THE IMMUNE MICROENVIRONMENT OF SARCOMAS: A COMPREHENSIVE EVALUATION OF INFILTRATING IMMUNE CELLS AND CHECKPOINT BIOMARKERS IN MUSCULOSKELETAL TUMORS

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Amanda R. Dancsok

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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Examining Committee:

Torsten Nielsen
Supervisor

Peter Watson
Supervisory Committee Member

Andrew Weng
University Examiner

James Lim
University Examiner

Additional Supervisory Committee Members:

Tony Ng
Supervisory Committee Member

Michael Underhill
Supervisory Committee Member

Abstract

Sarcomas are aggressive cancers of the connective tissues, such as bone, muscle, cartilage, and fat. Despite their diverse origins, sarcomas are predominantly treated by surgery and radiation, as conventional chemotherapy has limited benefit for most subtypes. When sarcomas recur or metastasize, there are few options for systemic therapy, and prognosis is very poor. Despite advancements in our understanding of the molecular drivers of sarcomas, almost no new treatments have proven benefit for metastatic sarcomas. Immunotherapy has shown value for other cancers, such as melanoma and lung cancer; however, sarcomas lag behind the common cancers in our understanding of their immune microenvironment and potential for treatment with immunotherapeutics. Early trials using single-agent immune checkpoint inhibitors in sarcomas delivered mixed results, but these studies somewhat indiscriminately lumped together different sarcoma subtypes that might have critical immunological differences. My study employs tissue microarrays incorporating 1360 sarcoma specimens (spanning 23 subtypes) to characterize immune infiltrates and expression of targetable immune biomarkers, using immunohistochemistry. Genomically-complex sarcoma types – driven by mutations and/or copy-number alterations – are found to have much higher levels of lymphocytic and phagocytic immune infiltrates than translocation-associated sarcomas. Across nearly all subtypes, tumor-associated macrophages outnumber tumor-infiltrating lymphocytes, predominately M2 (anti-inflammatory) macrophages. Expression of the target of first-generation immune checkpoint PD-(L)1 is uniformly low, but expression of LAG-3 and TIM-3 – emerging immune checkpoints – is significantly more common. Expression of anti-phagocytic immune checkpoint CD47 is yet more predominant, displaying all-or-nothing expression with 100% positivity seen in over half of positive cases. To further characterize the lymphocytic response, T-cell receptor (TCR)

sequencing was performed on specimens from 25 sarcoma patients on a clinical trial of tremelimumab (anti-CTLA-4) with durvalumab (anti-PD-L1). We found that the TCR repertoire is richer and more diverse among the genomically-complex sarcomas relative to the translocation-associated sarcomas, and following immune checkpoint blockade, we observed an overall increase in the clonality of the peripheral TCR repertoire. My study demonstrates a tangible positive relationship between genomic complexity and immunogenicity, and highlights novel immune checkpoints of relevance to sarcomas. As such, this work provides the essential translational background to direct the use of immunotherapy in sarcoma management.

Lay Summary

Sarcomas are aggressive cancers of bone and soft tissues that are especially common in children and adolescents. Chemotherapy is usually ineffective for sarcomas, so new treatments are desperately needed. The human immune system is designed to fight tumors, so when cancer develops, it is hiding from or fighting back against this immune attack. *Immunotherapy* is a type of cancer treatment that reactivates anti-cancer immunity. The type of immunotherapy needed depends on how the cancer has stifled the immune system. For this study, I looked at a large number of patient tumors to find out what type of immune response happens for the different sarcoma types. I found that the sarcoma types with more DNA damage are more likely to trigger a response from the immune system. This data will be used to help choose which sarcoma subtypes should be enrolled in future clinical trials for immunotherapy.

Preface

Chapters 2 and 3 are based on work conducted at the UBC Genetic Pathology Evaluation Centre (GPEC) as part of an international consortium "ImmunoSarc." The research program was identified by the ImmunoSarc Aim 1 principal investigators Dr. Torsten Nielsen and Dr. Elizabeth Demicco. My personal contributions to these chapters include the experimental design of the tissue microarray/immunohistochemistry experiments, antibody selection, optimization of each antibody's specific experimental parameters through control tissues, and performance of immunohistochemical staining on tissue microarrays. I performed all data calculations, normalization, and statistical analyses using SPSS Software (Kruskal-Wallis nonparametric comparison of distributions, 1-way ANOVA, Cox linear multiple regression, Kaplan-Meyer plots, multivariate regression analysis), matched data to clinical outcomes data (including quality assurance), generated and designed all tables, figures, and graphics, and wrote the manuscripts/chapter text. Histological scoring was performed by Dr. Nokitaka Setsu, Dr. Dongxia Gao, and Dr. Elizabeth Demicco. Clinical data was compiled from hospital records by Dr. Karama Asleh-Aburaya.

Chapter 4 is based on a clinical trial conducted by NHMRC Clinical Trials Centre (Sydney, NSW, Australia) and work conducted at the Princess Margaret Cancer Centre (Toronto, ON, Canada) as part of the ImmunoSarc international consortium. The research program was identified by Dr. Torsten Nielsen, Dr. Elizabeth Demicco, Dr. David Thomas, and Amanda Dancsok. My contributions were analysis of T-cell receptor repertoire data using SPSS software, generation and design of all tables and figures, and the writing of the manuscript/chapter text. Dr. Neal Poulin assisted with data analysis through R statistical software programming for generation of diversity and Shannon's entropy metrics for each sample. Clinical trial design and

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A version of the following sections of Chapter 1 have been published: "LAG-3," "TIM-3," and "CD47/SIRPα." Samantha Burugu, **Amanda R. Dancsok,** Torsten O. Nielsen. "Emerging targets in cancer immunotherapy." *Seminars in Cancer Biology* (2017);52(Pt 2):39-52. I researched and wrote these excerpts independently in addition to other manuscript sections, while Dr. Samantha Burugu wrote and researched other components of the manuscript. Dr. Torsten Nielsen was the primary advisor and provided advice on content and formatting, as well as editing of the final manuscript.

A version of Chapter 2 has been published: **Amanda R. Dancsok,** Nokitaka Setsu, Dongxia Gao, Jean-Yves Blay, David Thomas, Robert G. Maki, Torsten O. Nielsen, Elizabeth G. Demicco. "Expression of lymphocyte immunoregulatory biomarkers in bone and soft-tissue sarcomas." *Modern Pathology;* 1 Jul 2019. doi: 10.1038/s41379-019-0312-y. As stated above, my contributions to this manuscript were: the experimental design and optimization, technical laboratory performance, data analysis, figure design, and the writing of the manuscript. Histological scoring was performed by Dr. Nokitaka Setsu, Dr. Dongxia Gao, and Dr. Elizabeth Demicco. Dr. Torsten Nielsen and Dr. Elizabeth Demicco were the primary advisors. Authors Dr. Robert Maki, Dr. David Thomas, and Dr. Jean-Yves Blay provided advice and assistance with the development of the study design and troubleshooting and provided editing on the final manuscript.

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Human tissue accessions for these studies were reviewed and approved by the British Columbia Cancer Agency research ethics board (H17-01759).

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List of Abbreviations

ANOVA: analysis of variance

APC: antigen-presenting cell

B-ALL: B-cell acute lymphoblastic leukemia

Breg: regulatory B cell

BTLA: B And T Lymphocyte Associated

CAR-T cell: chimeric antigen receptor T cell

CCL: chemokine ligand

CCR: chemokine receptor

CT Antigen: cancer testis antigen

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DAB: 3,3'-diaminobenzidine

DAMP: damage-associated molecular pattern

DFSP: dermatofibrosarcoma protuberans

DNA: deoxyribonucleic acid

DSRCT: desmoplastic small round cell tumor

FOXP3: forkhead box P3

FNCLCC: Fédération Nationale des Centres de Lutte Contre le Cancer

GIST: gastrointestinal stromal tumor

GITR: glucocorticoid-induced TNFR family related gene

HDAC: histone deacetylase

HR: hazard ratio

HRD: homologous recombination deficiency

HER-2: human epidermal growth factor receptor 2

IBM: International Business Machines Corporation

ICOS: inducible T-cell costimulatory

IDO-1: Indoleamine-pyrrole 2,3-dioxygenase 1

IFN: interferon

IgG: immunoglobulin G

IgM: immunoglobulin M

IL: interleukin

Indel: insertion/deletion mutation

LAG-3: lymphocyte-activation gene 3

M0: macrophage type 0 (uncommitted)

M1: macrophage type 1 (classically-activated)

M2: macrophage type 2 (alternatively-activated)

mAb: monoclonal antibody

MDM2: mouse double minute 2 homolog

MHC: major histocompatibility complex

MMR: mismatch repair

MPNST: malignant peripheral nerve sheath tumor

MSH: Mount Sinai Hospital (New York)

mTOR: mammalian target of rapamycin

NF-1: neurofibromatosis type 1

NK: natural killer

NS: non-significant

NY-ESO-1: New York esophageal squamous cell carcinoma 1

OS: overall survival

OSa: osteosarcoma

PD-1: programmed-death-1

PD-L1: programmed-death-ligand-1

PFS: progression-free survival

Q1: quartile 1

Q3: quartile 3

RB: retinoblastoma gene

RNA: ribonucleic acid

SARC: Sarcoma Alliance for Research through Collaboration

SFT: solitary fibrous tumor

SHP-1/2: Src-homology-2 domain-containing protein tyrosine phosphatase 1/2

SIRPα: signal-regulatory protein alpha

SNV: single nucleotide variant

SPSS: Statistical Package for Social Sciences

SS: synovial sarcoma

TAM: tumor-associated macrophage

Tc: cytotoxic T cell

TCGA: The Cancer Genome Atlas

TCR: t-cell receptor

Th: helper T cell

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TIL: tumor-infiltrating lymphocyte

TIM-3: t-cell immunoglobulin and mucin-domain-containing-3

TMA: tissue microarray

TNF: tumor necrosis factor

TNM: tumor, nodes, metastasis

Treg: regulatory T cell

UBC: University of British Columbia

UPS: undifferentiated pleomorphic sarcoma

VISTA: V-domain Ig suppressor of T cell activation

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To my patients

May the lessons I learned in the challenges I faced to arrive here help me to care for you with insight, wisdom, and most of all, compassion.

Chapter 1: Introduction

1.1 Bone and Soft-Tissue Sarcomas

1.1.1 Etiology and Epidemiology of Sarcomas

"Sarcoma," from Greek *sark* ("flesh") and *oma* ("tumor"), describes a highly heterogeneous category of cancers, encompassing all solid malignant tumors of bone or soft tissues. The World Health Organization defines over 50 sarcoma types – genetically and pathologically distinct – originating from diverse tissues, including bone, cartilage, fat, fibrous tissues, nerve sheath, vasculature, and smooth and skeletal muscle.[1] By definition, sarcomas arise from cells of mesenchymal origin, and while no common cell-of-origin has yet been established, it is hypothesized that they occur as a result of transformation of multipotent mesenchymal stem cells.[2-5]

Some sarcomas are associated with rare genetic syndromes based on germline mutations in genes such as *TP53* (Li-Fraumeni syndrome),[6,7] *RB* (hereditary retinoblastoma),[8] and *NF-1* (neurofibromatosis type 1).[9] However, familial syndromes account for only 2-4% of all sarcomas.[10,11] The majority of sarcomas occur sporadically, due to spontaneous genomic alterations, including point mutations, copy-number alterations, simple DNA translocations, and complex chromosomal rearrangements. The exact etiology of sarcomas is usually unclear or unknown, but carcinogens, ionizing radiation, and viral infection can all be implicated.[12] Sarcomas are rare, accounting for less than 1% of all cancer diagnoses.[13,14] According to Statistics Canada's Canadian Cancer Registry, the incidence of soft-tissue sarcomas is approximately 3.7/100,000, and the incidence of bone/joint cancers is 0.9/100,000;[15] however, the actual incidence may have been underestimated here, as some visceral sarcomas were likely counted with their associated organs, rather than with soft-tissue sarcomas.

Sarcomas can occur from infancy through old age, but they are sometimes considered to be cancers of childhood and adolescence, as they disproportionately affect a young population, representing 20% of pediatric solid cancers.[13] The typical age of presentation varies widely with the spectrum of sarcoma subtypes. Some subtypes, such as rhabdomyosarcoma, typically arise in children, while others, such as synovial sarcoma and Ewing sarcoma, often present in adolescents and young adults.[16] Contrastingly, some subtypes, such as leiomyosarcoma and dedifferentiated liposarcoma, tend to present in middle-aged or elderly adults.[16]

1.1.2 Molecular Alterations and Classification of Sarcomas

Unlike most carcinomas, which are broadly categorized by site or organ of origin, sarcomas are classified according to characteristic pathological features and defined molecular alterations. Generally-speaking, sarcoma subtypes are commonly divided into two major categories: 1) those with relatively simple, near-diploid karyotypes on a comparatively quiet genomic background, and 2) those with complex karyotypes and severe genomic alteration and instability. [17-19] While some subtypes can be easily categorized as either karyotypically simple or complex, others are much more controversial, as our knowledge surrounding their molecular origins and genomic modifications continues to evolve.

Among the "karyotypically-simple" sarcomas, some subtypes are characterized by disease-specific chromosomal translocations that give rise to known fusion proteins. These subtypes are defined – and often diagnosed – by the presence of their respective translocations. Consequently, they are commonly grouped into a broader category referred to as "translocation-associated sarcomas." The common translocation-associated sarcomas discussed in the chapters of this thesis are outlined in Table 1.1. Translocation-associated sarcomas as a group tend to have little-to-no background genetic disruption aside from their initiating chromosomal

translocations.[20-24] The comparatively low degree of aneuploidy in these tumors is explained by the fact that most of the translocations involve genes encoding transcription factors or epigenetic modulators,[20,25,26] leading to a cascade of transcriptional dysregulation and uncontrolled growth.

Subtype	Translocations	Fusion gene
Fusion Transcriptional Modulators		
Alveolar rhabdomyosarcoma[27-29]	t(2:13)(q35:q14) t(1:13)(p36:q14)	*PAX3-FOXO1A* *PAX7-FOXO1A*
Alveolar soft part sarcoma[30]	der(17)t(X;17)(p11;q25)	*ASPL-TFE3*
Clear cell sarcoma[31,32]	t(12;22)(q13;q12) t(2;22)(q34;q12)	*EWSR1-ATF1* *EWSR1-CREB1*
Ewing sarcoma[33-37]	t(11:22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q21;q12) t(2;22)(q33;q12) t(16;21)(p11;q22)	*EWSR1-FLI1* *EWSR1-ERG* *EWSR1-ETV1* *EWSR1-ETV4* *EWSR1-FEV* *FUS-ERG*
Low-grade fibromyxoid sarcoma[38,39]	t(7;16)(q32-34;p11) t(11;16)(p11;p11)	*FUS-CREB3L2* *FUS-CREB3L1*
Myxoid liposarcoma[40-42]	t(12:16)(q13;p11) t(12;22)(q13;q12)	*FUS-DDIT3* *EWSR1-DDIT3*
Synovial sarcoma[43-46]	t(X;18)(p11:q11)	*SS18-SSX1/2/4*
Solitary fibrous tumor[47,48]	inv(12)(q13)	*NAB2-STAT6*
Fusion Growth Factors		
Dermatofibrosarcoma protuberans[49-51]	t(17:22)(q22:q13)	*COL1A1-PDGFB*

Table 1.1 Chromosomal translocations and fusion genes of some common translocation-associated sarcomas (non-exhaustive).

Alongside the translocation-associated sarcomas in the "karyotypically-simple" category is a group of subtypes with specific recurrent oncogenic mutations, deletions, or amplifications. They are often more aneuploid than translocation-associated sarcomas, but are associated with a recurrent genetic alteration and have a less unstable genome than the "karyotypically-complex" sarcomas discussed below. The common "karyotypically-simple," non-translocation sarcomas discussed in the chapters of this thesis are described in Table 1.2. The oncogenic mechanisms within this category vary by the specific genetic modifications, but generally relate to physical/functional loss of a tumor-suppressor gene or gain of function of an oncogene.

Subtype	Molecular alteration
Chordoma[52-54]	Copy-number losses leading to deletion of tumor-suppressor genes *CDKN2A* and/or *PTEN*
Embryonal rhabdomyosarcoma[55-60]	Loss of heterozygosity on a region of ch11 that contains *IGF2* and potential tumor-suppressor genes *H19* and *p57kip2*
Epithelioid sarcoma[61-65]	Loss of SMARCB1 (INI1) protein by *SMARCB1* gene deletion/mutation or other mechanisms
Gastrointestinal stromal tumor[66-69]	Gain-of-function mutations of tyrosine kinase receptor genes c-*KIT* and *PDGFRA*
Well-differentiated liposarcoma[70-72]	Amplification of ch12q13-15, containing oncogenes *MDM2, HMGA2,* and *CDK4*

Table 1.2 Recurrent molecular alterations underlying some common karyotypically-simple, non-translocation sarcomas.

Contrasting these, the "karyotypically-complex" sarcomas are a group of subtypes known to have multiple complex genomic abnormalities, without the recurrent or characteristic alterations seen among the "karyotypically-simple" subtypes. These sarcomas are characterized by complex structural changes involving multiple chromosomal rearrangements, duplications, and deletions – often resulting in supernumerary marker chromosomes formed from various chromosomes – and as such, are considered to be primarily copy-number-driven.[19,21,73,74] Aneuploid tumors represent about half of sarcoma diagnoses and are frequently characterized by a high-grade spindle or pleomorphic morphology.[75] The common "karyotypically-complex" sarcomas discussed in the chapters of this thesis (and any related precursor lesions) are listed in Table 1.3.

Subtype	Line of differentiation
Angiosarcoma	Vascular mesenchyme
Chondrosarcoma	Cartilage
Dedifferentiated liposarcoma	Adipose
Leiomyosarcoma	Smooth muscle
Malignant peripheral nerve sheath tumor	Nerve sheath
Myxofibrosarcoma	Fibrous tissue
Osteosarcoma	Bone
Undifferentiated pleomorphic sarcoma	Unknown

Table 1.3 Karyotypically-complex sarcomas and their associated line of differentiation.

