MFS) and OS. Similarly, the gene expression analysis involving 265 STS patients also displayed an

association between high MS4A1 expression and improved outcome. However, the favorable survival signal of MS4A1 was only observed under a setting of an $IL10^{low}$, $PTGS2^{low}$ or $CD163^{low}$ TME based on the transcriptomic data, indicating that immunosuppressive TAM may attenuate the anti-tumor activity by tumor infiltrated B cells (Tsagozis, Augsten et al. 2019).

In another transcriptome analysis of 608 STS cases, 5 distinct subgroups of STS were identified based on the composition of the TIME. Notably, tumors characterized by presence of TLS and particularly rich in B cells associated with improved survival and better response to pembrolizumab therapy. Even with a background of high or low CD8+ T cells and cytotoxic components, B cells remained as the strongest factor for improved prognosis (Petitprez, de Reynies et al. 2020). In the midst of exploring personalized immunotherapy in STS, B cells display great potential to be a key discriminative feature for response prediction. Deeper insight to unravel the underlying mechanism and identification of tumor-associated B cell phenotypes warrants further investigation.

1.3.3 Spatial profiling of B cells

Except for the sheer abundance of B cells, the spatial aggregation of B cells is also associated with immune activity and disease prognosis in various tumor types. In early-stage lung adenocarcinoma (LUAD), a spatially resolved single-cell atlas of TIL-Bs has been described by paired single-cell RNA and BCR sequencing with spatially defined methods. A gradual reduction in the proportion of naïve B cells and an increase in the proportion of class-switched memory B cells and plasma cells occur in the tumor proximity. Moreover, heightened BCR clonal diversity and SHM frequencies are observed in normal tissues closer to the LUADs. These findings suggest an active association between TIL-Bs and the development of LUAD, originating from a specific niche in the lung, rather than from other mutagenized areas influenced by smoking (Hao, Han et al. 2022).

In a study involving 104 human pancreatic ductal adenocarcinoma (PDAC) patients, IHC analysis revealed two distinct B cell location patterns: scattered infiltrations (CD20-TILs) or organized in TLS (CD20-TLT). The prolonged survival is observed only when there is an accumulation of B cells retained within CD20-TLT. A high density of B cells predicts an extended survival period, with a median survival of 16.9 months for $CD20\text{-}TLT^{hi}$ compared to 10.7 months for $CD20\text{-}TLT^{lo}$, suggesting their potential involvement in a protective antitumor role through a follicular immune response (Castino, Cortese et al. 2016). Additional understanding about B cells comes from a mIF study in 36 TNBC patients where patients with favorable clinical outcomes (recurrence free after 5 years) exhibit B cells distributed throughout the tumor in a dispersed spatial pattern. In contrast, those with unfavorable prognosis (recurrence within 3 years) have B cells that are spatially confined. Notably, the study demonstrated that LAs, whether composed solely of T cells or comprising a mixture of B and T cells, are more prevalent and exhibit a greater spatial dispersion in TNBC cases with favorable outcomes when compared to those displaying poor outcomes. In cases with better prognosis, heterotypic LAs, containing both B and T cells, are smaller in

size and more abundant. These heterotypic LAs are also located in closer proximity to cancer cell islands in cases with favorable outcomes, and their size diminishes as they approach these cancer cell islands (Wortman, He et al. 2021).

1.4 Future perspectives on clinical decision making

In the recent decades, the advent of immunotherapy has dramatically reshaped the treatment strategies against tumors. ICI targeting programmed cell death 1 (PD1), PD-ligand-1 (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) contribute to the major breakthrough of this therapeutic innovation. The remarkable success of immunotherapy is credited to the efficacy in a broad range of tumor types, including melanoma, renal cancer and lung cancer (Motzer, Escudier et al. 2015, Reck, Rodriguez-Abreu et al. 2016, Larkin, Chiarion-Sileni et al. 2019). Moreover, the long duration of responses and favored safety profile make immunotherapy extra attractive.

Inspired by the successful experience in various solid tumors, the application of ICI in STS has been actively explored. Regrettably, the ICI treatment yields an underwhelming efficacy in STS including all histological subtypes, with a low overall response rate (ORR) ranging from 5% to 23% and a median progression free survival (PFS) of around 3-4 months (Italiano, Bellera et al. 2020, Fazel, Dufresne et al. 2023). In the PEMBROSARC trial, the first phase 2 trial to explore the use of ICI in STS patients, 57 STS patients with various subtypes received a combination of pembrolizumab and metronomic cyclophosphamide. Unfortunately, the trial yielded a discouraging result, with only one out of 50 patients showing a partial response (PR).

It is tempting to speculate that the limited response may be attributed to the presence of an immunosuppressive TME characterized by macrophage infiltration and activation of the indoleamine 2,3-dioxygenase 1 (IDO1) pathway (Toulmonde, Penel et al. 2018). In another subsequent phase 2 trial (SARC028) evaluating pembrolizumab as a monotherapy in STS and bone sarcoma patients, seven out of 40 patients (18%) with STS demonstrated an overall response (OR) among all 86 enrolled patients. Although the primary objective of achieving an OR was not met, the study at least exhibited promising activity in patients with UPS or dedifferentiated liposarcoma (DDLPS) (Tawbi, Burgess et al. 2017).

In two other open-label, non-comparative, randomized phase 2 trials (Alliance A091401), researchers explored the use of nivolumab alone or in combination with ipilimumab as a treatment for sarcoma. The studies enrolled a total of 85 eligible patients, with 43 receiving nivolumab monotherapy and 42 receiving nivolumab plus ipilimumab. Among the 76 eligible patients analyzed, the nivolumab group displayed an ORR of 5%, with only two patients responding, while the nivolumab plus ipilimumab group exhibited an ORR of 16%, with six patients responding (D'Angelo, Mahoney et al. 2018). Interestingly, in the expansion cohorts enrolling UPS and DDLPS patients, improved ORR of around 23% and 10%, respectively, were observed in these selected population (Chen, Mahoney et al. 2020). Overall, these results suggest that immunotherapy may hold therapeutic potential for STS, particularly when combined with other treatment modalities, but the need for patient selection and identification of biomarkers remains essential.

Although the underlying mechanisms for the poor response to ICI remain unknown, a low level of tumor mutational burden (TMB) in STS suggests a potential explanation. TMB, which is defined as the number of somatic mutations per coding area in the tumor genome, is associated with a higher load of neoantigens and improved response rate to ICI treatment (Rizvi, Hellmann et al. 2015). In a large-scale study measuring the distribution of TMB across diverse tumors using a targeted comprehensive genomic profiling (CGP) assay, STS harbors a globally low TMB (mean TMB of 1.06 mut/Mb) with only 5% of the tumors carrying >20mut/Mb (Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research 2017).

In contrast, lung cancers and melanoma, which are more sensitive to ICI therapy, are characterized with higher median TMB load of 7.2 mutations/Mb and 13.5 mutations/Mb, respectively (Chalmers, Connelly et al. 2017). Although sarcoma generally have a low TMB, it is important to note the substantial variability across different subtypes. For instance, angiosarcomas (ANGS), UPS, and ULMS exhibit comparatively higher average TMBs, measuring at 3.0 mut/Mb, 2.6 mut/Mb, and 2.6 mut/Mb, respectively. Notably, ANGS and UPS also demonstrate a more substantial proportion of cases with a TMB of $\geqslant$10 mut/Mb, accounting for 7.6% and 6.7% of cases, respectively (Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research 2017).

However, the heterogeneity and rarity of STS shed light on finding specific subtypes or STS with a characteristic TME that is responsive to ICI therapies. Notably, with great genomic complexity and heterogeneity, UPS carries the highest level of copy number alteration (CNA) and TMB among STS, with frequent mutations of TP53 and RBI (Cancer Genome Atlas Research Network. Electronic address and Cancer Genome Atlas Research 2017, Chalmers, Connelly et al. 2017). Moreover, the TME of UPS seems to be more immunogenic compared to other STS characterized with high infiltration of TILs such as CD8+ cytotoxic T cells and FoxP3+ T cells (Klaver, Rijnders et al. 2020).

Consistent with the immune-reactive potential in UPS, the UPS subtype in the ICI clinical trials reports better treatment response with ORRs at around 15- 40% with ICI alone and over 50% for combined therapies based on ICIs (Italiano, Bellera et al. 2020). The selection of specific population who potentially benefit from ICI treatments based on biomarkers and application of personalized therapy regimens will therefore be the future step for exploring ICI treatments in STS.

Recent studies suggest that STS TME features can predict the response to ICIs and help stratifying appropriate patient populations. An extended cohort in PEMBROSARC trial enrolled 48 TLS-positive STS patients out of a total of 240 screened individuals. Among the 35 evaluable patients, the clinical benefit rate (CBR) reached 63% (with an OR of 30% and a stable disease (SD) of 33%), compared to the 2% OR observed in the entire group. The median PFS was 4.1 months, and the median OS was 14.5 months. (Italiano, Bessede et al. 2022). In the above-mentioned transcriptomic analysis encompassing 608 STS cases,

sarcoma patients with a high immune activity signature, characterized by the presence of TLS and plasma cells, was associated with a positive response to anti-PD1 therapy. This immune activity signature remains the most influential prognostic factor, regardless of high or low CD8+ T cell and cytotoxic signatures (Petitprez, de Reynies et al. 2020). Still, one of the major challenges in using ICI in STS is to define the best candidates and identify reliable biomarkers and response predictors. This inspires us to continue investigating B cell phenotypes and TLSs to aid STS patient selection and optimize personalized clinical decision making.

2 RESEARCH AIMS

This thesis encompasses the investigation of prognostic indicators in sarcomas and examination of the sarcoma TME with emphasis on spatial profiling. Specifically, the studies aim to:

Explore immune profiles in UPS: In paper I, we aimed to examine B cell profiles and LAs within the UPS TME and ascertain their potential associations with prognosis.

Characterize prognostic factors in soft tissue sarcoma: In paper II, we aimed to characterize the prognostic significance of CD11c-expressing APCs together with CD8-positive T cells in different molecular TMEs.