1.1.3 Clinical and Histopathological Features of Sarcomas

The clinical presentation of sarcomas varies with the subtype and site of origin. Sarcomas commonly develop in the extremities (40% of soft-tissue sarcomas, 45-55% of bone sarcomas), particularly the lower limb, but tumors can occur at virtually any site in the body.[76,77] Visceral sarcomas (22% of soft-tissue sarcomas) include gastrointestinal stromal tumor and uterine leiomyosarcoma, whereas retroperitoneal sarcomas (16% of soft-tissue sarcomas) are often soft-tissue leiomyosarcoma or well/de-differentiated liposarcoma.[77] Typically, patients with peripheral sarcomas present with a history of a suspicious mass that may or may not be increasing in size. Generally speaking, soft-tissue sarcomas tend to be painless (79% of cases) and bone sarcomas tend to be painful (88% of cases), but this is not a firm rule.[78] Constitutional symptoms are uncommon, though weight loss, malaise, and fever are sometimes seen.[79] Due to this relatively indolent nature of presentation, patients often do not seek medical attention until very late, when a suspicious mass has become quite large or painful and the sarcoma has progressed to a more advanced stage. In a study of 33 children with synovial sarcoma, patients waited an average of 43 weeks before seeing a doctor, and after the first appointment, it took an average of 50 weeks before a correct diagnosis was made.[80] Among a study of 10,000 soft-tissue sarcomas, 64% were high-grade at presentation and 38% had a diameter larger than 10cm, and outcomes were significantly worse among those who presented with large or high-grade tumors.[77]

Unlike most other cancers, the clinical staging of sarcomas is largely determined by histological grade. TNM staging for sarcomas includes the classic indicators of tumor size, lymph node status, and metastatic status, but additionally takes tumor grade into account.[81] Grade is typically assessed by a sarcoma-subspecialty pathologist using a standardized grading

system, most commonly the French (Fédération Nationale des Centres de Lutte Contre le Cancer; FNCLCC) system (Table 1.4 adapted from Trojani *et al*[82,83]), which evaluates mitotic activity, tumor necrosis, and differentiation.

Parameter	Score	Description
Tumor differentiation	1-3	Based on histologic type: low scores for subtypes that closely resemble normal tissue, higher scores for more pleomorphic/undifferentiated subtypes
Mitotic count	1	0-9 mitoses per 10 high-powered fields
	2	10-19 mitoses per 10 high-powered fields
	3	≥20 mitoses per 10 high-powered fields
Tumor necrosis	0	No tumor necrosis
	1	≤50% tumor necrosis
	2	>50% tumor necrosis
Combined score		**FNCLCC tumor grade**
2 or 3		Grade 1
4 or 5		Grade 2
6, 7, or 8		Grade 3

Table 1.4 The French (Fédération Nationale des Centres de Lutte Contre le Cancer; FNCLCC) sarcoma histological grading system: Definition of parameters.
1 High-powered field = area of tissue visible under 400-fold magnification.

The morphological appearance of sarcomas is, predictably, particularly diverse. The World Health Organization (WHO) categorizes bone and soft tissue lesions into umbrella categories, based on common tissue of origin and some morphological features.[1] Table 1.5 lists the WHO categories of subtypes discussed in the chapters of this thesis. Accurate diagnosis is essential for effective cancer management and is notoriously challenging in soft-tissue pathology, in which pathologists must distinguish among numerous soft tissue neoplasms (50+ distinct sarcoma types), many of which have nearly identical histological appearances.[84] Diagnosis relies upon recognition of characteristic features and/or patterns within cytology, architecture, stroma, and vascularization, some of which may not be represented in limited core needle biopsy or fine needle aspiration specimens.[85] Furthermore, the uncommonness of many soft-tissue lesions makes pattern-recognition difficult for non-subspecialty pathologists.

Immunohistochemistry and – in some cases – cytogenetics and molecular analysis have become indispensable adjuncts to standard morphological evaluation. [84,86]

Category	Examples
Soft-tissue sarcomas	
Adipocytic tumors	Dedifferentiated liposarcoma, myxoid liposarcoma, well-differentiated liposarcoma
Fibroblastic/myofibroblastic tumors	Dermatofibrosarcoma protuberans, low-grade fibromyxoid sarcoma, myxofibrosarcoma, solitary fibrous tumor
Gastrointestinal stromal tumors	Gastrointestinal stromal tumor
Nerve sheath tumors	Malignant peripheral nerve sheath tumor
Skeletal muscle tumors	Alveolar rhabdomyosarcoma, embryonal rhabdomyosarcoma
Smooth muscle tumors	Leiomyosarcoma
Vascular tumors	Angiosarcoma
Tumors of uncertain differentiation	Alveolar soft-part sarcoma, clear cell sarcoma, epithelioid sarcoma, synovial sarcoma
Undifferentiated/unclassified sarcomas	Undifferentiated pleomorphic sarcoma
Bone sarcomas	
Chondrogenic tumors	Chondrosarcoma
Ewing sarcoma	Ewing sarcoma
Notochordal tumors	Chordoma
Osteogenic tumors	Osteosarcoma

Table 1.5 The World Health Organization categories of some common sarcoma subtypes

1.1.4 Treatment and Prognosis of Sarcomas

1.1.4.1 Surgery

Surgical resection is the standard treatment for all localized bone and soft-tissue sarcomas, almost always performed by a sarcoma-subspecialty surgeon. [87-89] Complete excision with negative margins on all aspects of the tumor is the main objective of surgery, as positive margins are the main risk factor for local recurrence. [90,91] Therefore, for most cases, a wide local excision – removal of the tumor, the biopsy track, and a ~1cm border of normal tissue around it – is the preferred surgical option,[90] though marginal excision may be considered in some low-risk cases or in certain areas to preserve uninvolved major neurovascular structures. For sarcomas of the extremities, there has been a major shift away from amputational surgeries in favor of limb-

salvaging surgeries, in hopes of preserving limb function (without sacrificing long-term survival).[92] However, for some patients with very large or extensive tumors, wherein complete excision would severely compromise the likelihood of attaining good limb functionality (or in the case of major complications), amputation may be the only potentially curative option. Lymph node involvement is rare in soft-tissue sarcomas, so regional lymph node excision is not standard.[87] Ultimately, for many patients with soft-tissue sarcomas, surgery alone provides excellent disease control. In a study of 684 patients with primary nonmetastatic soft-tissue sarcoma treated with limb-sparing surgery alone, progression-free survival at 5 years was 87%.[93]

For sarcomas metastatic at the time of diagnosis – unlike the case in most other solid tumors – surgical resection of metastases is common-practice. Metastatic spread is primarily hematogenous, most commonly to the lung, but rarely to the skin, soft tissues, liver, or lymph nodes,[94] and the presence of clinically-apparent metastases does not necessarily signify the presence of sub-clinical ("micro") metastases.[95] Due to the relative resistance of sarcomas to either chemotherapy or radiotherapy, isolated pulmonary metastases are often managed surgically in bone and soft-tissue sarcomas.[96,97] Long-term results indicate that complete pulmonary metastatectomy is considered to be potentially curative in carefully selected cases of bone and soft-tissue sarcomas.[95,98,99]

1.1.4.2 Radiation Therapy

While surgery remains the mainstay treatment for sarcomas of all types, some patients are given complementary treatment based on factors associated with higher likelihood of recurrence: close/positive margin status, higher grade, deeper location, larger size (>5cm), and specific histologies.[93] Furthermore, studies have shown that combining limb-salvage surgery with radiation therapy in soft-tissue sarcomas provides comparable local control to that achieved by

amputation.[100] Addition of external beam radiation therapy to limb-sparing surgery for soft-tissue sarcomas reduces risk of local recurrence by 24% (absolute risk reduction) compared to surgery alone.[101] Brachytherapy has also been shown to reduce risk of local recurrence in soft-tissue sarcomas (12-15% absolute risk reduction),[102,103] but there have been no randomized trials to compare the two modalities. Outcomes are similar whether radiation is administered pre-operatively or post-operatively, but due to lower rates of long-term complications and better functional outcomes, pre-operative radiation therapy tends to be employed most commonly.[104] The strongest predictor for recurrence is positive surgical margins, even in patients who received radiation therapy.[105] In the event of grossly positive (also known as R2) margins, re-excision is considered to be the best option for management.[106]

The value of radiation therapy among bone sarcomas is much more limited.[107] The only clear role is in Ewing sarcoma, for which post-operative radiation therapy is given to patients with close margins and/or poor histologic response. [108,109] Proton beam radiation in combination with surgery has recently shown some benefit in skull base chordoma and chondrosarcoma.[110] For osteosarcoma, adjuvant radiation has fallen out of practice with the demonstrated benefit of chemotherapy in this disease.[111] Otherwise, adjuvant radiation therapy may be considered in bone sarcomas in the case of positive margins, for palliation of unresectable tumors, or for palliation of symptomatic primary tumors in patients with widespread metastatic disease. [112,113]

1.1.4.3 Chemotherapy

Despite excellent management of local disease, when treated with only surgery and radiation, metastasis occurs in >80% of osteosarcomas[114,115] and 25% of soft-tissue sarcomas.[105,116] In 1981, chemotherapy was introduced in a clinical trial for nonmetastatic osteosarcoma, improving 2-year relapse-free survival from 17% to 66% and 2-year overall

survival from 48% to 80%.[117] Since then, chemotherapy has become standard-of-care for conventional osteosarcoma, employing a regimen of high-dose methotrexate, cisplatin, and doxorubicin. [89,118,119] With the introduction of multimodal chemotherapy, 5-year overall survival for osteosarcoma improved from <20%[114] to 60-80%.[120,121]Comparable event-free survival is seen for adjuvant and neoadjuvant chemotherapy.[122] Similarly, chemotherapy also has proven benefit in the treatment of Ewing sarcoma. The standard regimen for Ewing sarcoma is vincristine, doxorubicin, and cyclophosphamide, alternated with ifosfamide and etoposide every 2 weeks. [89,119,123]

While the value of chemotherapy is well-established for conventional osteosarcoma and Ewing sarcoma, there is still no effective systemic therapy for other bone tumors like chondrosarcoma; furthermore, for management of soft-tissue sarcomas, use of chemotherapy might be most accurately described as "investigational."[119] Numerous clinical trials of cytotoxic agents in soft-tissue sarcomas have delivered conflicting results. A 2008 meta-analysis found a limited benefit of adjuvant chemotherapy in terms of both recurrence-free and overall survival, particularly among regimens combining ifosfamide with doxorubicin (absolute risk reduction of 11%).[124] However, in a subsequent phase III clinical trial that randomized patients to either adjuvant chemotherapy (doxorubicin with ifosfamide) or no systemic therapy, no survival benefit was observed.[125] As such, there is no consensus regarding cytotoxic therapy for soft-tissue sarcomas, and the administration of systemic treatment is largely dependent on institution.

Discrepancies in response among clinical trials that group together assorted subtypes under the "soft-tissue sarcoma" umbrella might very well be explained by discrepancies in response among specific subtypes. Unfortunately, due to the uncommonness of most subtypes, sarcoma clinical trials have a common practice of indiscriminately lumping together biologically

different subtypes to provide adequate numbers for statistical evaluation. As such, some specific subtypes may indeed be sensitive to chemotherapy. In retrospective analyses, adjuvant chemotherapy has been associated with improved outcomes in myxoid liposarcoma,[126,127] and synovial sarcoma,[128] but these associations have not been confirmed by randomized clinical trials or meta-analyses. Angiosarcoma has similarly shown improved outcomes associated with PEGylated liposomal doxorubicin and taxanes in the adjuvant setting,[129] and the latter was confirmed in a phase II clinical trial.[130] Another randomized phase II study reported benefit for adjuvant gemcitabine-containing therapy within patient subgroups of leiomyosarcoma and undifferentiated pleomorphic sarcoma.[131]

In advanced metastatic soft-tissue sarcomas, doxorubicin is the most effective cytotoxic agent and is considered to be the first-line therapy in this setting.[132,133] Doxorubicin has a response rate of 10–25% and median survival of ~1 year in metastatic soft-tissue sarcomas.[134] The value of adding ifosfamide to doxorubicin treatment is controversial. While the combination therapy shows improved response rates and progression-free survival, there is no significant difference in overall survival, and the toxicity profile is much worse.[135,136] A phase III study addressing this question concluded that due to toxicity and lack of survival advantage with the addition of ifosfamide, doxorubicin monotherapy remains the treatment of choice in metastatic soft-tissue sarcoma.[137] The most widely-used second-line treatment of advanced metastatic soft-tissue sarcoma is gemcitabine with or without docetaxel, particularly for leiomyosarcoma and undifferentiated pleomorphic sarcoma.[131,133,138] Additionally, a number of agents have been recently approved for use in specific sarcoma subtypes, mostly as second- or third-line, following failure of first-line treatment. Key examples of this include eribulin, which has

demonstrated improved overall survival for patients with liposarcomas,[139] and trabectedin, which has shown benefit for patients with leiomyosarcoma and liposarcoma.[140,141]

1.1.4.4 Targeted Therapy

With the elucidation of the molecular features of the various sarcoma subtypes has come a flood of efforts to identify systemic therapies that specifically target sarcoma cells and their pathogenic mechanisms. The most obvious success story is the treatment of gastrointestinal stromal tumors (GISTa) – which arise from gain-of-function mutations of tyrosine kinase receptor genes c-*KIT* and *PDGFRA* – by tyrosine kinase inhibitors. Imatinib was the first tyrosine kinase inhibitor to be investigated,[142,143] and by the time it reached standard-of care (following a highly successful phase III clinical trial), the median overall survival for advanced metastatic GIST increased from <1 year[144] to ~5 years.[145] For nonmetastatic GIST, treatment with imatinib in the adjuvant setting has a positive impact on progression-free survival,[146] particularly when treatment is continued in the longer-term, wherein 5-year progression-free survival is 90%.[147,148] For GISTs resistant to imatinib, patients are usually switched to newer multi-target tyrosine kinase inhibitors sunitinib[149] and then regorafenib.[150]

Following the introduction of multi-target tyrosine kinase inhibitors as standard treatments for GIST and other cancer types, a number of these agents were empirically studied in non-GIST soft-tissue sarcomas. Multi-target tyrosine kinase inhibitor pazopanib showed promise in phase II for soft-tissue sarcomas, except for the liposarcoma cohort, which was stopped early due to high rates of progression (only 26% reached 12-week progression-free survival).[151] In a subsequent randomized, placebo-controlled phase III trial of pazopanib in non-adipogenic soft-tissue sarcomas (the PALETTE trial), there was a modest improvement in meidan progression-free survival from 1.6 months to 4.6 months, but no difference in overall survival.[152] This led to

approval of pazopanib for non-adipogenic sarcomas failing conventional chemotherapy. However, the specific guidelines for which subtypes respond to pazopanib are still unclear, as the PALETTE trial grouped together a large number of disparate subtypes in a cohort called "other sarcomas," and excluded "adipogenic sarcomas" based preliminary results of the phase II trial, which lumped together 3 distinctly-different liposarcoma subtypes.[153] Adipogenic sarcomas may therefore have been inappropriately excluded, given that in a recent single-arm phase II clinical trial of pazopanib in medium- or high-grade liposarcomas (this excludes well-differentiated liposarcoma), 12-week progression-free survival was 69%.[154] The limitations of the PALETTE trial design again highlight the importance of reporting subtype-specific results in sarcoma clinical trials.[153] Other tyrosine kinase inhibitors under investigation in soft-tissue sarcomas have shown comparable outcomes in phase II trials: sorafenib,[155,156] sunitinib,[157] and regorafenib.[158] Also similar to gastrointestinal stromal tumors, many soft-tissue sarcomas overexpress PDGFR.[159] A phase 1b/2 trial of PDGFR inhibitor olaratumab combined with doxorubicin demonstrated an increased median overall survival of 26.5 months over doxorubicin alone, which was 14.7 months. This earned olaratumab the status of "breakthrough therapy" by the United States Food and Drug Administration in 2016, accelerating its approval for use in soft-tissue sarcoma.[160] However, results have not held up in preliminary results from the subsequent phase III ANNOUNCE trial.[161]

Other recent efforts have taken aim at targeting intracellular signaling pathways, some of which are depicted in Figure 1.1. The mTOR (mammalian target of rapamycin; part of the phosphatidylinositol-3-kinase pathway) inhibitor ridaforolimus was investigated for sarcomas (mainly soft-tissue), and a phase III trial showed improved progression-free survival in the maintenance setting following chemotherapy, but the clinical benefit was too small to change

standard of care.[162] Well- and de-differentiated liposarcoma are known to have amplifications of CDK4/6 (Cyclin-dependent kinase; part of the Rb pathway) and MDM2 (Mouse double minute 2 homolog; protein-ubiquitin ligase that regulates p53).[163,164] Phase II trials of CDK4/6 inhibitor palbociclib in well-dedifferentiated liposarcoma have given modest results, [165,166] and MDM2 inhibitors have so far only been tested in a phase I trial;[167] however, MDM2 and CDK4 might be synergistic, so combination therapy against these two targets is worth investigating.[168]

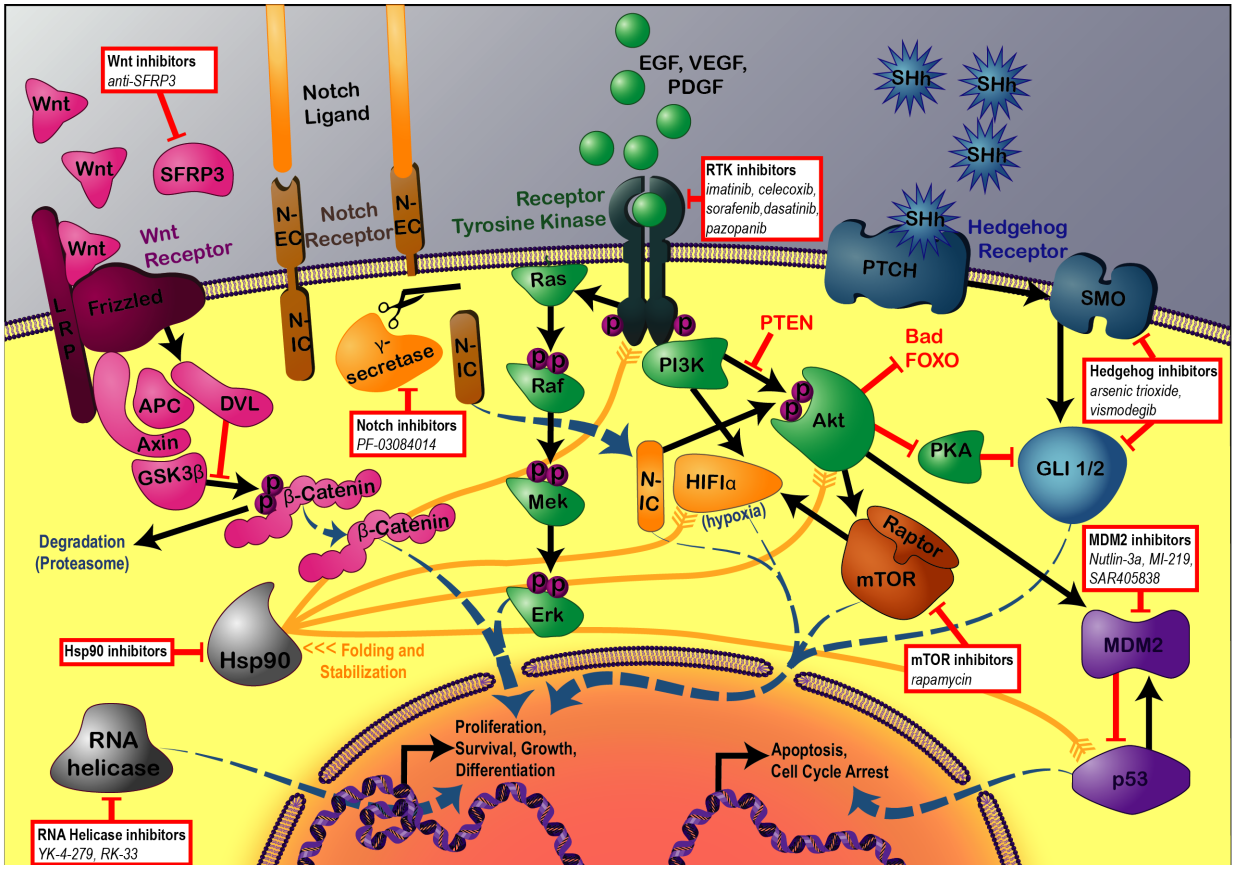


Figure 1.1 Signaling pathways currently targeted in sarcoma translational research.
Details of Wnt, Notch, Receptor Tyrosine Kinase, Hedgehog, MDM2, and mTOR signaling pathways, including common regulators and pathway interactions. Hsp90 protein clients also depicted. Co-inhibitory interactions are marked in red, and co-stimulatory interactions are marked in green. Figure taken from Figure 1 of Dancsok *et al.* 2017.[84]