Uncover heterogeneity in synovial sarcoma: In paper III, we aimed to profile and uncover the synovial sarcoma (SS) heterogeneity, identifying subtypes associated with distinct transcriptomic and genomic characteristics.

Develop a risk model in Ewing sarcoma: In paper IV, we aimed to investigate the role of RNA-binding proteins (RBPs) in Ewing sarcoma and develop a RBPs-based prognostic risk model to predict patient outcomes.

3 MATERIALS AND METHODS

The detailed methods are specified individually in each publication. Here, we provide an overview of the most used methods employed in this thesis.

3.1 Ethical consideration

Informed consent was obtained from all subjects participating in the studies of this thesis. All procedures involving human participants in this thesis were adhered to the ethical standards established by the institutional and/or national research committee (Stockholm region). They also conformed to the principles outlined in the 1964 Helsinki Declaration, including subsequent amendments and equivalent ethical standards. The studies conducted were approved by the Regional Ethics Committee in Stockholm, with document numbers as follows: for Paper I, Dnr: 2013/1979-31/3 and Dnr: 2018/2124-32; for Paper II, Dnr: 2012/1116-31/1 and 2013/1979-31/3; for Paper III, 2013/1979-31/3; and for Paper IV, 2013/1979-31/3.

3.2 Histological staining

3.2.1 Hematoxylin and eosin (H&E) staining

H&E staining is the cornerstone of pathological examination, serving as a universal staining protocol and a primary contrast method for diagnosing biopsy specimens in the field of medicine. Hematoxylin, with its deep blue-purple color, effectively stains nucleic acids through a complex reaction. On the other hand, eosin, appearing pink, nonspecifically stains proteins. Typically, H&E staining results in nuclei taking on a blue hue, while the cytoplasm and extracellular matrix display varying shades of pink staining. In well-fixed cells, intricate intranuclear details become visible. Notably, the staining of nuclei reveals distinctive patterns of heterochromatin condensation specific to cell types and cancer types, making it diagnostically valuable. In this thesis, H&E staining was performed for Paper I and Paper II.

3.2.2 Immunohistochemistry (IHC)

IHC is a technique used to visualize specific proteins within tissue samples. It involves several critical steps. Antigen retrieval is performed to expose target proteins. After blocking nonspecific binding, a primary antibody binds to the target protein, and a secondary antibody with a detectable label is applied. The visualization methods depend on the label used and can include color changes achieved with chromogenic substrates. IHC plays an essential role in the study of protein expression and abundance in biological samples, significantly aiding research and diagnostics. In this thesis, IHC staining was conducted for Paper II, Paper III, and Paper IV.

3.2.3 Opal™ multiplex immunofluorescent (mIF) staining

The Opal™ mIF staining system employs the Tyramide Signal Amplification (TSA) technology, which was developed over two decades ago to catalyze the conversion of TSA molecules into free radicals. These radicals then form covalent bonds with tyrosine residues situated in proximity to the protein epitope recognized by the primary antibody. This innovative technology has been fine-tuned for application in the multispectral mIF platform and is marketed under the Opal trademark. It enables the detection of proteins expressed at low levels, minimizes background tissue autofluorescence, and provides high photostability, allowing for slide storage and re-scanning without significant signal degradation. This system supports up to 8-plex staining (including 9 colors when including DAPI counterstain) and employs strategically chosen fluorophores to ensure effective spectral separation. The amplified detection signals streamline the process of slide scanning by enabling shorter camera exposure times. The staining process involves several sequential steps, which include slide preparation, epitope retrieval, blocking, primary antibodies incubation, introduction of Opal Polymer HRP, signal amplification, antibody stripping, as well as DAPI counterstaining and the mounting of the slides. In this thesis, mIF staining was performed for Paper I, Paper II, and Paper III.

3.3 Image acquisition and spatial analysis

3.3.1 Image acquisition and spectral unmixing

Imaging was performed with the Vectra Polaris scanning system at 20x magnification using the MOTIF scanning setting, followed by spectral unmixing by Inform software (Akoya Biosciences) with an individual reference spectrum. Spectral unmixing is a sophisticated analytical technique used in multispectral fluorescence imaging to deconvolute mixed fluorescent signals by identifying the source materials and their respective proportions. This method is crucial for distinguishing between similar fluorophores and effectively eliminating unwanted background noise and autofluorescence. It is necessary because the emissions of fluorescent light mix linearly, making it challenging to distinguish individual signals in the presence of multiple fluorophores. Inform software employs a linear unmixing process, which is a critical part of spectral unmixing, enables the differentiation of fluorophores with similar emission spectra. Additionally, it effectively removes unwanted signals, such as background noise and autofluorescence, from the fluorophore signal.

3.3.2 Cell segmentation and classification

We employed Qupath software version 0.3.2 (https://qupath.github.io/) for the tasks of cell segmentation and classification. Cell detection was executed utilizing the StarDist method (https://github.com/stardist/stardist) with its default parameters. Subsequently, a single classifier for each channel was set up by manual adjustment according to the training area and a combined classifier was established. The combined classifier was applied to all images and the results were exported into a .txt file for statistical analysis. Cells were categorized into different classes based on marker combinations, guided by biological knowledge.