Finally, epigenetic modulatory agents are also of interest in sarcomas, particularly among the translocation-associated sarcomas, many of which involve epigenetic machinery in their

pathogenic mechanism. Histone deacetylase (HDAC) inhibitors showed great promise in pre-clinical work,[169-171] particularly in combination with proteasome inhibitors.[172,173] However, in phase II clinical trials of monotherapy HDAC inhibition for sarcomas, the best outcome attained in all 3 studies was stable disease.[174-176] As such, it is thought that strategies combining other therapies with of HDAC inhibitors will be more likely to achieve adequate disease response.[177] Histone methyltransferase EZH2 (enhancer of zeste homolog 2) is involved in the disease processes of a number of sarcoma subtypes, but in a phase II trial, only epithelioid sarcomas saw a durable response.[178]

1.2 Cancer Immunology

Our understanding of the immune system's role in cancer has increased dramatically over the past 20 years, but the idea that the human immune system has a fundamental duty to target and destroy cancer is not new. It is generally accepted that patrolling innate and adaptive immune cells will recognize and – most often – destroy cancer precursor cells before they become a clinically-apparent cancer. Tumors growing in immunocompetent mice can be grown in immunodeficient mice of the same strain, but not vice versa;[179] that is, unless variant tumor cells possess selected-for traits to survive immune surveillance, either via decreased immunogenicity (immunoselection) or via disruption of the antitumor immune response (immunosubversion).[180] Described as "cancer immunoediting," this ability to evade immune surveillance has been proposed as the seventh hallmark of cancer. [181,182] The process of immunoediting has been described in three phases, known as "the three Es": 1) elimination of tumor cells by the immune system; 2) equilibrium of tumor cell destruction and proliferation;

and 3) escape from immune response. [182-184] Once immune escape has occurred, the aberrant cells can proliferate quickly to form a malignant neoplasm.

1.2.1 Immune Surveillance of Cancer

In 1957, Burnet – considered the "architect" of the cancer immunosurveillance hypothesis[185] – theorized that curative treatment for cancer might be able to draw upon "the body's own [immunological] resources" due to "sufficient antigenic difference" between a cancer cell and "the body's own pattern" as a result of somatic mutation.[186] His concept of immunological surveillance, published in 1970,[187] declared:

> "It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating (...) dangerous mutant cells, and it is postulated that this mechanism is of immunological character."

While investigated extensively, the concept was nearly abandoned when immunologically deficient nude mice showed no differences in primary tumor development from syngeneic wild-type mice.[188-190] However, subsequent studies showed that athymic nude mice produce low numbers of functional T cells (as well as natural killer cells) and are therefore not perfectly immunologically deficient.[191,192] Interest in anti-tumoral immunity was renewed following observations of enhanced tumor growth in mice with inhibition of immune system components IFNγ[193,194] and perforin.[195-197] Burnet's theory wasn't definitively validated until three decades after its publication, fueled by technological advances in the genetic engineering of mice and laboratory monoclonal antibody production. Gene-targeted mice lacking recombination-activating gene 2 (RAG-2: rearranges lymphocyte antigen receptors) and therefore lacking mature lymphocytes,[198] developed sarcomas more rapidly and frequently than syngeneic wild-type mice.[179] Taken together with epidemiological evidence of higher incidences of cancer

among immunosuppressed or immunodeficient patients,[199-201] the concept of anti-tumor immunity in humans came into mainstream acceptance.

Both the innate and adaptive immune systems function to protect the host against foreign materials – either pathogenic or neoplastic – by differentiating between "self" and "non-self." In an evolving tumor, numerous foreign antigens may be generated, as a result of nonsilent mutations and other genetic abnormalities. Accordingly, numerous components of the immune system assemble to identify and eliminate the abnormal cells, with a target of preventing or destroying malignant neoplasia.

1.2.1.1 Adaptive Immune Response to Cancer

The adaptive immune system is the body's latent, specific response to foreign materials, acting via recognition of "non-self" antigens by (nearly) infinite possible antigen receptors created through genetic rearrangements in the development of adaptive immune cells. Tumor-associated antigens are identified based on altered protein structure or abnormal expression patterns:[202] 1) Gene mutations (or aberrant splicing) give rise to foreign protein products, known as a "tumor-specific antigens" (*e.g.,* mutant KRAS in lung and colorectal cancer); 2) Chromosomal translocations result in expression of a foreign "fusion protein" product (*e.g.,* BCR-ABL in chronic myeloid leukemia); 3) "Cancer-testis antigens" (CT antigens) are proteins normally expressed only in (immune-privileged) testes, so expression elsewhere is abnormal (*e.g.,* NY-ESO-1); 4) "Differentiation antigens" are lineage-specific proteins expressed by the tumor's tissue of origin but over-expressed in the tumor (*e.g.,* PSA – prostate-specific antigen); 5) "Over-expression antigens" are proteins expressed by normal tissues (below a threshold for immune activation) but over-expressed by tumor cells, overriding immune tolerance (*e.g.,* HER2 –in breast cancer).

The presence of foreign antigens alone is insufficient to trigger an adaptive immune response. [203] In order to activate adaptive immune cells, tumor-associated antigens must first be presented to effector cells. Antigens are processed into short antigenic peptides (epitopes), which are loaded onto major histocompatibility complex (MHC) molecules on the surface of professional antigen presenting cells (APCs: dendritic cells, macrophages; MHC class II), as well as normal and transformed cells (MHC class I). [204] Recognition of antigens presented by professional APCs leads to clonal expansion of T cells, which secrete cytokines to recruit other immune cells, including those of the innate immune system. T helper (Th) cells recognize antigens presented by professional APCs (MHC class II) and secrete cytokines and chemokines to regulate different cell types; Th1 helper T cells activate cytotoxic T cells, favoring cellular immunity, whereas Th2 helper T cells act on B cells, favoring humoral immunity. [204,205] Cytotoxic T cells are activated both by direct presentation of antigen (by MHC class I) or by Th1-mediated activation. Ultimately, the tumor cell is destroyed by direct (cell-mediated) and/or indirect (antibody-mediated) cytotoxicity.

1.2.1.1.1 Tumor-infiltrating lymphocytes

Lymphocytes found among the cells of a tumor (or at the invasive margin) are called "tumor-infiltrating lymphocytes" (TILs), and these can be divided into subpopulations with various roles in tumor immunity.

Cytotoxic T-cells (Tc, cytotoxic T lymphocytes, CTL) are the main effector cells of the adaptive immune system, usually identified by their expression of CD8, a co-receptor for MHC class I. CD8+ T cells recognize their cognate antigenic peptides by direct binding of their T-cell receptor (TCR) and CD8 to antigen-loaded class I MHC presented on the surface of tumor cells. Cytotoxic T cell activation also requires the co-stimulatory interaction between CD28 on the T

cell and CD80 (B7-1) or CD86 (B7-2) on the APC. If there is no co-stimulation – or if there is a co-inhibitory signal on the APC – T cells become anergic or apoptotic.[204,206] Once activated, cytotoxic T cells release "lytic granules," which are modified lysosomes that contain cytotoxic effector proteins, such as perforin and granzymes.[207,208] In the presence of calcium, perforin polymerizes to form channels that become transmembrane pores in target cell membranes, which facilitate the entry of granzymes, serine proteases that activate caspase-mediated apoptosis of target cells.[208,209] Activated CD8+ T cells also express Fas ligand, for inducing calcium-independent target cell apoptosis. While activation of apoptosis is the main way by which cytotoxic T cells eliminate target cells, most also release cytokines such as IFN-γ, which upregulates expression of MHC class I and activates tumoricidal macrophages.[179,210]

Helper T cells (Th) are the mediators of the adaptive immune system – enabling different functions, depending on the cytokines they are exposed to – that are usually identified by their expression of CD4, a co-receptor for MHC class II.[211] CD4+ T cells also recognize their cognate antigenic peptides by direct binding of their T-cell receptor (TCR) and CD4 to antigen-loaded MHC, but in the case of helper T cells, it is class II MHC presented on the professional APCs. Helper T cell activation also requires the co-stimulatory interaction between CD28 and CD80/86. In response to different cytokines, naïve CD4+ T cells differentiate into different subsets (Th1, Th2, Th17) through epigenetic modifications and transcriptional activation of cytokine genes.[212-214] Activated type 1 helper T cells (Th1 cells) produce IFN-γ, IL-2, and TNFβ, and are responsible for activating and regulating CD8+ cytotoxic T cells[213-216] They also support the function of antigen presenting cells and activate tumoricidal macrophages. Differentiation of naïve helper T cells to Th1 depends on high antigen density on APCs, high TCR-antigen affinity, and the presence of IL-12 during antigen presentation.[215,216] Type 2 helper T cells (Th2 cells)

produce IL-4, IL-5, IL-10, and IL-13, and they stimulate B cells to produce most classes of antibodies.[213-216] Th2 cells are major contributors to the immune response against extracellular pathogens and parasites, but are not considered major players in anti-tumor activity. Th2 differentiation is promoted by IL-4 and blocked by IL-12.[215,216] Helper T cells are not plastic; once a naïve Th cell differentiates into Th1 or Th2, it inhibits the differentiation of the other type of helper T cell.

Regulatory T cells (Tregs) are another specialized subset of CD4+ T cells, which are responsible for inhibiting the immune response to maintain "self-tolerance."[217] While Tregs are necessary to protect against autoimmune disease, they can also inhibit anti-tumoral immunity.[218] Naïve helper T cells differentiate into Tregs when exposed to TGF-β or B7-H1.[219] A common marker of regulatory T cells is FOXP3 (forkhead box P3), which is an important nuclear transcription factor in Treg differentiation.[220] Regulatory T cells suppress the function of immune effector cells either through direct contact or through secretion of cytokines IL-10, IL-35, and TGF-β. By direct contact, Tregs suppress cytotoxic T cell activation by inhibiting proliferation and effector cytokine (IFN-γ and IL-2) production.[221] They can also directly inhibit proliferation, differentiation, and activation of naïve T cells.[222] Secretion of IL-10 functions to limit and ultimately terminate inflammatory responses by inhibiting tumoricidal macrophage activation and proliferation, inhibiting helper T cell proliferation, stimulating differentiation of Tregs, inhibiting MHC-II expression, and, perhaps most impactfully, inhibiting production of pro-inflammatory cytokines.[223] IL-35 suppresses helper T cell proliferation and enhances Treg proliferation. [224,225] TGF-β function is less straightforward, but it involves direct suppression of effector T cells and induction of regulatory T cells. [226,227]

B cells are not considered dominant effectors in the anti-tumoral immune response, but they are capable of generating tumor-specific antibodies against tumor-associated antigens. [228-230] Tumor-specific antibodies can coat tumor cells and destroy them via cell- or complement-mediated cytotoxicity. In cell-mediated cytotoxicity, the Fc domains of antibodies coating target cells are recognized by receptors on natural killer cells, macrophages, and neutrophils, triggering their respective cytotoxic effector functions.[231] In complement-mediated cytotoxicity, the immune complement pathway is activated, resulting in the formation of the membrane attack complex, which induces direct cytolysis by forming a pore in the target cell membrane. There is also some evidence of direct cytotoxic activity of B cells on tumor cells through activation of apoptotic pathways. [232,233] Like T cells, there are a subset of B cells that are immunosuppressive, called regulatory B cells (Bregs), that produce IL-10 and likely contribute to immune tolerance of tumors.[234]

1.2.1.2 Innate Immune Response to Cancer

The innate immune system is the body's initial rapid response to foreign materials, acting through various mechanisms, including physical barriers (skin, mucous membranes), effector cells (macrophages, natural killer cells, dendritic cells, mast cells, neutrophils, eosinophils), and humoral mechanisms (complement proteins, cytokines). While the role of the adaptive immune response has been a major emphasis across the majority of studies, increasing evidence supports a role for innate immune effector cells as well. Whereas the adaptive response uses rearranged antigen receptors to identify almost infinite possible tumor antigens, innate immune cells express a fixed set of germline-encoded receptors, which identify nonspecific molecular patterns, such as STING (stimulator of interferon genes) or other DAMPs (damage-associated molecular

patterns).[235] Furthermore, in addition to interacting with tumor cells directly, innate immune cells function to attract and augment the activities of other immune cells.

1.2.1.2.1 Natural Killer Cells

Natural killer (NK) cells are cytolytic immune effector cells that carry out their effector function on target (neoplastic and virally-infected) cells through perforin-mediated cell membrane perforation and granzyme-mediated intracellular proteolysis.[236] NK cells are activated upon recognition of cellular stress molecules by activating receptors, the most well-described of which is NKG2D (natural-killer group 2, member D).[237] NKG2D recognizes several major histocompatibility complex (MHC)-related ligands that are poorly expressed by normal cells but upregulated on tumor cells. [238-240] In addition to NKG2D, several other receptors can induce NK cell activation, such as other natural cytotoxicity receptors (NCRs: NKp46, NKp44, and NKp30),[241] SLAM (signaling lymphocyte activating molecule),[242] 2B4, [243,244] CD38,[245] and NKp80.[246] Upon activation, direct tumor cell lysis by NK cells is mediated primarily by perforin, [195,207,247,248] which is released from the NK lysosome into target cells through an NK-cell-to-tumor-cell immune synapse, where it polymerizes into channels that form pores in the target cell membrane.[249] Perforation facilitates the entry of granzymes into the target cell, which are serine proteases that activate caspase-mediated apoptosis of target cells.[208,209] Natural killer cells can also induce tumor cell elimination through their cell-surface expression of tumor necrosis factor (TNF) – and related ligands TRAIL (TNF-related apoptosis-inducing ligand), FasL (Fas ligand) – that induce target cell apoptosis upon recognition by TNF receptors. [250-253]

Activated NK cells also secrete cytokines (IFN-γ, TNF-α, G-CSF, GM-CSF) and numerous chemokines that encourage anti-tumoral immunity both directly on the target cells and through communication with other immune cells.[254] For example, interferon-gamma (IFN-γ) has

powerful anti-tumor activities,[255] including inducing tumor cell MHC class I expression, sensitizing tumor cells to CD8+ T cell killing, priming Th1 cells, and polarizing macrophages towards their tumoricidal M1 state.[256] TNF-α can directly induce tumor cell death via caspase 8-mediated apoptosis.[257] Furthermore, the combination of IFN-γ and TNF-α can direct tumor cells down a senescence pathway.[258]

1.2.1.2.2 Gamma/delta (γδ) T Cells

Gamma/delta T-cells – sometimes called "natural killer T-cells" or NKT cells – are another class of cytolytic innate lymphocytic cells. These differ from the NK cells of the previous section because they are T cells and therefore mature in the thymus, whereas NK cells mature in circulation.[259] Unlike NK cells, γδ T cells have no cytoplasmic granules and express a rearranged T-cell receptor (TCR).[260] In contrast to typical (alpha/beta) T cells, which have a TCR made up of one α and one β chain and are activated by antigens presented by major histocompatibility complex (MHC), the γδ TCR is made up of one γ and one δ chain and can be activated in a MHC-independent manner.[261,262] The absence of a need for MHC-presented antigens to activate γδ T cells is the reason they are considered part of the innate immune system, and it makes them particularly suited to targeting tumor cells.[260] Some γδ T cells also express NKG2D, which induces activation in the absence of TCR-mediated ligand recognition.[263] Once activated, γδ T cells function like both cytotoxic T cells and NK cells, exerting their effector function through both death receptor-dependent (*e.g.*, Fas/Fas-ligand) and perforin/granzyme-dependent pathways. [260,264,265] Like NK cells, γδ T cells also secrete massive amounts of pro-inflammatory cytokines and chemokines including IFN-γ, TNF-α, G-CSF, and GM-CSF.[254] Like conventional αβ T cells, not all γδ T cells exert effector functions, but rather, a subset are known as regulatory/suppressor γδ T cells (γδ Tregs).[266] γδ Tregs modulate the immune response

and assist in maintaining immunological tolerance by targeting dendritic cells and naïve T cells to induce senescence.[266,267] Interestingly, unlike conventional αβ T cells, γδ T cells are plastic and can shift (or "polarize") from one phenotype to another in response to different cytokines.[268,269] Therefore, depending on the cytokines present in the tumor's microenvironment, they can change their function toward being pro- or anti-tumor.[270]

1.2.1.2.3 Tumor-Associated Macrophages (TAMs)

Tumor-associated macrophages (TAMs) are derived from peripheral blood monocytes and make up a prominent proportion of the immune component of tumors.[271] While this would intuitively suggest strong antitumor immunity, high frequencies of TAMs are generally associated with poor prognosis in most cancer types.[272,273] Physiologically, macrophages are known to have extremely diverse functions, so unsurprisingly, TAMs have numerous roles within a tumor, including influencing tumor cell proliferation/growth/metastasis, stromal formation and dissolution, vascularization, and mediating both pro- and anti-neoplastic inflammation.[274] TAMs are conventionally divided into two subgroups: M1 (classically-activated) and M2 (alternatively-activated) macrophages. M1-polarized macrophages are considered to be pro-inflammatory, as they have high levels of phagocytic activity and play an important role in amplifying the immune response through secretion of pro-inflammatory cytokines.[275] M2-polarized macrophages are considered to be anti-inflammatory, as they are mainly involved in tissue repair and the resolution of inflammation.[275] The M1/M2 nomenclature was designed to mirror the Th1/Th2 designation of helper T cells.