3.3.3 Definition of a B-index for enrichment and maturity analysis

To quantify B cell density combined with B cell differentiation, a whole-section (WS) B-index and an intratumoral B-index (In-T) were developed with the following formula:

$$\text{WS B-index}_i = \frac{DenWB_i - \min(DenWB_c)}{\max(DenWB_c) - min(DenWB_c)} * 0.5 + \frac{PWmB_i}{\max(PWmB_c)} * 0.5$$

where $DenWB_i$ represents the B cell density within the entire section of each sample; $DenWB_c$ represents the B cell density within the entire section of the whole cohort; $PWmB_i$ indicates the percentage of mature B cell (defined as activated/GC B cell, memory B cell, IgM+ plasma cell and plasmablast/plasma cell) out of all B cell subsets within the entire section of each sample; $PWmB_c$ indicates the percentage of mature B cell (defined as activated/GC B cell, memory B cell, IgM+ plasma cell and plasmablast/plasma cell) out of all B cell subsets within the entire section of the whole cohort.

$$\text{In-T B-index}_i = \frac{DenIB_i - \min(DenIB_c)}{\max(DenIB_c) - min(DenIB_c)} * 0.5 + \frac{PImB_i}{\max(PImB_c)} * 0.5$$

where $DenIB_i$ represents the B cell density within the tumor core of each sample; $DenIB_c$ indicates the B cell represents within the tumor core of the whole cohort; $PImB_i$ indicates the percentage of mature B cell (defined as activated/GC B cell, memory B cell, IgM+ plasma cell and plasmablast/plasma cell) out of all B cell subsets within the tumor core of each sample; $PImB_c$ indicates the percentage of mature B cell (defined as activated/GC B cell, memory B cell, IgM+ plasma cell and plasmablast/plasma cell) out of all B cell subsets within the tumor core of the whole cohort.

3.3.4 B cell desert area analysis

3.3.4.1 Spatial clustering analysis by TissUUmaps

In order to explore the spatial features of B cell distribution beyond B cell abundance, we utilized the TissUUmaps Points2Region toolbox for B cell desert analysis (https://tissuumaps.research.it.uu.se/). This analysis involved a cell phenotype dataset with cell coordinates as input. Based on the frequency of B cells in small local neighborhoods, three region masks were generated as follows: B cell hot area, regions with high B cell infiltration; low B cell area, regions with few B cell infiltration; B cell desert area, regions with very low or no B cell infiltration. A pilot cohort of 20 sample images were used to optimize the parameter settings, afterwards the same setting was applied for the whole cohort. Masks of each region were exported with json files and subsequent area size was calculated in the R software.

3.3.4.2 Distance transform (DT)

DT is a fundamental concept in the field of image processing and computer vision, with applications spanning various domains, such as image registration, template matching, classification, and segmentation. Its primary objective is to calculate the distance from every background point in an image to the nearest object point. In DT analysis, a binary image is considered as a mathematical function which assign a value of either 0 or 1 to each pixel. Pixels with a value of 0 are typically regarded as representing the background, while those with a value of 1 represent the foreground.

In this study, the Euclidean distance transform was computed for a binary image representing a B cell desert area using an algorithm proposed in reference (Maurer, Calvin et al., 2003). This computation involved assigning each pixel in the binary image a value corresponding to the Euclidean distance between that pixel and the nearest nonzero pixel in the binary image. In the context of this study, the nonzero pixels represent areas associated with B cell presence. The result of this computation was a distance map, where pixel values were higher the further they were from any B cell infiltrated regions. To identify and capture extensive desert areas that were distant from B cell infiltrated regions, a thresholding process was applied to the distance map. Specifically, a manually chosen threshold value (t=0.45) was used to distinguish between desert regions with significant separation from B cell infiltrated regions. We calculated and reported the ratio of the combined area after thresholding to the total area of the original desert image.

4 RESULTS AND DISCUSSION

4.1 Investigating Spatial Ecosystems of B Cells and Lymphocyte Aggregates in Undifferentiated Pleomorphic Sarcoma (Paper I)

In this study, we centered on investigating the composition and variability of B cells within UPS. Our inquiry involved analyzing the intricate spatial patterns of B cells within the TIME, understanding the contributions of LAs to B cell maturation and differentiation, and assessing the significance of tumor-associated LAs and B cell profile in predicting clinical outcomes.

To gain a comprehensive understanding of the immune cell landscape, we utilized a seven-color mIF staining panel, including CD20, CD27, CD38, CD138, IgM, CD163, and DAPI, to identify various B cell subsets, CD27+/CD38+ cells (representing major subsets of non-B activated immune cells), and M2-like macrophages. The immune cell profiling revealed a TME dominated by M2-like macrophages and heterogenous B cell fractions. B cells displayed varying maturation profiles with immature B cells representing the most prevalent subset, followed by activated/GC B cells, memory B cells, IgM+ plasma cells, and plasmablasts/plasma cells.

We defined LAs as the clustering of more than 200 infiltrating lymphocytes in tumor tissue stained by H&E. The presence of LAs emerged as a significant prognostic factor in our UPS cohort, associated with better OS and MFS.

Our findings revealed that LA-positive tumors had significantly higher fractions of B cells and CD27+/CD38+ cells (non-B). In contrast, they exhibited significantly lower fractions of M2-like macrophages. This trend was consistent when examining cell density, as LA-positive tumors also demonstrated higher B cell and CD27+/CD38+ cell densities. Notably, the enrichment of activated immune cells was not confined solely in the area of LAs; it extended to the tumor mass and invasive margin outside the LA structures in LA-positive tumors. This indicated that LAs were associated with increased immune cell infiltration not only within the LAs but also throughout the tumor mass and in the vicinity of the invasive margins.

Furthermore, LA-positive tumors presented a more mature (well-differentiated) B cell profile across all regions. In the entire tumor section, LA-positive tumors had significantly lower fractions of immature B cells and higher fractions of mature B cells (including activated/GC B cells, memory B cells, IgM+ plasma cells, and plasmablasts/plasma cells). However, despite the lower fraction of immature B cells, LA-positive tumors displayed significantly higher densities of both immature and mature B cells throughout the entire section. This trend of decreased immature B cells and increased mature B cells was consistent across all regions.

To comprehensively describe the B cell profile in the TME, we developed the B-index allowing a combined assessment of B cell abundance and maturity. Patients with a high B-index exhibited better OS and MFS. IgG-labeled cells were next investigated to assess the amount of tumor-directed antibodies. Tumors with high B-index presented significantly

higher percentages of IgG-labeled cells. Since IgG production is primarily attributed to plasma cells, we also explored the relationship between in-situ plasma cell infiltration and bound IgG. Tumors with high intratumoral plasma cell density exhibited significantly higher percentages of IgG-bound cells. However, no significant differences in the percentage of intratumoral IgG-labeled cells between LA-positive and LA-negative cases were observed. These results suggested that although LA was associated with increased B cell abundance and maturity, immature LAs could not support intratumoral plasma cell development. In contrast, a higher intratumoral plasma cell density and B-index, indicating a more abundant and mature B cell profile, were essential for sustaining the presence of tumor-bound IgG. This result implied that immature or functionally limited LAs may fail to generate tumor-specific plasma cells. The maturity of TLSs could significantly impact these effector responses, warranting further investigation to assess the maturity or functional status of LAs for a more comprehensive understanding of their anti-tumor activities.

In order to explore the spatial features of B cell distribution beyond abundance, we utilized the TissUUmaps Points2Region toolbox to conduct B cell desert analysis. Based on B cell frequency in small local neighborhoods, three region masks were generated as follows: (1) B cell hot area, regions with high B cell infiltration; (2) Low B cell area, regions with few B cell infiltration; (3) B cell desert area, regions with very low or no B cell infiltration. The allocation of regions was based on a relative density-dependent method rather than an absolute density-dependent approach. Our analysis of B cell distribution patterns revealed that LA-positive tumors contain smaller and more fragmented B cell desert areas. Essentially, LAs appeared to regulate B cell infiltration patterns, facilitating B cells spreading within the tumor mass.

In summary, our study provides a comprehensive examination of the B cell spatial profile within the UPS TME. A comprehensive assessment of the B cell profile holds the potential to stratify prognosis in UPS. The identification of distinct B cell subsets, their association with LAs, and their potential impact on B cell infiltration patterns enhance our understanding of the complex immune landscape within UPS. These findings highlight the therapeutic potential of B cells in STS immunotherapy, paving the way for tailored interventions. Furthermore, delving into the detailed mechanisms governing LA formation and B cell maturation holds promise for targeted therapies aimed at enhancing their presence and function.

4.2 Prognostic Significance of Direct Spatial Interactions Between CD11c+ Antigen-Presenting Cells and CD8+ Cells in Soft Tissue Sarcoma (Paper II)

The potential role of professional APCs in STS remains relatively unexplored. These APCs, including dendritic cells and macrophages, can present external antigens to CD8+ T cells, a process known as cross presentation. Our study investigated the hypothesis that the direct cell-cell interactions between CD11c+ cells and CD8+ T cells within the primary tumor, is associated with a dynamic anti-tumor microenvironment and a more favorable prognosis.

We assessed CD11c and CD8 expression in whole tissue sections from an STS cohort with patients treated at Karolinska University Hospital. Clinicopathological correlations showed that the sole expression of CD11c or CD8 was not associated with patient MFS or OS. However, the presence of direct cell-cell interactions between CD11c+ and CD8+ cells associated with improved MFS and OS. This finding aligns with the hypothesis that a specific spatial distribution of APCs and CD8+ T cells indicated a beneficial TIME.

CD11c is expressed not only by conventional dendritic cells but also by pro-inflammatory macrophages, which are also capable of cross-presenting antigens to CD8+ T cells in tumors. Further characterization identified a subset of CD11c+ cells as macrophages, confirmed by markers of CD68 and CD163. CD8+ cells were characterized as T cells through flow cytometry analysis.