M1-polarized macrophages are defined by their ability to direct acute inflammatory responses. In cancer, M1 macrophages are activated primarily by interferon-gamma (IFNγ) secreted by NK cells and other macrophages (classical activation),[276,277] and can also be activated

by GM-CSF (granulocyte macrophage colony-stimulating factor).[278] Once activated, M1 macrophages are directly tumoricidal via complement-mediated phagocytosis. [275] Activated M1 macrophages also secrete high levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12, IL-18, IL-23, type I IFN), chemokines (CXCL1, CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CXCL13, CXCL16, CCL2, CCL3, CCL4, CCL5, CCL8, CCL15, CCL11, CCL19, CCL20, CX3CL1), MHC molecules, and effector molecules, such as nitric oxide synthase. [275,279-281] These secreted molecules recruit more M1 macrophages and, importantly, activate the Th1 adaptive immune response.[282]

M2 macrophages are known for their anti-inflammatory role in wound-healing and clearance of damaged tissues. M2 macrophages are activated primarily by IL-4, IL-10, IL-13, and M-CSF (alternative activation). [277,283] All activated M2 macrophages characteristically downregulate expression of the pro-inflammatory cytokine IL-12 and upregulate expression of the anti-inflammatory cytokine IL-10.[284] While still capable of phagocytosis, M2 macrophages target only apoptotic cells in this manner (efferocytosis). [285,286] M2 macrophages can be further subdivided into M2a ("alternative- activated"), M2b ("type 2"), and M2c ("deactivated") macrophages, based on differential stimuli and cytokine products.[287] Tumor-associated M2 macrophages are sometimes categorized as a novel subset, "M2d," based on their ability inhibit pro-inflammatory M1 macrophages. In addition to the primary M2 stimuli, M2d macrophages can be activated by IL-6 and adenosines[288,289] and secrete chemokines CXCL10, CXCL16, and CCL5.[290] An important function of all activated M2 macrophages is massive production of the important anti-inflammatory cytokine IL-10. As mentioned in the discussion of regulatory T cells, IL-10 is a major immunosuppressive cytokine that inhibits multiple immune functions: M1 macrophage proliferation/activation, helper T cell proliferation, MHC-II expression, and

production of pro-inflammatory cytokines.[223] In addition to IL-10, M2 macrophages produce high levels of IL-8, IL-13, IL-1R, MCP-1 (monocyte chemo-attractant protein-1), MIP-1β (macrophages inflammatory protein-1β), and numerous chemokines (CCL1, CCL2, CCL13, CCL14, CCL17, CCL18, CCL22, CCL23, CCL24, CCL26) in order to recruit anti-inflammatory neutrophils, monocytes, and T cells. [281,291-293]

Unlike their namesake Th1 and Th2 cells, M1 and M2 macrophages are plastic, with polarization defined by their gene expression profiles rather than by differentiation pathways and lineage choices.[282] Prior to polarization, macrophages first exist as M0 (uncommitted) macrophages, and polarization is largely determined by cytokine stimuli. *In vitro*, polarized macrophages grown in cytokine-deficient medium revert back to their M0 state within 12 days.[291] Macrophages can switch polarization in response to external stimuli. IFNγ has been shown to switch M2 macrophages to M1,[210] and IL-10 has been shown to switch M1 macrophages to M2.[294] The heterogeneity and plasticity of macrophage functionality suggest that M1 and M2 macrophage "types" are in fact extremes of a spectrum of macrophage functional states.

1.2.2 Cancer Immunoediting

Despite potent anti-tumoral mechanisms from both the adaptive and innate immune systems, any tumor that progresses to malignancy has managed to escape immunosurveillance and immune destruction.[180] This immunological "escape" is usually a result of tumors hijacking physiological mechanisms that control self-tolerance, wound-healing, and tissue repair. Much like with pathogenic infections, it is classic Darwinian selection; these immunoevasive mechanisms are induced as a result of repeated attacks by the immune system, which generate selective pressure for tumor variants better suited to survive anti-tumoral immunity.[295] For

example, passaging of transplantable tumors through immunocompetent hosts generates less antigenic tumor variants. [296,297] Immune escape mechanisms exploit all areas of tumor immunosurveillance: antigenicity, cytokine signaling, cell trafficking, regulatory cells, and immune cell activation.[298]

1.2.2.1 Loss of Tumor Antigenicity

A fundamental component of anti-tumoral immunity is the ability to discriminate between healthy and malignant cells; therefore, a fundamental mechanism of immune escape is down-regulation of the components of antigen processing and presentation: major histocompatibility complex class I, [298-301] antigen proteasome subunits LMP2/7 (latent membrane protein 2/7),[302] and tapasin and other components with the TAP (transporter associated with antigen processing) complex. [303-305] Loss of MHC-I expression has been found to be a poor prognostic marker in multiple cancer types.[306-308] As a result, expression of tumor-associated antigens is reduced, and immune effector cells can no longer recognize the "non-self" antigens of tumor cells.[304]

1.2.2.2 Immune Checkpoints

Beyond tumor-associated antigen recognition, immune cell activation is heavily controlled by antigen-presenting cell (tumor or professional) expression of both co-stimulatory and co-inhibitory molecules. These molecules, called "immune checkpoints," function to maintain self-tolerance, with low expression of co-stimulatory signals and high expression of co-inhibitory signals present in normal tissues. However, cancer cells evolve to exploit this safeguard, and upregulation of immune checkpoint molecules is now recognized as major mechanism to suppress anti-tumoral immunity.[309]

As discussed above, T cell receptors (TCRs) require co-stimulatory binding of CD28 and CD80/86 in order to activate. Tumor cells can downregulate (or fail to express) costimulatory molecules, thereby engaging the TCR without activating the T cell, inducing anergy and/or tolerance.[206] In addition to CD28, other key T cell co-stimulatory receptors include CD27, CD137, OX40, and CD40-L. Decreased expression of co-stimulatory receptors has been observed in most cancers.[310-314]

Tumor cells can similarly evolve to overexpress co-inhibitory molecules that, when present in the immune synapse, prevent effector cell activation and stimulate pathways that lead to immune cell exhaustion through reduced activation or proliferation and/or through induction of apoptosis. For example, CTLA-4 (cytotoxic T-lymphocyte associated antigen-4), the first target investigated for immune checkpoint inhibitor therapy, competitively inhibits the binding of the T cell co-stimulatory receptor CD28 to target cell CD80 or CD86, hindering the activation of T cells.[315] Overexpression of CTLA-4 has been shown to predict poor survival in some cancer types.[316,317] The ultimate consequence of co-inhibitory immune checkpoint signaling is T cell exhaustion, a phenotype defined by reduced anti-tumoral effector functions. There have been multiple other co-inhibitory immune checkpoint molecules found to be overexpressed in various cancers, such as LAG-3, TIM-3, CD47, TIGIT, BTLA, and VISTA,[309] some of which are discussed in detail later in this chapter.

1.2.2.3 Recruitment of Immunoregulatory Cells

Immune suppression in the tumor microenvironment by regulatory immune cells (Tregs, Bregs, γδ Tregs, M2 macrophages) is another major mechanism by which tumors escape immunosurveillance.[318] Tumors can attract regulatory cells (and also impede effector cells) through production of immunosuppressive cytokines – by either the cancer cells themselves or

by nearby immune or epithelial cells – including TGF-β, TNF-α, CSF-1, IL-1, IL-6, IL-8, IL-10, and type I IFN. [319-325] In fact, evidence suggests that tumor-derived Tregs have higher suppressive activity compared to naturally-occurring Tregs. [326,327] Furthermore, tumor-derived TGF-β has been shown to help convert CD4+ T cells into Tregs,[218] and with IL-10 expression, to convert helper T cells from Th1 to Th2.[321] Tumor-associated macrophages are considered to be predominantly M2,[328]and tumor cells have been shown to promote this polarization shift through production of CSF1, CCL2, CCL3, CCL14, IL-4, and/or IL-10. [329-332]

1.3 Cancer Immunotherapy

As we come to understand the widespread capacity for cancer cells to exploit immune mechanisms and evade immunosurveillance, it apparent that a fast-growing malignant tumor can evolve well beyond the capacity of the human body's limited immune response. It is therefore a compelling strategy to find a way to trigger/reactivate the anti-tumoral immune response, equipping the body with strategies to fight the ever-changing cancer. In 2013, *Science* championed cancer immunotherapy as the "Breakthrough of the Year,"[333] and in the past year, the 2018 Nobel Prize for Physiology/ Medicine went to James P. Allison, PhD (University of Texas MD Anderson Cancer Center) and Dr. Tasuku Honjo (Kyoto University) for their work on understanding and targeting co-inhibitory immune checkpoints (CTLA-4 and PD-L1, respectively).

Cancer immunotherapy strategies are thematically divided into two main categories: 1) immunostimulatory therapies, which aim to stimulate an immune response in tumors with no/low immune activation, and 2) immunomodulatory therapies, which target the immunoediting mechanisms by which cancer cells inactivate the anti-tumoral immune response.

1.3.1 Immunostimulatory Therapy

1.3.1.1 Cytokine Therapy

Cytokines are the molecular messengers of both the innate and adaptive immunity systems, functioning to recruit, activate, and incite specific functions in various immune cell types. In the context of immunoquiescent tumors – those with little-to-no immune infiltration – an appealing therapeutic option has been the administration of pro-inflammatory cytokines, in hopes of attracting immune effector cells to the tumor to seek out and destroy cancer cells. Indeed, multiple cancer models in mice treated with regimens of IFNα, IFNγ, GM-CSF, IL-2, IL-12, IL-15, or IL-21 showed lots of promise in the early preclinical setting.[334] In 1986, IFNα – a pro-inflammatory cytokine that upregulates MHC class I, polarizes helper T cells to Th1, enhances cytotoxicity and survival of NK cells, and induces survival of CD8+ T cells – was the first cytokine approved for the treatment of human cancer (hairy cell leukemia),[335] but it has since been relegated to second-line therapy. IFNα has also been approved under select settings for melanoma, renal cell carcinoma, and Kaposi's sarcoma. [336,337] However, generally speaking, cytokine monotherapy in clinical trials did not fulfill the initial excitement from *in vivo* studies.

Several factors contribute to the limited clinical efficacy of cytokine monotherapy. Cytokines are not tumor-specific, so in the absence of tumor-antigen-directed immunity, the host initiates only a generalized immune response. Furthermore, cytokine stimulation can induce the secretion of immunosuppressive cytokines (IL-10 and TGF-β) and the activation of regulatory T cells.[338] Newer cytokine therapy approaches include: 1) cytokine engineering to generate "superkines" with improved binding affinity for select receptors to increase antitumor responses and decrease stimulation of Tregs,[339] and 2) chimeric antibody-cytokine fusion proteins "anticytokines" to improve their cytokine localization to the tumor.[340] However, the main

involvement of cytokine therapy in today's clinical trials is in the contex of combinations of cytokines, predominately IL-2, with other immunotherapeutics, such as cancer vaccines, adoptive cell therapy, and immune checkpoint inhibitors. [341-343]

1.3.1.2 Cancer Vaccines

Cancer vaccines are designed to introduce cancer antigens – either by whole tumor lysate or known tumor-associated antigens – to the cells of the adaptive immune system, in hopes of activating a cytotoxic T-cell response. The first documented attempt to vaccinate humans against cancer was in 1902 by von Leyden and Blumenthal, inoculating patients with an autologous tumor suspension, with limited success.[344] Over the next century, repeated attempts have been made to create a successful vaccine, with variable and largely disappointing results, largely due to a lack of understanding of the complexity of the immune response. [344] Modern cancer vaccines include more than bare tumor lysate, such as cytokine "adjuvants" and other proteins to help activate antigen-presenting cells; however, the optimal design for cancer vaccines is yet to be established.[345] Numerous preclinical *in vivo* mouse studies have demonstrated the potential efficacy of cancer vaccines, but in clinical trials, it has been difficult to design protocols that give reproducible results.[346] Many experimental treatment regimens are complex, multi-step protocols involving "priming" of a patient's autologous dendritic cells with tumor-associated antigens. [347,348] Identification of candidate tumor-associated antigens has proven particularly challenging, and research is turning towards "personalized" cancer vaccines that target autologous tumor antigens, as predicted by RNA-based next-generation sequencing and MHC-binding algorithms. Two recent phase I trials of personalized cancer vaccines in advanced melanoma gave promising results, with 2-year progression-free survival at 67% and 62%, respectively. [349,350]

As much as cancer vaccines seem an appealing and elegant option, the reality of their administration is complex. Further studies are needed to optimize the method by which tumor-associated antigen targets are accurately determined (*e.g.,* peptide vs. RNA discovery), as well as the components and method of delivery of the vaccine.

1.3.1.3 Chimeric Antigen Receptors and Adoptive Cell Transfer

Adoptive cell transfer is the transfer of immune cells into a patient to target tumor cells. The potential to apply adoptive cell transfer to treat cancer was first attempted in mice in 1955,[351] then in humans in 1991, where cytomegalovirus-specific T cells were expanded *ex vivo* and infused into patients, resulting in the establishment of an anti-cytomegalovirus immune response.[352] In modern immunotherapy, adoptive cell transfer most often refers to autologous T cell therapy, which is the extraction of the patient's own T-cells, *ex vivo* expansion (of all T cells or of select clones, based on TCR specificity), and re-infusion for targeted tumor cell killing. In recent years, this protocol has been frequently expanded to include a step in which the patient's T cells are engineered, by viral vector transduction, to express a pre-specified TCR. The engineered T cells, called chimeric antigen receptor T cells (CAR-T cells), can then specifically target the cells expressing tumor-associated antigen(s) they have been programmed to recognize.

CAR-T cell immunotherapy was named the "2018 Advance of the Year" by the American Society of Clinical Oncology, following two pivotal phase II trials of CAR-T cell therapy targeting CD19 in B cell malignancies. In one trial of children and young adults with B cell acute lymphoblastic leukemia (B-ALL), 81% demonstrated a complete response at 28 days, but relapse-free survival was 59% by the 12-month follow-up.[353] Among relapsed patients, 94% occurred due to therapy resistance based on antigen loss, which occurs by selective pressure for CD19 mutants/isoform variants that lack the CAR-T targeted antigen.[354-356] In the second trial,

this time in refractory large B cell lymphoma, complete response was observed in 54% of patients, and only 14% experienced relapse (27% due to antigen loss). [357,358] More recently, a phase 1 trial in pediatric B-ALL targeting another B cell antigen, CD-22, found a complete response rate of 73%.[359] Resistance by antigen escape also occurred in this trial, but in this case, it was a result of downregulated expression of CD22.

The development of antigen escape in CAR-T therapy is a major barrier to therapeutic efficacy, and as such, it has driven the development of multi-antigen-targeted CAR-T cells. A bivalent CD19/CD22-targeted CAR T cell was able direct T cell activity against both cell surface molecules in cell line- and patient-derived xenografts.[360] It is likely that multi-specific CAR-T cells will become increasingly important as a means to circumvent acquired resistance to single-target CAR-T cell therapy.

1.3.2 Immunomodulatory Therapy: Immune Checkpoint Modulators

Immune checkpoint modulators are perhaps the fastest-growing branch of immune-oncology, due to our rapidly expanding knowledge of the effects of co-stimulatory and co-inhibitory molecules on the immune response. The first agent targeting a co-inhibitory immune checkpoint to gain U.S. Food and Drug Administration (FDA) approval – in 2011 – was ipilimumab, a human monoclonal antibody targeting CTLA-4 (cytotoxic T-lymphocyte associated antigen-4). In the seminal trial for ipilimumab in metastatic melanoma, tumor regression was seen in 22% of patients, with 2 partial responses and 1 complete response. [361,362] The subsequent phase II trial in metastatic melanoma showed that treatment with ipilimumab extended median overall survival from 6.4 months to 10.0 months.[363] However, in most of the latest clinical trials, it has become apparent that CTLA-4 inhibitor monotherapy will likely be insufficient to control cancer alone. Figure 1.2 depicts a non-exhaustive schematic of the most

common immune checkpoints – both co-stimulatory (targeted by agonists) and co-inhibitory (targeted by inhibitors) – that are being targeted by recent clinical trials, and their main associated effector cells. Because of the numerous immune checkpoints that a tumor might subjugate to evade immunosurveillance, these alternate immune escape routes are thought to be common mechanisms of resistance to immune checkpoint blockade. New trials of CTLA-4 (and other immune checkpoint) inhibitors are largely trending toward combinatorial therapy regimens, either with another immune checkpoint inhibitor or with other types of immune and/or non-immune treatments.