CD11c-CD8 interactions remained statistically significant for improved MFS and OS within the heterogeneous Karolinska cohort, in the multivariable cox-regression analysis including established prognostic factors such tumor size and grade while adjusting for age and sex. This suggested that CD11c-CD8 interactions served as an independent biomarker with prognostic value beyond existing markers.

We also performed a comprehensive investigation of intratumoral cell-cell interactions between CD11c+ APCs and CD8+ T cells in an extended liposarcoma cohort. These interactions were more common in tumors with TA-TLSs. Interestingly, smaller tumors more frequently exhibited mature TA-TLSs featuring germinal centers. However, only a limited number of patients had TA-TLSs, indicating that mature TA-TLSs were less common compared to CD11c-CD8 interactions. Importantly, the presence of CD11c-CD8 interactions was not significantly influenced by tumor size.

Tregs, marked by Foxp3 expression, have been known to suppress effector T cell regarding the anti-tumor responses. To investigate whether these cells might interfere with CD11c+ APC function, we examined and quantified Foxp3+ cell distribution in four liposarcoma cases. Our analysis revealed that Foxp3+ cells were predominantly located within the intra-TLS regions, though they could also be sparsely distributed in tumors lacking mature LAs. This observation aligns with the previous literature suggesting that Foxp3+ Tregs are present where immune regulation is required.

In our validation analysis of gene expression data from the TCGA sarcoma cohort, we uncovered strong associations between the ITGAX gene, responsible for encoding the CD11c protein, and classical MHC class II molecules such as HLA-DRA, HLA-DPB1, and HLA-DPA1. Additionally, we detected correlations between ITGAX and critical molecules like CD86, CD48, and CD5, all of which play essential roles in the activation and differentiation of immune cells. These findings offerred compelling evidence for the involvement of CD11c+ cells in processes related to antigen presentation and T cell activation.

In a subgroup analysis of leiomyosarcoma in the TCGA cohort, we found that the $ITGAX^{high}CD8A^{high}$ gene signature remained prognostic. Intriguingly, when we factored in FOXP3 levels, the $ITGAX^{high}CD8A^{high}$ gene signature still maintained its prognostic value in a tumor microenvironment with high FOXP3 levels. This suggested that while Foxp3+ cells may temper lymphocyte function, they did not entirely extinguish the anti-tumor activities stemming from CD11c-CD8 interactions. Furthermore, the $ITGAX^{high}CD8A^{high}$ gene signature remained a significant prognostic factor even in a tumor microenvironment rich in CD274 (PD-L1). The results demonstrated that the prognostic value of $ITGAX^{high}CD8A^{high}$ remained intact, seemingly undeterred by the presence of PD-L1.

In summary, our work introduces a novel biomarker for immune surveillance, where the presence of spatial cross-presentation at tissue-level resolution is strongly linked to better OS in STS.

4.3 Unveiling Pertinent Subtypes of Synovial Sarcoma through Transcriptomic and Genomic Profiling (Paper III)

Molecular profiling holds the potential to distinguish distinct biological subgroups within SS and offer invaluable insights for prognosis and personalized treatment. To this end, we analyzed SS by both whole transcriptome sequencing (WTS) and target gene DNA-seq, with validation by mIF in 55 SS patients.

The SS18-SSX fusion was identified in nearly all RNA-sequenced samples, with only three exceptions. We extensively characterized the SS18-SSX fusion variants and observed the typical variants, with SS18-SSX1 and SS18-SSX2 being the most prevalent, while SS18-SSX4 was detected in only seven samples. Patient outcomes were similar for SSX1 and SSX2 fusions in terms of OS and MFS. Secondary fusion events were rare and lacked clinical significance, supporting the primary role of the SS18-SSX fusion in tumorigenesis.

We classified SS into three clusters (SSC-I, II, and III) based on their transcriptomic characteristics. This stratification revealed distinct differences in oncogenic activity and prognosis. SSC-I, characterized by heightened core oncogenic program upregulation, was associated with metastasis and poor survival. In contrast, SSC-II, the least aggressive group, exhibited lower mortality and metastasis rates, primarily consisting of monophasic cases. SSC-III, predominantly comprising biphasic tumors, showed a favorable response to neoadjuvant treatment but often led to later metastasis.

Genomic analysis unveiled the distinct genetic landscape of these clusters, with SSC-III demonstrating the highest levels of CNAs and TMB. Somatic copy number alterations (SCNAs) analysis highlighted a higher frequency of amplifications and deletions in SSC-III.

Single-cell RNA-seq analysis from an external dataset showed that most SS patients lacked T cell infiltration. An SS-specific gene signature for TME revealed varying cell fractions among the three subtypes. SSC-II exhibited the highest proportions of mesenchymal and endothelial cells, SSC-III had the greatest fractions of epithelial cells, and SSC-I featured elevated fractions of cycling mesenchymal cells, with tumor cells predominating.