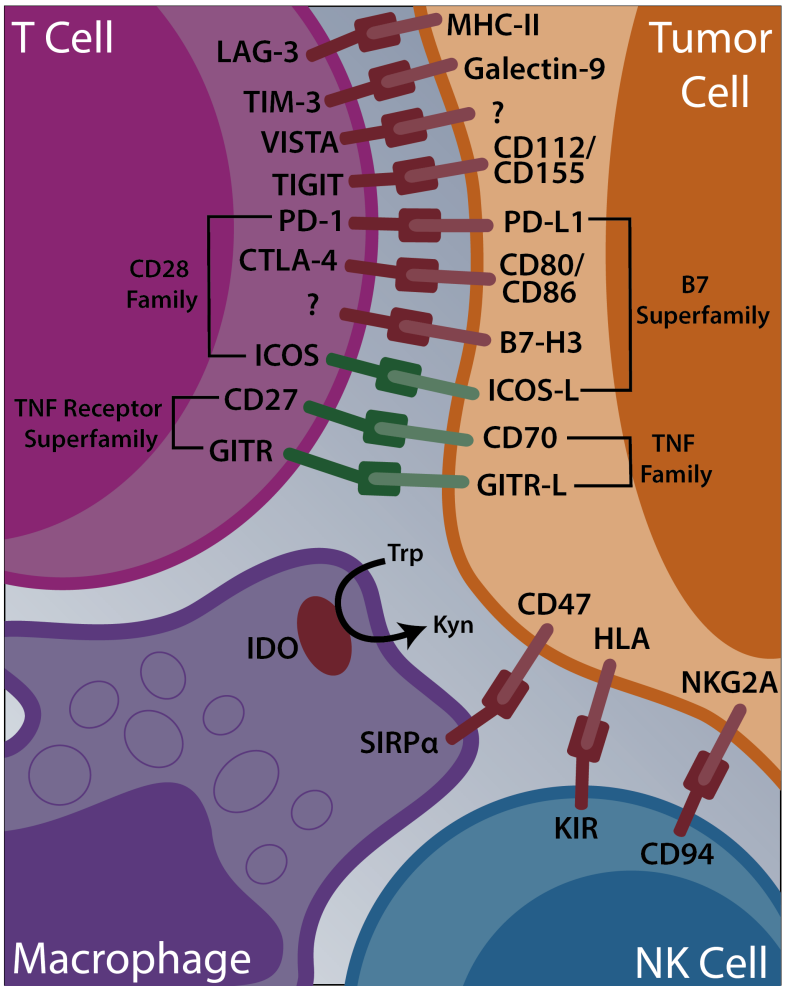


Figure 1.2 Overview of emerging targets for immune checkpoint inhibitor therapy.
Co-inhibitory interactions are marked in red, and co-stimulatory interactions are marked in green. Figure taken from figure 1 of Burugu and Dancsok *et al.* 2017.[364]

1.3.2.1 Programmed-Death 1 (PD-1/PD-L1)

Programmed-death-1 (PD-1, CD279) is an immunosuppressive cell-surface receptor[365] that is expressed by all T cells upon T cell receptor (TCR) activation.[366] Other cell types can express PD-1, such as B cells,[367] dendritic cells,[368] and mast cells.[369] PD-1 has two ligands, PD-L1 (B7-H1, CD274)[370,371] and PD-L2 (B7-DC, CD273),[372,373] both of which can be found on the surface of professional antigen-presenting cells (dendritic cells, macrophages) as well as non-lymphoid tissues. PD-L1 and PD-L2 are upregulated in the presence of IFN-γ.[374] In T cells, PD-1 activation initiates a signaling cascade that dephosphorylates the TCR co-stimulator CD28, inactivating it.[375-378] Following loss of CD28 activity, the T cell undergoes a reduction in cytokine production (IL-2, IFN-γ, TNF-α), cell cycle progression, and, notably, pro-survival Bcl-xL gene expression, leading to T cell apoptosis (Fig. 1.3).[371,375,377,379]

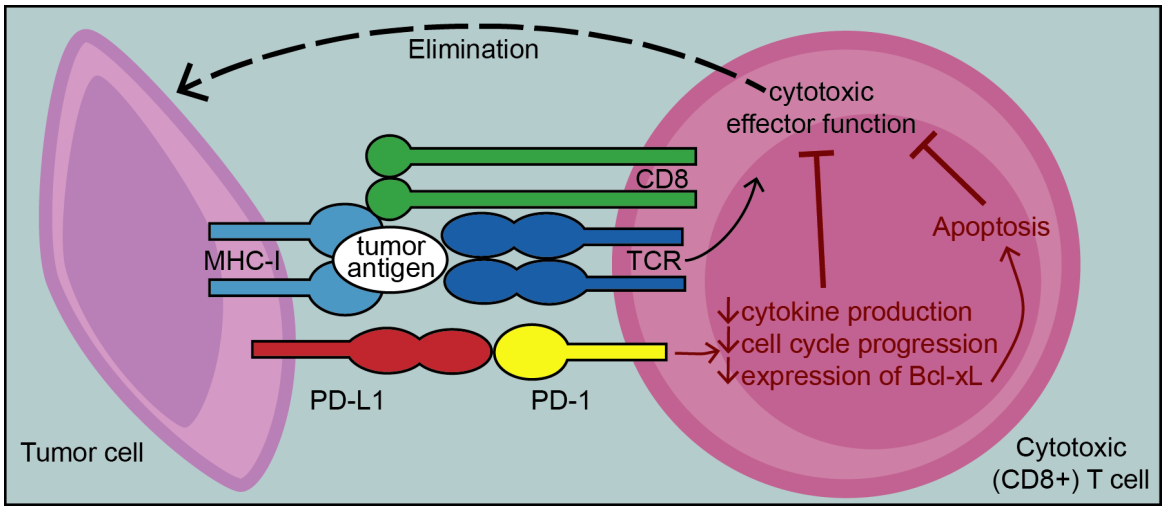


Figure 1.3 Mechanism of action of the programmed-death 1 (PD-1) immune checkpoint.
When T-cell-expressed PD-1 recognizes the co-inhibitory signal PD-L1 on the target cell, a T cell signaling cascade leads to inactivation of CD28, which modulates cytokine expression, cell cycle progression, and pro-survival gene expression.

PD-1 and PD-L1 overexpression have been observed in many cancer types (melanoma, renal cell carcinoma, lung, esophageal, gastric, and ovarian cancers), but studies do not agree on whether it is associated with good or bad prognosis, even within the same diseases; [380-385]

however, a 2013 meta-analysis of 29 studies examining PD-(L)1 expression across carcinomas found both PD-1 and PD-L1 expression to be markers of poor prognosis.[386] PD-1 mediates immune self-tolerance; mice genetically deficient in *Pdcd1* (the mouse PD-1 gene) exhibit accelerated development of autoimmune diseases like type I diabetes, pneumonitis, cardiomyopathy, or lupus.[365,387-389] High expression of PD-(L)1 is common during chronic infections and cancer, and *in vivo* PD-(L)1 blockade reduces tumor burden through improved T cell function in mouse models of melanoma, myeloma, breast, ovarian, and head and neck cancers.[390-394]

The first clinical trial to test a PD-1-targeted monoclonal antibody, nivolumab, was initiated in 2006 for 44 patients with advanced metastatic melanoma, colorectal cancer, castrate-resistant prostate cancer, non-small-cell lung cancer, or renal cell carcinoma.[395] They observed 1 complete response (colorectal cancer) and 2 partial responses (melanoma and renal cell carcinoma). [395] This was followed up by a second phase I trial in advanced melanoma, non–small-cell lung cancer, castration-resistant prostate cancer, or renal-cell or colorectal cancer, which saw a 17% objective response rate (22/129 patients).[396,397] A subsequent phase II trial comparing nivolumab against CTLA-4 inhibitor ipilimumab in metastatic melanoma observed a longer median progression-free survival with nivolumab monotherapy (6.9mos vs. 2.9mos), and the combination of nivolumab+ipilimumab did the best overall (11.5mos median progression-free survival).[398] Since the initial trials, 6 agents targeting either PD-1 or PD-L1 have been approved by the FDA (described in Table 1.6). The number of active clinical trials testing anti-PD(L)1 agents has been growing exponentially, with 2,250 studies registered with the U.S. National Institute of Health clinical trials database as of September 2018.[399]

PD-(L)1 blockade has become central to the diverse therapeutic approaches and combinations in the field of immune-oncology. Among the 2,250 active clinical trials of PD-(L)1 inhibitors, 1,716 are in combination with other cancer therapies: 339 with anti-CTLA4 agents, 283 with chemotherapy, 114 with radiotherapy, and 58 with chemoradiotherapy.[399] CTLA-4 inhibitors are the most common combinatorial agent with PD-(L)1 inhibitors, likely partially owing to the famous success of the phase III trial of nivolumab and ipilimumab in advanced metastatic melanoma, which saw 58% 3-year overall survival in the combination arm, compared to 34% in the ipilimumab-only group.[400] PD-(L)1 inhibitors are beginning to be combined with novel immune checkpoints as well, such as LAG-3 and TIM-3, discussed below.

Agent	Target	Highest Phase	Diseases included in clinical trials
Nivolumab	PD-1	FDA-approved	Melanoma, non-small cell lung cancer, renal cell carcinoma, Hodgkin lymphoma, pancreatic and endometrial cancers, sarcoma, solid tumors
Pembrolizumab	PD-1	FDA-approved	Melanoma, non-small cell lung cancer, renal cell carcinoma, breast, ovarian, bladder, rectal, and Merkel cell cancers, CML, glioma, head and neck cancer, sarcoma, solid tumors
Atezolizumab	PD-L1	FDA-approved	Non-small cell lung cancer, renal cell carcinoma, breast, colorectal, ovarian, bladder, and pancreatic cancers, CLL, non-Hodgkin lymphoma, malignant mesothelioma, multiple myeloma, solid tumors
Avelumab	PD-L1	FDA-approved	Non-small cell lung cancer, breast cancer, bladder cancer, endometrial cancer, follicular lymphoma, malignant mesothelioma, Merkel cell cancer, head and neck cancer, osteosarcoma, solid tumors
Durvalumab	PD-L1	FDA-approved	Lung, colorectal, breast, pancreatic, thyroid, endometrial, prostate, liver, and head and neck cancers, B-cell cancers, solid tumors
Cemiplimab	PD-1	FDA-approved	Lung cancer, squamous cell carcinoma, cervical, prostate, ovarian, and head and neck cancers, glioma, lymphoma, myeloma, solid tumors
Spartalizumab (PDR001)	PD-1	Phase III	Non-small cell lung cancer, gastric, breast, ovarian, and pancreatic cancers, hematologic cancers, non-Hodgkin lymphoma, solid tumors
Tislelizumab	PD-1	Phase III	Squamous cell carcinoma, hepatocellular carcinomas, lymphoma
TSR-042	PD-1	Phase III	Ovarian cancer, solid tumors
Pidilizumab	PD-1	Phase II	Melanoma, renal cell carcinoma, hepatocellular carcinoma, pancreatic and prostate cancers, glioma, AML, multiple myeloma, lymphoma
MEDI0680	PD-1	Phase I/II	B-cell lymphomas, advanced malignancies, solid tumors
AMP-224	PD-L2	Phase I	Colorectal cancer, advanced cancer
BMS936559	PD-L1	Phase I	Melanoma, hematologic malignancy
CK301	PD-L1	Phase I	Advanced cancers
Sym021	PD-1	Phase I	Lymphoma, solid tumors

Table 1.6 Select clinical trials for PD-1/PD-L1 inhibitors.

1.3.2.2 Lymphocyte-Activation Gene 3 (LAG-3)

Lymphocyte activation gene 3 (LAG-3) is a surface receptor expressed on activated T cells, with immunosuppressive activity. Major histocompatibility complex class II (MHC-II) is the main ligand for LAG-3, though additional ligands (L-selectin, galectin-3) have also been identified.[401] Regulatory T cells (Tregs) expressing LAG-3 have enhanced suppressive activity, whereas cytotoxic CD8+ T cells expressing LAG-3 have lower proliferation rates and reduced effector cytokine production (Fig. 1.4).[402-404] A splice variant of LAG-3 secreted in the cellular microenvironment has immune-activating properties when bound to MHC-II on antigen presenting cells.[405]

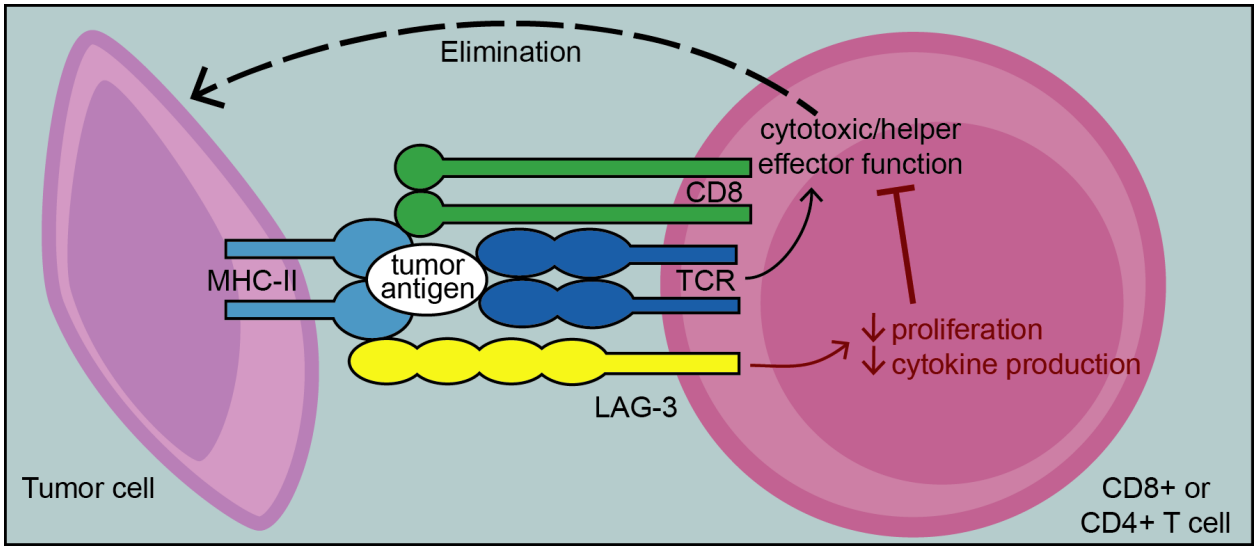


Figure 1.4 Mechanism of action of the lymphocyte activation gene 3 (LAG-3) immune checkpoint. T-cell-expressed LAG-3 interacts with major histocompatibility (MHC) class I, downregulating CD8+ T cell proliferation and cytokine production, leading to reduced capability of exerting its effector function.

LAG-3+ tumor-infiltrating lymphocytes (TILs) have been reported in melanoma, colon, pancreatic, breast, lung, hematopoietic, and head and neck cancers, [406-409] in association with aggressive clinical features. Antibody-based LAG-3 blockade *in vivo* restores CD8+ effector T cells and diminishes Treg populations, an effect enhanced when combined with anti-PD-1.[410,411] A study in a metastatic ovarian cancer mouse model showed that LAG-3 blockade leads to

upregulation of other immune checkpoints (PD-1, CTLA-4, and TIM-3), and combination therapy targeting LAG-3, PD-1, and CTLA-4 increases functional cytotoxic T cell levels while reducing Tregs and myeloid-derived suppressor cells.[412]

Multiple phase I and II clinical trials are testing anti-LAG-3 agents, mostly in combination with anti-PD-1 and/or anti-CTLA-4 therapy (Table 1.7). Initial LAG-3 trials focused on eftilagimod alpha ("efti," formerly IMP321), a recombinant LAG-3-IgG1 antibody fusion protein that acts by binding to MHC class II to mediate antigen-presenting cell activation, and therefore, cytotoxic T-cell activation.[413] Efti was tested in advanced solid malignancies,[414] and demonstrated sufficient tolerability and efficacy to warrant advancement to phase II. The most recent clinical results for efti report the results of a phase II trial in breast cancer (NCT02614833), in which 7 patients (47%) had a partial response to a combination treatment of efti and paclitaxel.[415] The first monoclonal antibody therapy developed against LAG-3, relatlimab (formerly BMS03440437), was tested in a phase I/IIa trial in combination with PD-1 inhibitor nivolumab in a variety of advanced solid and hematologic malignancies (NCT01968109). They reported the results for the cohort of patients with advanced melanoma who had previously progressed on anti-PD-(L)1 therapy, and observed an objective response (1 complete response and 6 partial responses) in 11.5% of the cohort.[416] The results from a phase I/II trial of another anti-LAG-3 monoclonal antibody, LAG525 (NCT02460224), in advanced malignancies reported durable objective responses (1 complete response and 11 partial responses) among 121 patients receiving a combination of LAG525 and a PD-1 inhibitor.[417] Phase II trials will select disease types that responded in this trial, including triple-negative breast cancer and mesothelioma.[417] Recently, a phase 1 trial opened up for FS119, a bispecific

antibody that targets both PD-1 and LAG-3 in patients with advanced cancer that has progressed on/after PD-(L)1 therapy (NCT03440437).

Agent	Phase	Disease(s)	Other agents	Identifier
Relatlimab (BMS986016)	1/2	Advanced solid + hematologic cancer	Anti-PD-1	NCT01968109
	1/2	Hematologic malignancies	Anti-PD-1	NCT02061761
	2	Microsatellite stable colorectal cancer	Anti PD-1	NCT03642067
	2	Mismatch repair deficient cancer	Anti-PD01	NCT03607890
	2	Colorectal cancer	Anti PD-1, Anti-CTLA-4 Anti-MEK, Anti-CD38	NCT02060188
	2	Metastatic melanoma	Anti-PD-1	NCT03743766
	2	Advanced surgically-resectable melanoma	Anti-PD-1, Anti-CTLA-4	NCT02519322
	2	Advanced chordoma	Anti-PD-1	NCT03623854
	1/2	Virus-associated tumors	Anti-PD-1	NCT02488759
	2	Advanced gastric cancer	Anti-PD-1	NCT02935634
	2	Advanced gastric cancer	Anti-PD-1, chemotherapy	NCT03662659
	1	Gastric and esophageal cancer before systemic ablative radiation	Anti-PD-1, chemotherapy	NCT03044613
	1/2	Gastroesophageal cancer after systemic ablative radiation	Anti-PD-1	NCT03610711
	2	Advanced non-small cell lung cancer	Anti-PD-1	NCT02750514
	2	Advanced renal cell carcinoma	Anti-PD-1	NCT02996110
	1	Recurrent brain neoplasms	Anti-PD-1	NCT02658981
	1	Advanced solid malignancies	Anti-PD-1	NCT02966548
	1/2	Advanced malignant tumors	Anti-PD1, Anti-CTLA4	NCT03459222
	1/2	Advanced malignant tumors	Anti-PD-1, Anti-CTLA-4, Anti-CD-38	NCT02488759
Eftilagimod alpha (IMP321)	1	Metastatic breast cancer	Paclitaxel	NCT00349934
	2	Metastatic breast cancer	Paclitaxel	NCT02614833
	1	Solid malignancies	-	NCT03252938
	1	Metastatic renal cell carcinoma	-	NCT00351949
	2	Non-small cell lung cancer, head and neck squamous cell carcinoma	Anti-PD-1	NCT03625323
	1	Advanced melanoma	Anti-PD-1	NCT02676869
LAG525	1/2	Advanced solid malignancies	Anti-PD-1	NCT02460224
	2	Advanced solid + hematologic cancer	Anti-PD-1	NCT03365791
	1	Triple-negative breast cancer	Anti-PD-1, Anti-A2A-R, Anti-HGFR, Anti-M-CSF, Anti-IL-1β	NCT03742349
REGN3767	1	Progressive, immune-checkpoint-naive	Anti-PD-1	NCT03005782
Sym022	1	Lymphoma, solid tumors	-	NCT03489369
	1	Lymphoma, solid tumors	Anti-PD-1, Anti-TIM-3	NCT03311412
TSR-033	1	Advanced solid malignancies	Anti-PD-1	NCT03250832
	1	Advanced solid malignancies	Anti-PD-1, Anti-TIM-3	NCT02817633
INCAGN02385	1	Advanced malignancies	-	NCT03538028

Table 1.7 Clinical trials for LAG-3 inhibitors.

1.3.2.3 T-cell Immunoglobulin and Mucin-Domain-Containing 3 (TIM-3)

T-cell Immunoglobulin- and Mucin-domain-containing molecule 3 (TIM-3) is an immune-inhibitory molecule first identified on CD4+ Th1 (helper) T-cells and CD8+ Tc1 (cytotoxic) T-cells,[418] then later on regulatory T-cells[419,420] and innate immune cells.[421-423] TIM-3 is activated primarily by its widely-expressed ligand galectin-9, which can be secreted or expressed on the cell surface,[424] leading to immune cell death through calcium influx, cellular aggregation, and apoptosis (Fig. 1.5).[425] When TIM-3 signaling is active, T-cells become exhausted, resulting in Th1 suppression and immune tolerance.[425-427] TIM-3 expression is observed during chronic infection, as a characteristic marker of exhausted T cells.[428-432]

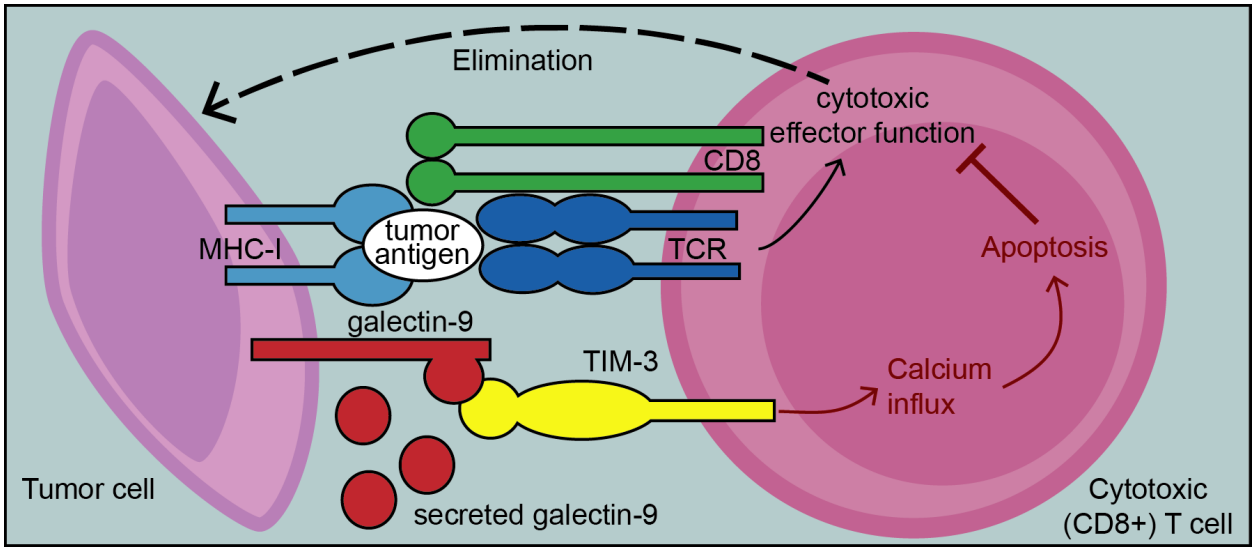


Figure 1.5 Mechanism of action of the T-cell Immunoglobulin- and Mucin-domain-containing molecule 3 (TIM-3) immune checkpoint.
T-cell-expressed TIM-3 interacts with target-cell-expressed galectin-9 (free or membrane-bound), causing an influx of calcium, cellular aggregation, and apoptosis.

In cancer, tumor-infiltrating lymphocytes expressing TIM-3 have been observed in melanoma,[433,434] non-Hodgkin lymphoma,[435] lung,[419] gastric,[436,437] breast,[438] and other cancers.[439-442] In these studies, TIM-3 is co-expressed with PD-1 and associated with effector T-cell exhaustion and dysfunction. This phenomenon is also observed in mouse models of solid[443]

and hematologic[444] cancers, where TIM3+PD1+CD8+ T-cells exhibit an exhausted phenotype characterized by reduced proliferation and defective production of IL-2, TNFα, and IFN-γ. In contrast, TIM-3+ regulatory T-cells display increased expression of effector molecules and are more immunosuppressive than their TIM-3- counterparts.[445,446]

Inhibition of TIM-3 alone tends to have little effect on tumor growth in pre-clinical mouse models, despite some evidence supporting a reversal of immune cell exhaustion.[433,443,447-449] However, combined targeting of PD-1 and TIM-3 leads to a substantial reduction in tumor growth – better than either pathway alone – in numerous preclinical *in vivo* models,[433,443,444,449] supporting the concept that malignant cells become resistant to PD-1 checkpoint blockade by activating another immune checkpoint. Indeed, mouse models partially responsive to PD-L1 inhibition upregulated TIM-3 expression in resistant tumors,[441,450] and addition of TIM-3 blockade was successful in overcoming that resistance. Upregulation of TIM-3 has also been observed in patients receiving PD-L1 monotherapy.[450]

Several early phase clinical trials are underway that attempt to combine anti PD-L1 therapy with agents targeting TIM-3, but results have yet to be presented (Table 1.8). Like with LAG-3, a phase I clinical trial has just been launched for a bispecific antibody that targets both TIM-3 ad PD-1, RO7121661, in advanced and/or metastatic solid tumors (NCT03708328). Additionally, a phase 1a/1b clinical trial has opened for patients with advanced solid tumors for a different bispecific antibody, LY3415244, which targets TIM-3 and PD-L1 (NCT03752177).

Agent	Phase	Disease(s)	Other agents	Identifier
TSR-022	1	Advanced solid malignancies	Anti-PD-1, Anti-LAG-3	NCT02817633
	2	Adult primary liver cancer	Anti-PD-1	NCT03680508
	1	Advanced or metastatic malignancies	Anti-PARP, Anti-PD-1, Anti-VEGFA, chemotherapy	NCT03307785
LY3321367	1	Advanced relapsed/refractory solid malignancies	Anti-PD-L1	NCT03099109
MBG453	1-1b/2	Advanced/metastatic solid malignancies	Anti-PD-1	NCT02608268
	1	Acute myeloid leukemia or high-risk myelodysplastic syndrome	Anti-PD-1	NCT03066648
Sym023	1	Lymphoma, solid tumors	-	NCT03489343
	1	Lymphoma, solid tumors	Anti-PD-1, Anti-LAG-3	NCT03311412
BGB-A425	1/2	Advanced or metastatic solid tumors	Anti-PD-1	NCT03744468
INCAGN02390	1	Advanced malignancies	-	NCT03652077

Table 1.8 Clinical trials for TIM-3 inhibitors.

1.3.2.4 Signal-Regulatory Protein alpha (SIRPα) / CD47

CD47, first identified as Integrin-Associated Protein (IAP),[451-454] is a cell-surface immunoglobulin that negatively regulates anti-tumor immunity through suppression of phagocytosis. Expressed ubiquitously in normal tissues,[455] CD47 functions in part to protect viable erythrocytes from phagocytosis.[456-461] Signaling occurs by interaction with its receptor SIRPα (signal-regulatory protein alpha), a cell-surface immunoglobulin mainly expressed by macrophages and dendritic cells.[462] Activation of SIRPα by CD47 suppresses phagocytosis by activating SHP1/2 (SH2-domain-containing tyrosine phosphatases 1 and 2), which inhibits myosin-II, preventing contraction with F-actin for macrophage phagocytic movement (Fig. 1.6).[463] T-cell activation is secondarily decreased as an indirect result of reduced tumor cell ingestion by antigen-presenting cells;[464] furthermore, activation of CD47 on naïve T-cells promotes the formation of Tregs[465,466] and inhibits formation of T helper 1 effector cells.[467]

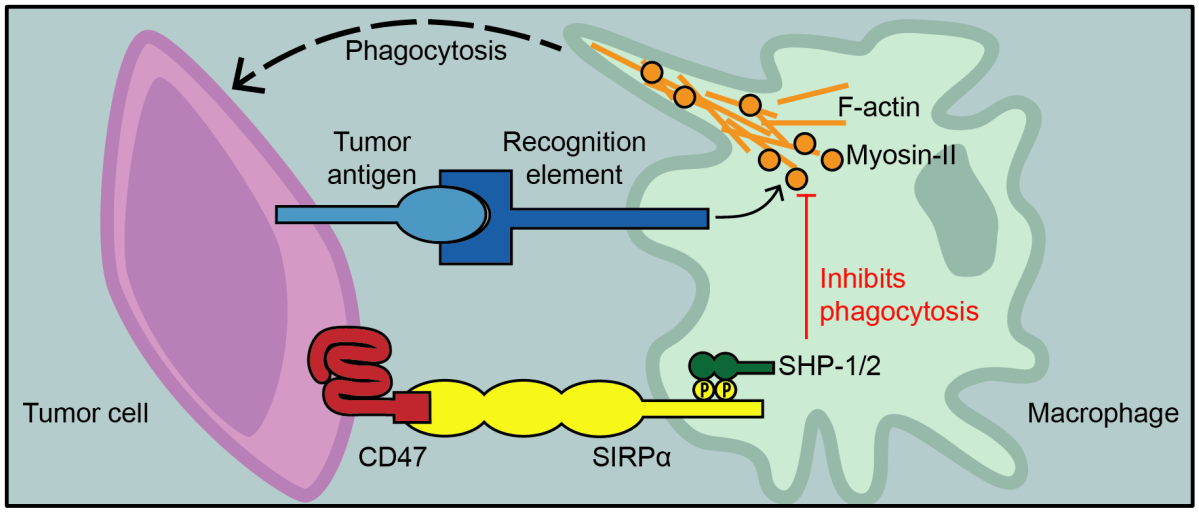


Figure 1.6 Mechanism of the macrophage-associated "Don't eat me" immune checkpoint CD47/SIRPα. Tumor-expressed CD47 ligand binds to SIRPα receptor on macrophages, inducing cytoplasmic tyrosine-phosphorylation. Phosphorylated SIRPα recruits and activates SH2-domain-containing tyrosine phosphatases SHP-1 and SHP-2, which in turn dephosphorylate specific protein substrates – such as myosin-II – thereby inhibiting Factin-myosin contractility.

Overexpression of CD47 has been observed across most cancers,[468-475] suggesting that malignant cells exploit the CD47/SIRPα "don't eat me" signal to evade phagocytosis. In translational studies, high *CD47* mRNA expression levels correlate with poor clinical outcomes.[473,475-483] *In vitro,* CD47/SIRPα blockade induces phagocytosis of cancer cells by human and mouse macrophages.[471-473,475,476] Anti-CD47 monoclonal antibodies have impressive activity in xenograft models,[471-476,484,485] although because human CD47 binds exceptionally well to the mouse SIRPα,[486,487] some studies may overestimate the degree of efficacy.[488]

Many early phase clinical trials are in progress targeting the CD47/SIRPα axis (Table 1.9), either by monoclonal antibody directed against CD47 or by an engineered fusion protein between the CD47-binding domain of SIRPα and the Fc domain of an IgG antibody. One such anti-CD47 antibody, Hu5F9-G4 has generated positive results in two different clinical trials. In a phase Ib study of 22 non-Hodgkin lymphoma patients treated with Hu5F9-G4 with or without CD20 inhibitor rituximab, 50% of the patients had an objective response (36% complete

response) (NCT02953509).[489] In another phase I trial in 16 patients with advanced solid tumors, Hu5F9-G4 as monotherapy was well tolerated, and stable disease was observed for two patients, lasting 8 and 16 months, respectively (NCT02216409). [485,490] Results of a phase I study of TTI-621, one of the recombinant SIRPα-Fc fusion proteins, in relapsed/refractory hematologic malignancies report progression-free survival of 161 and 70 days respectively in a patient with diffuse large B-cell lymphoma and a patient with follicular lymphoma.[491]

Agent	Mechanism of Action	Phase	Disease(s)	Other Agents	Identifier
Hu5F9-G4	Anti-CD47 mAb	1	Advanced solid malignancies	-	NCT02216409
		1	Relapsed/refractory acute myeloid leukemia	-	NCT02678338
		1b/2	Solid malignancies, advanced colorectal cancer	Anti-EGFR	NCT02953782
		1b/2	Relapsed/refractory B-cell non-Hodgkin's lymphoma	Anti-CD20	NCT02953509
		1	Acute myeloid leukemia, myelodysplastic syndrome	chemotherapy	NCT03248479
TTI-621	SIRPα-IgG Fc fusion	1	Relapsed/refractory solid malignancies	Anti-PD-(L)1, peg-IFN-α2a, radiation, oncolytic virus	NCT02890368
		1a/1b	Hematologic malignancies	Anti-PD-1, Anti-CD20	NCT02663518
TTI-622	SIRPα-IgG Fc fusion	1	Advanced lymphoma or myeloma	Anti-PD-(L)1, Anti-CD20, Proteasome-i	NCT03530683
CC-90002	Anti-CD47 mAb	1	Advanced solid and hematologic malignancies	Anti-CD20	NCT02367196
		1	Acute myeloid leukemia, myelodysplastic syndrome	-	NCT02641002
ALX148	SIRPα-IgG Fc fusion	1	Advanced solid malignancies, lymphoma	Anti-PD-L1, Anti-HER2	NCT03013218
IBI188	Anti-CD47 mAb	1	Advanced malignancies	Anti-CD20	NCT03717103
		1	Advanced malignant tumors and lymphomas	-	NCT03763149
AO-176	Anti-CD47 mAb	1	Solid malignancies	-	NCT03834948
SRF231	Anti-CD47 mAb	1	Advanced solid and hematologic cancers	-	NCT03512340

Table 1.9 Clinical trials for CD47/SIRPα inhibitors.

1.3.3 Markers of Response to Immunotherapy

The clinical promise shown by immune checkpoint inhibitors in clinical trials has raised a lot of excitement around these still-poorly-understood new therapies. Responses to immune checkpoint inhibition have been heterogeneous, even within a specific diagnosis. Development of predictive biomarkers to select patients likely to respond to therapy is badly needed, to help minimize the risk of toxicities, inform combinatorial therapies (when choosing from an ever-growing list of options), and maximize patient benefit,[492] particularly within a public healthcare system with limited resources (and presently, a very large price tag on immunotherapeutics). Most recent clinical trials of immune checkpoint inhibitors have been orchestrating large correlative study plans in an effort to identify a reliable, generalizable biomarker to differentiate responders from non-responders. Some commonly used approaches and investigated biomarkers include target levels determined by immunohistochemistry (PD-1 expression, PD-L1 expression, TIL count), tumor mutational burden, and T cell receptor repertoire.

1.3.3.1 Immunohistochemistry

Direct assessment of tumor cell PD-L1 expression is a logical and heavily-researched biomarker for predicting response to anti-PD-(L)1 therapies. The first phase 1 trial of nivolumab showed promise for PDL1 as a predictive biomarker. The study defined PD-L1 positivity as >5% tumor cell staining, and they found that 36% (9/25) of the PDL1-positive patients – but 0% of the PDL1-negative patients – showed an objective response.[397] Unfortunately, subsequent studies have revealed that use of PD-L1 as a predictive biomarker is not as straightforward as expected,[493] particularly after the pharmaceutical companies sponsoring the different PD-(L)1 inhibitors each developed their own proprietary companion assays for measuring PD-L1 "positivity", all with different methods, thresholds, and antibody clones. For nivolumab, because

of the strong association between PD-L1 expression and response to therapy, the companion assay defined the positivity threshold as > 5% tumor cell membrane PD-L1 expression with clone 28-8. [494] The pembrolizumab assay also defined PD-L1 positivity as tumor cell expression, but their cutoff was > 1% tumor cell membrane expression with clone 22c3.[495] The atezolizumab assay measures PD-L1 expression on immune cells (as opposed to tumor cells) by clone SP142, because that metric predicted benefit for their agent in patients with bladder cancer.[496] Adding yet more complexity, studies have repeatedly shown that "PDL1-negative" patients can still benefit from PD-(L)1 therapies, with objective responses within this population ranging from 11% to 41%.[493] Despite these limitations, PD-L1 immunohistochemistry still plays a role in the stratification of patients included in PD-(L)1 inhibitor trials.

Tumor-infiltrating lymphocytes (TILs) and/or TIL subsets are another immunohistochemically-detected measure commonly explored as a potential prognostic biomarker. Lymphocyte infiltration has been shown to be associated with clinical benefit from immunotherapies such as the MAGE-A3 cancer vaccine and high-dose IL-2 therapy.[497] The KEYNOTE-001 study[498] was a phase I clinical trial of pembrolizumab in melanoma. Pretreatment tumor samples showed higher CD8+ TIL densities in responding patients than in patients with disease progression.[499] Furthermore, there was an increase in CD8+ TIL density in serial biopsy samples of tumors over the course of treatment in the responding group, but not in the disease progression group.[499] This trend was confirmed by another study of melanoma patients on anti-PD-1 therapy, which showed a modest association of pretreatment CD8+ TIL densities between responders and non-responders, and after anti-PD-1 treatment, the associations were more significant.[500] However, the challenge in this study is demonstrative of the main issue with using TILs as a predictive biomarker: baseline CD8+ TIL densities overlapped between

responders and those with disease progression, hindering the establishment of an absolute cutoff as a clinically useful predictive biomarker. Furthermore, TILs do not uniformly infiltrate tumors, as studies have shown greater TIL densities at tumor invasive margins than at the centre, the location from which most core-needle biopsies are taken.[501] It is also difficult by immunohistochemistry alone to decipher the functional roles of infiltrating lymphocytes and whether they are involved in anti-tumoral immunity or are simply bystanders.

1.3.3.2 Tumor Mutational Burden

Somatic mutations in tumor cells generate neoantigens capable of activating the adaptive immune response.[502] Intra-tumoral levels of perforin-1 and granzyme-B (presumably produced by NK and T cells) have been shown to correlate with high tumor mutational burden,[503] suggesting that tumors with high mutational loads might be more responsive to immunotherapy. Melanoma and non-small cell lung cancer are among the most responsive cancer types to immune checkpoint therapy, and both are known for having high levels of somatic mutations.[504] In a phase II trial of pembrolizumab in colorectal cancer,[505] the objective response among patients with mismatch repair (MMR)-deficient tumors (and therefore, >20 times the mutational burden) was 50%, versus 0% among patients with MMR-proficient tumors.[506] In that trial, patients with MMR-deficient tumors also had longer overall and progression-free survival on pembrolizumab, compared to MMR-proficient tumors.[506]

The use of mutational/neoantigen burden has been studied as a predictive biomarker in checkpoint inhibitor clinical trials. In a study of advanced melanoma treated with ipilimumab or tremilimumab, high mutational burden (>100 non-synonymous somatic mutations by whole-exome sequencing) was associated with long-term clinical benefit (responsive or stable disease for 6months) and better overall survival.[507] A similar study of ipilimumab in melanoma also

showed an association between mutational burden (>100 non-synonymous somatic mutations) and clinical benefit; however, of the 75,179 unique neoantigens they identified, only 28 (0.04%) were common to >1 patient, suggesting that the neoantigens associated with benefit from immunotherapy are largely patient-specific.[508] This association has also been demonstrated with anti-PD-(L)1 therapy. A study of non-small cell lung cancer treated with pembrolizumab showed an association between high mutational burden (≥178 non-synonymous mutations) and clinical benefit (responsive or stable disease >6 months),[509] and a study of advanced melanoma treated with nivolumab, pembrolizumab, or atezolizumab showed an association between high mutational burden (by next-generation sequencing) and better overall and progression-free survival (as well as objective response).[510] With these studies and more, tumor mutational burden has emerged as a strong predictor of response to immune checkpoint blockade; however, there is not yet a consensus regarding methods or threshold for determining high mutational burden, and the cost and workload involved in measuring mutational/neoantigen load may be too high for routine clinical use.