To gain insights into potential factors influencing treatment responses and to delve into the biology of SSC-III, characterized by low proliferation and immune suppression profile, we undertook a comprehensive analysis of genome-wide differences in chromosome arm-level copy-number events. By comparing SSC-III to SSC-II, we identified two significantly up-regulated genes, PD1 and TMPRSS2. Further examination of their prognostic impacts showed that in SSC-III, patients with PD1 or TMPRSS2 amplification had better outcomes. Patients with high amplification status of both PD1 and TMPRSS2 exhibited the highest predictive value for metastasis or death.

Subsequent mIF staining in selected patients confirmed these findings, showing low immune cell infiltration and significant PD-1 expression in tumor cells, while TMPRSS2 expression was predominantly observed in epithelial cells. Amplification of TMPRSS2 was associated

with early estrogen response and a decrease in MTORC1 and KRAS signaling. This suggested that both PD1 and TMPRSS2 amplification may serve as positive prognostic indicators for patients with biphasic tumors.

Our findings propose a model of molecular subtyping in SS, leading us to the following conclusions for each subtype: SSC-I is characterized by hyperproliferation, immune evasion, and an unfavorable prognosis. SSC-II is marked by the predominance of vascular-stromal components, which are associated with better outcomes. SSC-III exhibits biphasic differentiation, genomic complexity, and immune suppression mediated by checkpoints. These insights have the potential to foster the development of innovative therapeutic strategies and deepen our comprehension of SS.

4.4 Development and Validation of an RNA-Binding-Protein-Based Prognostic Model for Ewing Sarcoma (Paper IV)

We conducted a comprehensive analysis of transcriptomic signatures from previously published cohorts of 155 ES patients, to investigate the role of RNA-binding proteins (RBPs) in prognosis and identify transcriptional subtypes. The data were processed to remove batch effects, and overlapping genes across multiple datasets were identified.

We identified 22 differentially expressed RBPs (DERBPs) by comparing gene expression between deceased and surviving patients in the training cohort. Based on these DERBPs, we classified the ES patients into three distinct subtypes (RS1, RS2, and RS3) using consensus k-means clustering, each with varying prognostic outcomes. Notably, patients in Cluster 1 exhibited the best prognosis, while those in Cluster 3 had the worst.

Further analysis of 1542 quantified RBPs revealed significant differences in expression among these subtypes. A total of 90 up-regulated and 125 down-regulated DERBPs were identified, forming the basis for subsequent functional enrichment analysis, which indicated that these DERBPs were associated with RNA and protein metabolism processes.

A protein-protein interaction (PPI) network was constructed to identify key components, and hub genes within the network were identified. The prognostic relevance of 171 RBPs from the PPI network was assessed, leading to the identification of 29 prognostic-associated hub RBP genes.

Finally, a prognostic risk model (RPRM) was developed based on the identified RBPs, exhibiting strong prognostic performance in predicting OS. High-risk patients had significantly worse survival outcomes. Furthermore, we examined the relationship between RPRM risk scores and hallmark gene sets, identifying specific gene sets associated with better or worse prognosis. Notably, the reactive oxygen species pathway and glycolysis were strongly correlated with risk scores.

Six key RBPs (DCP18, DDX23, GRATCH8, NSUN7, RPL6, and ZCCHC6) were found to be significantly associated with OS in the training cohort, providing further insights into the prognostic relevance of these genes. NSUN7, in particular, was significantly correlated with survival status. Immunoreactivity of NSUN7 was assessed based on IHC staining and found to be associated with OS. NSUN7-negative cases had a shorter OS compared to positive ones, with implications for treatment response.

In summary, our study provides valuable insights into the role of RBPs in ES prognosis, identifies transcriptional subtypes, and offers a prognostic risk model that can be applied to predict patient outcomes. The study also highlights the significance of specific RBPs, particularly NSUN7, in ES prognosis and treatment response.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis represents a comprehensive and in-depth exploration of the sarcoma TME through advanced in situ spatial analysis, coupled with an investigation into the molecular and genetic characteristics of sarcomas using transcriptomic and genomic sequencing techniques.

In **paper I**, we utilized advanced mIF staining and spatial analysis to identify specific B cell profiles in UPS. The results revealed that LAs have a significant impact on B-cell maturation, activity, and distribution within the tumor mass, ultimately leading to a more functional immune microenvironment. Several clinical trials have suggested that the presence of TLS in STS predicts a more favorable response to ICI therapies (Petitprez, de Reynies et al. 2020, Italiano, Bessede et al. 2022). Here, we are the first to report that LAs, which represent a loosely-defined form of TLS at various maturation stages, can also stratify patient survival in the context of conventional chemotherapy.

The lack of a precise tool for stratifying patient survival remains a significant challenge in the clinical management of STS. A recent successful effort in this regard is the development of Sarculator, a predictive tool that uses a nomogram to estimate the risk of survival for patients with STS undergoing standard treatment (Callegaro, Miceli et al. 2016). Sarculator is a robust predictor of OS and has been well-validated in large external patient populations (Voss, Callegaro et al. 2022). It incorporates patient features such as age, tumor size, histology, grade, depth, and anatomic location. However, the lack of TIME features in this model limits its ability to provide a comprehensive evaluation of the overall tumor characteristics. Therefore, the combination of LA scoring and Sarculator holds great potential for improving the differentiation of patient prognosis. Additionally, LA evaluation is relatively straightforward and requires only H&E staining. We anticipate that the increased application of LA in the diagnosis and prognosis prediction of STS will contribute significantly to clinical decision-making.