1.3.3.3 T-cell Receptor (TCR) Repertoire

Another potential biomarker currently under investigation for use in clinical trials is the quantitative sequencing of variable (antigen recognition) regions of the T cell receptors (TCR) of TILs and/or peripheral lymphocytes. T cell repertoire diversity is commonly quantified using the Shannon entropy metric, which has 2 complementary components: 1) "richness," the number of unique sequences, and 2) "clonality" or "evenness," the distribution of unique sequences, wherein low clonality/high evenness indicates equal numbers of all clones, and high clonality/low evenness indicates a skewed population, with a few clones predominating.[511,512] In a study by Tumeh *et al,* melanomas treated with pembrolizumab underwent targeted TCR β-

chain sequencing, and they found that among responders, the pre-treatment TCR repertoire was more clonal (less diverse) than in those with disease progression.[499] This was supported by another study in melanoma that showed that higher baseline TCR clonality was predictive of response to PD-1 (but not CTLA-4) blockade[513] and by a study in non-small cell lung cancer treated with nivolumab, wherein responders had higher TCR clonality than non-responders at the time of resection (after initiating therapy).[514] Furthermore, Tumeh *et al* also found that comparison of the pre-treated TCR repertoire with that obtained from post-treatment biopsy samples showed a 10-fold increase in clonal TCR sequences (reflecting the clonal expansion of those T cells) in the responding group, suggesting a tumor-specific response to therapy.[499] A similar observation was made in nivolumab-treated metastatic melanoma.[515]

Contrastingly, one study in melanoma found no associations between baseline TCR repertoire and anti-PD1 response or survival, but the investigators did not examine on-therapy changes.[516]

A retrospective analysis of the trial of advanced melanoma treated with nivolumab, pembrolizumab, or atezolizumab saw no correlation between TCR diversity and response to PD-1 inhibitor therapy;[510] however, the investigators speculated that their archival tumor samples might not have accurately represented the tumor microenvironment. Further complicating matters, prior exposure to immunotherapy may influence TCR dynamics during checkpoint inhibitor therapy. In a study of nivolumab in patients with advanced melanoma, investigators compared patients who had previously progressed on ipilimumab with those were ipilimumab-naïve.[511] While they observed no significant difference in baseline TCR diversity between responders and non-responders or between the cohorts (ipilimumab-naïve vs ipilimumab-progressive), on-therapy biopsies demonstrated significant changes in TCR richness and

clonality that were dependent on prior therapy. On-therapy TCR richness increased among responders that had prior exposure to ipilimumab, but not among non-responders or ipilimumab-naïve patients. On-therapy TCR clonality also increased among responders, but only those who were ipilimumab-naïve.[511]

Assessment of peripheral T-cell populations might also have a potential role as a predictive biomarker, but presently, sequencing data from the peripheral blood is somewhat limited. In a pilot study, the pretreatment peripheral blood TCR repertoire diversity was assessed for a trial of ipilimumab in metastatic melanoma.[517] Peripheral TCR richness and evenness (*i.e.,* low clonality) were both significantly associated with clinical benefit (responsive or stable disease for ≥9 months), but not with overall survival, based on β-chain TCR sequencing.[517] A study of atezolizumab in urothelial cancer found that baseline peripheral TCR evenness was associated with improved overall and progression-free survival (though intratumoral TCR clonality did not associate with survival).[518] Similarly, a study in metastatic pancreatic cancer found that peripheral TCR evenness was associated with survival in patients treated with anti-CTLA-4 (not anti-PD-1) therapy.[519]

Clearly, the TCR repertoire is closely involved in response to immune checkpoint blockade; however, similar to the other predictive markers discussed, it is challenging to find a defined threshold that could serve as a clear clinical predictive biomarker.[520]

1.4 Immunotherapy in Sarcomas

Sarcomas played a key role in the foundational studies of cancer immunotherapy. William B. Coley, considered the "father of immunotherapy," discovered in 1891 that injection of streptococcal bacteria into a malignant tumor (a periosteal round-cell sarcoma of the

metacarpal bone) caused an infection, with the "side effect" of tumor shrinkage.[521] Coley, a sarcoma surgeon, proceeded to treat over 1000 patients with bacteria or bacterial products – known as Coley's Toxins – and reported such excellent outcomes that many of his contemporary doctors did not believe his results.[522] However, modern immunology research has since confirmed that Coley's principles were in fact correct. However, despite having been the first cancers to be successfully treated by Coley's immunotherapy, adoption of the exciting modern immunotherapies has been slow in sarcomas.[523]

1.4.1 Sarcoma Immune Microenvironment

Sarcomas have been shown to have fewer tumor-infiltrating lymphocytes (TILs) than renal cell carcinoma, breast cancer, or colorectal cancer.[524] Studies characterizing the tumor immune microenvironment in sarcomas have generally lagged behind other diseases, and are mainly limited to evaluations of small numbers of cases with unequal (and disproportionate) coverage of the sarcoma subtypes. As such, the "big picture" for the sarcoma immune microenvironment has yet to be fully described.

The largest study that descriptively quantifies immune infiltrates in sarcomas by immunohistochemistry is a mixed-subtype study of 50 cases (14 GIST, 5 synovial sarcoma, 4 leiomyosarcoma, and 1-3 cases of 18 other subtypes) and found TILs (CD3+ staining by immunohistochemistry) in 49 cases (98%) and TAMs (determined qualitatively) in 45 cases (90%).[525] They also characterized TIL subsets (by CD4, CD8, and FOXP3 staining), but with such low numbers for the various sarcoma subsets, it is difficult to draw any tangible conclusions. A larger mixed-subtype study of 208 cases that looked only at CD8 staining (as well as PD-1 and PD-L1, discussed later) included 46 osteosarcoma, 32 Ewing sarcoma, 20 alveolar rhabdomyosarcoma, 77 embryonal rhabdomyosarcoma, 22 synovial sarcoma, and 11 DSRCT

(desmoplastic small round cell tumors).[526] They noted that CD8 expression varied by subtype, with the most prominent expression in DSRCT (82% CD8-high) and osteosarcoma (35% CD8-high). [526] Isolated subtype-specific studies have been done in angiosarcoma (n=55),[527] chondrosarcoma (n=26),[528] chordoma (n=54, n=78), [529,530] Ewing sarcoma (n=217),[531] GIST (n=129),[532] leiomyosarcoma (n=11),[533] MPNST (n=53), [534] myxofibrosarcoma (n=150),[535] osteosarcoma (n=129, n=150) [536,537], synovial sarcoma (n=29),[538] UPS (n=17),[539] and well/de-differentiated liposarcoma (n=8); [540] however, differences of methodology and study goals make the results difficult to compare, as most of these studies discuss the survival correlations of the biomarkers, without reporting biomarker expression in detail.

The presence of TILs has been associated with improved outcomes in studies of angiosarcoma,[527] chordoma,[530] Ewing sarcoma,[541] synovial sarcoma,[542] and GIST,[543] and as well as cohorts of mixed subtypes including leiomyosarcoma, synovial sarcoma, liposarcoma, and undifferentiated pleomorphic sarcoma, among others.[544] One such study looked at 249 soft-tissue sarcomas (68 UPS, 67 leiomyosarcoma, 34 liposarcoma, 20 malignant fibroblastic/myofibroblastic tumors, 16 rhabdomyosarcoma, 16 synovial sarcoma, 13 angiosarcoma, 11 MPNST, and 4 other) for expression of CD3+ (to represent T cells), CD4+, CD8+, CD20+, and CD45+ lymphocytes by immunohistochemistry. CD20+ B cells associated with improved disease-free survival, whereas low CD3+ and CD4+ T-cell infiltrations were associated with better overall survival.[544] Notably, some studies have shown no effect or worse survival with presence of TILs, including GIST, leiomyosarcoma, and undifferentiated pleomorphic sarcoma.[545,546]

Tumor-associated macrophages were assessed by gene expression profiling of 51 various soft tissue sarcomas (10 tenosynovial giant cell tumor, 8 GIST, 8 desmoid-type fibromatosis, 8

leiomyosarcoma, 7 synovial sarcoma, 5 solitary fibrous tumor, and 5 DFSP), and high levels of TAM-associated genes were expressed in all 8 cases of leiomyosarcoma.[547] TAM infiltration in 149 leiomyosarcomas was subsequently assessed by IHC (CD68 and CD163), and a high density of TAMs was associated with significantly worse disease-specific survival among the soft-tissue leiomyosarcomas.[547] Later studies from the same group found a significant association between expression of a TAM-associated 4-gene signature and poor prognosis in leiomyosarcoma. [548,549] Similarly, poor prognosis was associated with high levels of CD68+ TAMs in studies of both myxoid liposarcoma (n=78)[550] and Ewing sarcoma (n=41).[551] In GIST, M2 macrophages were found to be the most abundant immune infiltrate by CD163 immunohistochemistry.[552] In a study in 36 synovial sarcomas, higher levels of CD163+ TAMs were associated with worse overall- and progression-free survival.[542]

Gene expression profiling studies have also attempted to characterize the sarcoma microenvironment. Pollack *et al* performed gene expression and TCR sequencing on 81 soft tissue sarcomas (27 liposarcoma, 20 UPS, 19 leiomyosarcoma, and 15 synovial sarcoma).[553] Antigen presentation and T-cell infiltration genes were most highly expressed in UPS and leiomyosarcoma.[553] Based on TCR sequencing, UPS was found to have the highest TIL infiltration, while synovial sarcoma had the lowest.[553] In a study focusing on the sarcomas in the Cancer Genome Atlas (TCGA), gene expression signatures[554] were used to identify immune infiltrates for 206 soft-tissue sarcomas (80 leiomyosarcoma, 50 dedifferentiated liposarcoma, 44 UPS, 17 myxofibrosarcoma, 10 synovial sarcoma, and 5 MPNST).[21] Macrophage scores were highest among dedifferentiated liposarcoma, myxofibrosarcoma, and UPS, and the CD8 score correlated with improved survival in gynecologic leiomyosarcomas. High NK cell scores were associated with disease-specific survival in leiomyosarcoma, myxofibrosarcoma, and UPS. In

dedifferentiated liposarcoma, a higher Th2 score correlated with worse disease-specific survival.[21]

1.4.2 Immune Checkpoint Expression in Sarcomas

Studies in sarcomas generally report low, diagnostic-subtype-dependent expression of PD-L1. [525,542,546,555,556] The study of 50 soft-tissue sarcoma cases described in the previous section found that PD-L1 expression in tumor cells, lymphocytes, and macrophages was 12%, 30%, and 58%, respectively, with the highest scores observed in GIST.[525] They did not find any association between PD-L1 expression (in tumor or immune infiltrates) and clinical features or overall survival.[525] Contrastingly, another study of 105 soft-tissue sarcomas reported that expression of PD-1 and PD-L1 predicts worse outcomes, and patients positive for both markers had the shortest survival time.[546] In that study, PD1+ lymphocytes and PD-L1 tumor cell expression were seen in 65% and 58% of cases, respectively.

Of course, PD-1 and PD-L1 expression are highly subtype-dependent. In the study by Pollack *et al*., UPS was found to have the highest levels of PD-L1 and PD-1, while synovial sarcoma had the lowest.[553] PD-L1 and PD-1 expression in that study were not associated with overall or progression-free survival.[553] In the TCGA analysis, the highest PD-L1 score was observed in leiomyosarcoma, and TIM-3 expression was highest in dedifferentiated liposarcoma, myxofibrosarcoma, and undifferentiated pleomorphic sarcoma.[21] IDO (indolamine-2,3-dioxygenase) is an enzyme of tryptophan catabolism whose activity enhances Treg activity, and as such, it can be upregulated by tumors for immunoevasion.[557] In an immunohistochemistry study of 371 soft-tissue sarcomas, IDO was positive on tumor cells in 152 samples (41%).[558]

1.4.3 Immunotherapy Clinical Trials in Sarcomas

1.4.4 Cancer Vaccines

A number of small trials have used a variety of different cancer vaccine strategies in sarcomas, ranging from whole tumor cells, tumor lysates, to isolated or recombinant proteins and peptides. An early placebo-controlled phase II trial in 136 patients (with advanced melanoma and sarcoma) treated patients with a trivalent peptide vaccine against the ganglioside antigens GD2, GD3, and GM2.[559] A serological response toward GM2 and GD2 was observed in 98% of patients in the treated group, compared to 21% in the placebo group; however, there was no observed difference in progression-free survival between the trial arms.[559] Subsequent trials attempted to treat sarcomas with tumor-lysate-loaded dendritic cells, and while they saw no objective responses, there was evidence of delayed hypersensitivity reactions. [560,561] A study in 52 patients with Ewing sarcoma that treated patients with a dendritic cell vaccine, paired with high-dose chemotherapy, showed improved outcomes compared to historical survival data.[562] In another Ewing sarcoma trial using dendritic cells pulsed with tumor lysate or peptide for 5 pediatric Ewing sarcoma patients, one patient had a durable complete response.[563] Another study of tumor-lysate-pulsed dendritic cells in pediatric solid tumors observed regression of pulmonary metastases in 1 fibrosarcoma patient.[564]

In theory, some sarcomas are particularly well-suited to cancer vaccine strategies, based on expression of highly specific neoantigens, such as the fusion proteins generated by translocation-associated sarcomas. A pilot study using a 9-mer peptide spanning the SS18-SSX fusion was run in 21 patients with synovial sarcoma, and while 9 patients had a >2-fold increase in CD8+ T cells, only 1 patient had transient shrinkage of a metastatic lesion.[565] Cancer testis antigens, another highly specific antigen choice, are highly expressed in some sarcomas,

particularly NY-ESO-1 in synovial sarcoma and myxoid liposarcoma.[566-568] LV305 is part of a new class of cancer vaccines (that use a lentiviral vector to selectively target dendritic cells to present select antigens) that encodes NY-ESO-1. [569,570] The first clinical trial of LV305 included 24 sarcoma paients, [571,572] among whom one patient had a partial response and 13 had stable disease.[571,573] The trial also demonstrated increased NY-ESO-1-specific CD8 and/or CD4 T cells following treatment.[572] The partial responder was a patient with treatment-refractory synovial sarcoma, and the response was durable at 3 years post-treatment.[574] A follow-up phase I trial assessed the CMB305 'prime-boost' regimen in 49 patients (25 sarcomas: 14 synovial sarcoma, 9 myxoid liposarcoma, and 2 other), demonstrating an impressive 76% overall survival at 18 months.[575] CMB305 was next evaluated in combination with atezolizumab,[576] but due to disappointing results, the company decided to halt the planned phase III SYNOVATE trial (NCT03520959) in favor of pursuing other vaccine strategies.

In most of these studies, it has been difficult demonstrate meaningful clinical benefit, despite evidence of immune engagement. This could be due to a number of factors, including tumor escape by down-regulation of the target antigens, suboptimal vaccine adjuvant combinations, or poor target antigen selection.

1.4.5 Adoptive T-Cell Therapies

Groups have also attempted to exploit the high NY-ESO-1 expression of synovial sarcoma through adoptive T cell therapies. NY-ESO-1–specific T cells can be isolated from the peripheral blood (following peptide-pulsed dendritic cell stimulation) using NY-ESO-1 tetramer-based cell sorting.[577] In a study of six patients with NY-ESO-1-expressing sarcomas, oligoclonal antigen-specific CD8 T cells were generated in all patients, which were capable of lysing NY-

ESO-1–expressing tumor cells *ex vivo*.[577] Phase I trials of this adoptive cell therapy have been conducted, with results not yet reported.(NCT01477021, NCT02319824).

NY-ESO-1 has also been targeted using chimeric antigen receptor (CAR)-T cells. In a pilot study testing the activity of high-affinity NY-ESO-1-specific CAR-T cells in synovial sarcoma and melanoma patients with proven NY-ESO-1 expression, objective responses were seen in 11/18 synovial sarcomas, and 3- and 5-year survival rates were 38% and 14%, respectively. [578,579] In another study, CAR-T cells targeting HER-2 (human epidermal growth factor receptor 2) were used to treat HER-2-expressing sarcomas (16 osteosarcoma, 1 Ewing sarcoma, 1 primitive neuroectodermal tumor, 1 desmoplastic small round cell tumor), and 4/17 evaluable patients had stable disease for 12 weeks to 14 months, with a median overall survival of 10.3 months.[580] The investigators observed tumor necrosis in excised tumors, and CAR-T cells were detected at tumor sites of 2/2 patients examined.[580] CAR-T cells targeting GD-2, as well as the immune checkpoint B7-H3 are currently being tested in clinical trials for sarcomas (NCT00902044, NCT02107963, NCT01953900).

1.4.6 Immune Checkpoint Inhibitors

Clinical trials targeting CTLA-4, PD-(L)1, LAG-3, TIM-3, or CD47 in sarcomas are outlined in Table 1.10. Three major clinical trials, SARC028 (NCT02301039), Alliance A091401 (NCT02500797), and PEMBROSARC (NCT02406781), have so far published results. [581-583] SARC028 was a multi-centre, single-arm, open-label, phase II study of pembrolizumab in 2 sarcoma cohorts: bone sarcomas and soft-tissue sarcomas.[581] The soft-tissue sarcoma cohort recruited 10 leiomyosarcomas, 10 UPS, 10 liposarcomas, and 10 synovial sarcomas, while the bone sarcoma cohort recruited 22 osteosarcomas, 13 Ewing sarcomas, and 5 chondrosarcomas. Among the soft-tissue sarcomas, 18% (7/40) patients had an objective response: 4/10 UPS, 2/10

liposarcomas, and 1/10 synovial sarcomas. Among the bone sarcomas, 5% (2/40) objective responses were observed, 1/22 (5%) in osteosarcoma and 1/5 (20%) in chondrosarcoma. No patients with leiomyosarcoma or Ewing sarcoma had an objective response.

The Alliance trial was a multi-centre, open-label, non-comparative, randomized, phase II study of nivolumab (anti-PD1) with or without ipilimumab (anti-CTLA4) in 85 sarcoma patients, including 29 leiomyosarcomas, 9 osteosarcomas, 6 liposarcomas, 11 spindle cell sarcomas, 11 UPS, and 19 other sarcomas.[582] In the combination therapy group (nivolumab + ipilimumab), objective response was seen in 6 (16%) of 38 patients, compared to nivolumab alone, wherein objective responses were seen in 2 (5%) of 38 patients. Responses in the combination group were seen in leiomyosarcoma (n=2), myxofibrosarcoma (n=1), UPS (n=3), and angiosarcoma (n=1).[582]

PEMBROSARC was a multi-centre, open-label, phase II study of pembrolizumab with metronomic cyclophosphamide in 4 cohorts: leiomyosarcomas (n=15), UPS (n=16), GIST (n=10), and other sarcomas (n=16).[583] Three patients experienced tumor shrinkage (1 GIST, 1 solitary fibrous tumor, 1 endometrial stromal sarcoma), but the best objective response observed was stable disease for 16 patients (3 leiomyosarcoma, 5 UPS, 3 GIST, 5 other) and 1 partial response (solitary fibrous tumor). The partial responder was the only case with strong PD-L1 staining, and analyses of all tumor samples showed strong infiltration by CD163+ (M2) macrophages expressing IDO.[583] While the signal was generally low across all three of these trials, genomically complex sarcomas, such as UPS and dedifferentiated liposarcoma, tended to have higher rates of response, except for leiomyosarcoma, which had unexpectedly low response rates. There is likely potential for immune checkpoint blockade in sarcomas, but future trials need to involve informed design of patient cohorts in order to demonstrate clinical benefit.