Previous studies on quantifying B cells within the TME often relied on gene expression levels or cell counts derived from IHC staining. However, there has been a lack of attempts to integrate both the abundance and maturation profile of B cells to create a comprehensive B cell maturation map. In **paper I**, we introduced the concept of a B-index, a novel idea in tumor research. Importantly, solely measuring the density or fraction of B cell subsets was insufficient for effective prognostic stratification. The innovative B-index demonstrated robust potential for prognostic stratification, prompting a shift towards a more comprehensive examination of TME components. This approach goes beyond the conventional method of assessing only the density of specific cell types and incorporates an evaluation of their maturity profiles, which inspires us to explore TME components in a more holistic manner. Nonetheless, the reliability and validity of the B-index require further validation. Moreover, the optimal balance between the weightings of B cell abundance and maturation level remains to be explored for defining a B cell profile with robust anti-tumor activity.

It has been suggested that TLS creates a conducive niche for antigen presentation, lymphocyte maturation and proliferation in the TME. This process leads to the development of effector memory T and B cells, and plasma cells (Sautes-Fridman, Petitprez et al. 2019). However, limited evidence exists regarding how TLS influences the spatial distribution of B cells in the TME. Inspired by the immune-excluded (I-E) TME, characterized by the exclusion of T cells, we introduce the concept of measuring a "B cell desert," representing areas with extremely low B cell infiltration. B cells can serve as APCs and modulate immune responses through direct cell-to-cell interactions, especially when located near T cells and other immune components. B cell desert regions, especially with extensive and continuous areas, may hinder such interactions may impede these interactions, potentially leading to altered immune activation, suppression, or dysregulation within specific tumor regions. Therefore, we delve into studying B cell deserts in two dimensions, examining their size and fragmentation level. Importantly, this study marks the first attempt to establish a visualized and multidimensional approach to evaluate the spatial distribution of B cells in TME. This discovery opens new avenues for exploring the spatial organization of other immune cells within the TME and their impacts on anti-tumor immunity.

As suggested in **paper II**, cell-cell interactions between APCs and effector T cells are likely to promote anti-tumor immunity. Conversely, direct interactions between immunosuppressive cells and effector cells might also create an immunosuppressive TME, diminishing the anti-tumor effect. We can further investigate how the direct interaction between M2-like macrophages and B cells alters B cell activity. To provide a comprehensive and detailed depiction of the UPS TME, combining spatial clustering analysis and cell-cell interaction analysis contributes to understanding how the spatial organization of immune cells influences anti-tumor immunity. The innovative spatial analysis methods in **paper I** can also be applied to study other immune cell types in various tumor settings.

While the mIF staining technique robustly assesses immune cell subsets, the incorporation of exploratory methodologies enabling the investigation of additional cell types within the TME can significantly enhance our understanding of the spatial composition of TIME. Advanced spatial omics methods, such as spatial transcriptome sequencing and imaging mass cytometry (IMC), have the potential to provide a more comprehensive and detailed depiction of the UPS TME. In addition to spatial analysis, we conducted transcriptomic and genomic studies to explore sarcoma tumor biology and mechanisms of tumorigenesis. In **paper IV**, we observed that the RPRM risk score showed a significant association with the cell cycle and the PI3K/mTOR pathway. Interestingly, in **paper III**, we observed that SSC-I patients exhibited a high enrichment in proliferation and cell cycle-related pathways, suggesting a potential response to CDK4/6 inhibitors. So far, the clinical evaluation for the administration of CDK4/6 inhibitors in the treatment of these patients has not been reported (Hsu, Seligson et al. 2022). It would be valuable to conduct clinical trials to assess the administration of CDK4/6 inhibitors, either in a single drug or in combination with PI3K/mTOR inhibitors, in this patient population.

In **Paper III** and **Paper IV**, we have developed prognostic prediction tools and subtyping clustering based on transcriptomic data. However, the challenge lies in translating these findings into clinical applications. It is impractical to sequence every clinical sample due to cost-efficiency considerations. In **paper III**, SSC-III was characterized with an immunosuppressive profile indicating a potential to respond to ICI treatment. Interestingly, we have observed that SSC-III is predominantly characterized by a biphasic phenotype and exhibiting the best response to neoadjuvant therapy. Traditionally, patients undergoing neoadjuvant chemotherapy are frequently presumed to have more aggressive tumors during preoperative assessment. This raises a significant question: Can we accurately evaluate the TME solely through histological observations? We intend to explore this hypothesis by examining the connections between epithelial cells, mesenchymal cells, and immune cells at single cell level omics studies. If our hypothesis is substantiated, it could open the door to including biphasic patients in clinical trials involving ICIs, potentially enhancing the chances to reach favorable response.

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