Agent	Phase	Disease(s)	Other agents	Identifier	Status
CTLA-4 Inhibitors					
Ipilimumab	1	Sarcoma, Wilm's tumor, lymphoma, neuroblastoma	-	NCT01445379	Completed
	1	GIST, unresectable sarcoma	Dasatinib	NCT01643278	Completed
PD-(L)1 Inhibitors					
Pembrolizumab	2	Bone and soft-tissue sarcoma	-	NCT02301039	Active
	2	Alveolar soft-part sarcoma	Axitinib	NCT02636725	Active
	2	Advanced sarcomas	Cyclophosphamide	NCT02406781	Recruiting
	1/2	Leiomyosarcoma and UPS	Gemcitabine	NCT03123276	Recruiting
	2	Advanced/metastatic sarcoma	T-VEC	NCT03069378	Recruiting
	2	Soft-tissue sarcoma	Eribulin	NCT03899805	Planning
Nivolumab	2	Metastatic soft-tissue sarcoma	Trebectedin	NCT03590210	Recruiting
	2	Advanced/metastatic sarcoma	CD122 agonist	NCT03282344	Recruiting
	2	Sarcoma	Trabectedin, T-VEC	NCT03886311	Planning
Atezolizumab	2	Localized soft-tissue sarcoma	-	NCT03474094	Recruiting
Durvalumab	2	Advanced solid tumors (leiomyosarcoma cohort)	Olaparib, cediranib	NCT03851614	Recruiting
SHR-1210	2	Advanced osteosarcoma	Apatinib	NCT03359018	Active
Spartalizumab	1	Advanced solid tumors (chordoma and ASPS cohort)	FAZ053 (Anti-PD-L1)	NCT02936102	Active
GB226	2	Alveolar soft part sarcoma	-	NCT03623581	Recruiting
Camrelizumab	2	Soft-tissue sarcoma	Apatinib	NCT03711279	Planning
Combination of PD-(L)1 and CTLA-4 inhibitors					
Nivolumab + Ipilimumab (anti-CTLA-4)	2	Uterine leiomyosarcoma	-	NCT02428192	Completed
	2	Metastatic sarcoma	-	NCT02500797	Active
	1/2	Advanced soft-tissue sarcoma	Trabectedin	NCT03138161	Recruiting
	1/2	Pediatric solid tumors	-	NCT02304458	Recruiting
	2	Rare tumors	-	NCT02834013	Recruiting
	2	Non-resectable sarcoma and uterine carcinoma	-	NCT02982486	Planning
Durvalumab + Tremelimumab (anti-CTLA-4)	1/2	High-risk soft-tissue sarcoma	-	NCT03116529	Recruiting
	1/2	Advanced solid tumors (sarcoma cohort)	TLR agonist	NCT02643303	Recruiting
LAG-3 Inhibitors					
LAG525	2	Advanced solid malignancies (soft-tissue sarcoma cohort)	Spartalizumab (Anti-PD-1)	NCT03365791	Active
CD47/SIRPα Inhibitors					
TTI-621	1	Relapsed/refractory solid tumors (sarcoma cohort)	Anti-PD1, IFN-α2a, T-VEC	NCT02890368	Recruiting

Table 1.10 Immune checkpoint inhibitor clinical trials in sarcomas, accessed March 28, 2019.

1.5 Thesis Objectives and Chapter Overview

Immunotherapy clinical trials for sarcomas have delivered disappointing results to date, due in part to small numbers for any one subtype, as well as their pragmatic but biologically-inappropriate grouping of disparate subtypes. The lack of comprehensive data on the immune microenvironment in sarcomas represents a major gap in knowledge that would be instrumental to a more rational design of immunotherapy clinical trials. My work is a detailed analysis of the immune cells in a large series of bone and soft-tissue sarcomas, with the objective of fully characterizing the subtype-specific immune microenvironments. I hypothesized that the "karyotypically-complex" sarcoma subtypes (as described in section 1.1.2 and table 1.3) would have the highest degree of immune infiltration (both lymphocytic and phagocytic), and following with this, that these tumor types would be stimulated to express higher levels of immune checkpoint biomarkers and have more diverse T-cell repertoires. This study aims to inform the rational design of sarcoma cohorts and subtype inclusion for forthcoming immunotherapy clinical trials.

Chapter 2 examines tumor-infiltrating lymphocytes across sarcoma subtypes. This chapter focused on quantifying lymphocytes and T-cell subsets by sarcoma type via immunohistochemistry. Tissue expression of lymphocyte-related immune checkpoints PD-1, LAG-3, and TIM-3 was also characterized.

Chapter 3 examines tumor-associated macrophages across sarcoma subtypes. This chapter focused on quantifying macrophages and their polarity by sarcoma type via immunohistochemistry. Results were validated using RNA expression data from The Cancer Genome Atlas. Tissue expression of macrophage-related immune checkpoints CD47 was also characterized.

Chapter 4 examines the T-cell receptor repertoire for sarcomas on an Australian clinical trial of PD-1 inhibitor durvalumab with or without CTLA-4 inhibitor tremelimumab. Pre- and post-treatment tumor and peripheral blood samples were analyzed for T cell receptor richness and clonality.

Finally, Chapter 5 summarizes the findings of this work in the context of the burgeoning field of immunotherapy, and also provides future directions for extending the findings of this work.

Chapter 2: Tumor-Infiltrating Lymphocytes and Lymphocyte-Related Immune Checkpoint Expression in Sarcomas

2.1 Abstract

Despite advances in our understanding of the underlying molecular drivers of sarcomas, few treatments are available with proven benefit for advanced metastatic sarcomas. Immunotherapy has value in this setting for some types of cancer, but sarcomas, with their multiplicity of rare types, have not been characterized in detail for their expression of targetable immune biomarkers. This study provides the most systematic evaluation to date of tumor-infiltrating lymphocytes (TILs) and immune checkpoint biomarker expression in sarcomas. We examined by morphology and immunohistochemistry 1079 sarcoma specimens representing 23 types, in addition to 236 benign bone and soft-tissue tumors. Genomically-complex sarcoma types – those driven by mutations and/or copy-number alterations – had much higher levels of TILs than translocation-associated sarcomas (median 54 vs. 34 TILs/mm^2 tumor tissue, $p<0.0001$). Prior exposure to radiotherapy was associated with increased immune infiltrates ($p<0.01$). Higher lymphocytic infiltration was associated with better overall survival among the non-translocation associated sarcomas ($p=0.01$). Expression of PD-1 and CD56 were associated with worse overall survival ($p=0.03$, $p=0.05$, respectively). Expression of LAG-3 and TIM-3, two emerging immune checkpoints, was more common than expression of PD-1 or PD-L1. Indeed, most cases positive for PD-(L)1 co-expressed one or both of these novel biomarkers, providing a potential explanation for resistance to PD-(L)1 monotherapy seen in sarcoma clinical trials and support for trials targeting LAG-3 and/or TIM-3.

2.2 Introduction

Sarcomas are malignant tumors of the bone or soft-tissue that demonstrate mesenchymal differentiation. The World Health Organization defines over 50 distinct sarcoma types[1] that, while very heterogeneous in apparent histogenesis, tumor site, and molecular etiology, are most often treated primarily through surgery and radiation. Generally speaking, cytotoxic chemotherapy is not particularly effective for sarcomas, aside from some type-specific benefits.[584,585] Despite advances in our understanding of the underlying molecular drivers of sarcomas and the development of rational therapies to target them, effective systemic treatment strategies for metastatic sarcomas, which occur in about half of patients[94], remain largely elusive. When metastasis occurs, cure is rare, resulting in a median overall survival (OS) of 12-18 months for advanced metastatic sarcomas.[131,586-591] New systemic treatment strategies are clearly needed, and immunotherapy provides a compelling approach.

Cancer immunotherapy is an emerging field encompassing both immunostimulatory and immunomodulatory approaches, which have shown remarkable promise in clinical trials, bringing about durable responses in some types of cancer previously considered "incurable." Immunostimulatory therapies, which function to initiate an anti-tumoral immune response, saw early successes in melanoma and leukemia, [592-594] and have since shown efficacy in synovial sarcoma and myxoid/round cell liposarcoma[572,574,578,579] in studies employing autologous T-cells engineered with receptors to target cancer testis antigen NY-ESO-1. Immunomodulatory therapies, particularly those employing immune checkpoint inhibitors, have produced dramatic, practice-changing results in melanoma, lung and renal cell carcinomas.[363,397,400,595-598] Clinical trials of immune checkpoint inhibitors have yielded multiple successes in other tumor types, but

few studies have been undertaken for sarcomas and no practice-changing benefits have been observed. [581-583,599-601]

In the Phase 2 clinical trial of the anti-PD-1 checkpoint inhibitor pembrolizumab in sarcomas (SARC028)[581] and the Alliance randomized trial[582] of nivolumab (anti-PD1) ± ipilimumab (anti-CTLA4), the most consistent and impressive responses were observed in patients with undifferentiated pleomorphic sarcoma, dedifferentiated liposarcoma, or myxofibrosarcoma. These findings generally support the widespread speculation that pleomorphic sarcomas, by nature of their higher mutational burden than translocation-associated sarcomas, exhibit increased immunogenicity, and are more likely to respond to immune checkpoint inhibitors than their genetically simpler counterparts. However, patients with some pleomorphic sarcomas, notably leiomyosarcoma or osteosarcoma, showed far less activity of PD1-based immunotherapy. Though the relevance of PD-L1 expression to predict response to these agents remains unclear, studies in sarcomas generally report low, histotype-dependent expression of PD-L1.[525,546,555,556] Recent publications have highlighted the existence of alternative immune checkpoint pathways,[364,602,603] such as pro-apoptotic TIM-3 or anti-proliferative LAG-3, that contribute to T-cell exhaustion and could explain resistance to checkpoint inhibitor monotherapy. Drugs targeting these pathways are in active development[364], and anti-TIM-3 antibodies have shown some promise in murine models of sarcoma in preclinical studies.[449]

The purpose of this study is to quantify and characterize tumor-infiltrating lymphocytes across a large, comprehensive cohort of bone and soft-tissue sarcomas. We employ immunohistochemistry to identify important T-cell subsets within immune infiltrates and to assess expression of immune checkpoint biomarkers PD-1, PD-L1, TIM-3, and LAG-3. This

study aims to describe, in a systematic manner, the relative immunogenicity of many diverse sarcoma types, and to assess their expression of key immune checkpoint biomarkers.

2.3 Materials and Methods

2.3.1 Patient Tumor Samples

Formalin-fixed, paraffin-embedded tissue microarrays (TMAs) were constructed at the University of British Columbia (Vancouver, BC, Canada) and at Mount Sinai Hospital New York (NY, USA). From the University of British Columbia, 14 TMAs were included: TMA 01-003 (synovial sarcoma and differential diagnoses, 82 cases in duplicate)[604]; TMA 03-008 (chondroid tumors, 121 cases in duplicate)[605]; TMA 06-001A (gastrointestinal stromal tumors, 148 cases in duplicate)[606]; TMA 06-007 (myxoid liposarcomas, 69 cases in triplicate)[607]; TMA 09-006 (epithelioid sarcoma and differential diagnoses, 53 cases in duplicate)[608]; TMA 10-004 (28 chordomas, in duplicate); TMA 10-009 (8 alveolar soft part sarcomas, 2 alveolar rhabdomyosarcomas, 2 desmoplastic small round cell tumors, in triplicate)[608]; TMA 12-004 (BCL2-positive tumors, 35 cases in triplicate)[609]; TMA 12-005 (pediatric spindle cell lesions, 134 cases in duplicate)[609]; TMA 12-006 (translocation-associated sarcomas, 10 cases in duplicate)[609]; TMA 12-010 (5 dedifferentiated liposarcomas and 5 undifferentiated pleomorphic sarcomas, in duplicate)[609]; TMA 14-006 (4 myxoid liposarcomas, 3 myxofibrosarcomas, 3 chondrosarcomas, 1 synovial sarcoma, 1 malignant peripheral nerve sheath tumor, in duplicate)[566]; TMA 14-007 (dedifferentiated liposarcomas with well-differentiated areas, both components for 57 cases in duplicate)[610]; and TMA MPNST (malignant peripheral nerve sheath tumor and differential diagnoses, 176 cases in duplicate)[611]. From the Mount Sinai Hospital, 3 TMAs were included: MSH-OSa (osteosarcomas, 280 cases in duplicate); MSH-SS (synovial

sarcomas, 70 cases in duplicate); and MSH-UPS (74 undifferentiated pleomorphic sarcomas, 52 myxofibrosarcomas, 18 leiomyosarcomas, 13 dedifferentiated liposarcomas, 9 dermatofibrosarcoma protuberans, and differential diagnoses; 210 cases total in duplicate).

2.3.2 Tissue Microarray Preparation

All tissue specimens were derived from surgical resection specimens from Mount Sinai Hospital, New York, NY (MSH TMAs) [612], 20 centres throughout Norway (TMA 06-001), or Vancouver General Hospital, Vancouver, BC (all other TMAs). Cores with a diameter of 1.0 mm (TMA 14-007, all MSH TMAs) or 0.6 mm (all other TMAs) were extracted from representative viable tumor tissue, at the centre of the tumor, targeting areas of most representative morphology, as identified by a bone and soft tissue subspecialty pathologist (TO Nielsen, EG Demicco). TMAs were cut to 4-µm-thick sections, mounted to Fisherbrand™ Superfrost™ Plus charged glass slides (Thermo Fisher Scientific Inc, Waltham, MA), and incubated for 1 hour at 60ºC.

2.3.3 Immunohistochemistry

Immunohistochemical staining was performed on serial TMA sections. All TMA blocks were batch cut within 6 months of staining in order to prevent PD-L1 degradation.[613] All UBC TMAs were stained within 7 days of batch cutting, and MSH TMA blocks were cut immediately prior to shipping to UBC for staining. All antibodies, except for PD-L1, were applied using the Ventana DISCOVERY® ULTRA semi-automated staining system (Ventana Medical Systems Inc, Tucson, AZ), as described previously[610]. Briefly, heat-induced antigen retrieval was performed using the standard Cell Conditioning 1 (CC1, Ventana) protocol. Slides were incubated with primary antibodies (described in Table 2.1) in DISCOVERY antibody diluent (Ventana) for 2 hours at room temperature. For CD4, CD8, CD56, and FOXP3 slides were

incubated for 16 minutes at 37°C in DISCOVERY Universal secondary antibody (Ventana), and chromogen visualization was performed by DAB map detection (Ventana). For PD-1, LAG-3, and TIM-3, slides were incubated for 2 hours at room temperature with the UltraMap anti-mouse (PD-1 and LAG-3) or anti-rabbit (TIM-3) secondary antibody (Ventana) and visualized using the UltraMap DAB Kit (Ventana).

Marker	Clone	Details	Vendor	Dilution
CD8	C8/144	mouse mAb	Dako, Agilent Technologies, Santa Clara, CA, USA	1:200
CD4	SP35	rabbit mAb	Ventana Medical Systems Inc, Oro Valley, AZ, USA	undiluted
FOXP3	236A/E7	mouse mAb	Abcam, Cambridge, UK	1:20
CD56	123C3.D5	mouse mAb	CellMarque Corporation, Rocklin, CA, USA	1:200
PD-1	NAT105	mouse mAb	Abcam, Cambridge, UK	undiluted
PD-L1	SP142	rabbit mAb	Ventana Medical Systems Inc, Oro Valley, AZ, USA	1:100
TIM-3	D5D5R	rabbit mAb	Cell Signalling Technology, Danvers, MA, USA	1:50
LAG-3	17B4	mouse mAb	Lifespan Biosciences, Seattle, WA, USA	1:100

Table 2.1 Details of primary antibodies used for tumor-infiltrating lymphocyte immunohistochemistry.

PD-L1 staining was performed using the Intellipath FLX Automated Staining system, as described previously.[614] Briefly, heat-induced antigen retrieval was performed using Diva Decloaker (Biocare Medical, Pacheco, CA, USA) for 30 seconds at 125°C. Following the standard Intellipath FLX Protocol, slides were blocked with Peroxidazed-1 (Biocare Medical) and Background-Sniper (Biocare Medical), then incubated with primary antibody (Table 2.1) in DaVinci Green diluent (Biocare Medical) for 30 minutes at room temperature. Slides were incubated with secondary antibody (LLC MACH3 rabbit HRP-Polymer, Biocare Medical) for 30 minutes at room temperature, followed by Intellipath FLC DAB chromogen kit (Biocare Medical) for 5 minutes at room temperature.

Following immunohistochemistry, all slides were counterstained with hematoxylin and mounted. Digital images of immunostained tissue microarrays were acquired using the Olympus BLISS high-definition virtual microscope and slide scanner (Olympus Life Science Solutions: Bacus

Laboratories, Lombard, IL, USA) or the Aperio digital pathology slide scanner (Leica Biosystems, Wetzlar, Germany).

Positive control tissue for all antibodies was normal lymph node, negative control tissue was cerebellum. Our CD4, CD8, FOXP3, CD56, PD-1, and PD-L1 antibodies are validated, clinically-available antibodies, while LAG-3 and TIM-3 are research-grade commercial antibodies that were validated by the manufacturers. We did not perform independent validation studies beyond the use of appropriate positive and negative tissue controls.

2.3.4 Histological Scoring

Scoring was performed by pathologists experienced in scoring biomarkers in bone and soft tissue tumors (N Setsu, D Gao, EG Demicco). Replicate cores were scored separately, with the pathologist blinded to replicates and final histological diagnosis, and the mean score from all replicates was calculated. Tumor-infiltrating lymphocytes (TILs) were counted directly from hematoxylin- and eosin-stained TMA slides (Fig. 2.1A-B). Lymphocyte biomarkers (TIL counts, CD8, CD4, FOXP3, CD56, PD-1, TIM-3, LAG-3) were scored by counting the number of positive-staining lymphocytes per TMA core (Fig. 2.1C-J). Different core sizes were normalized by dividing scores by the area of the respective core, to give TILs per mm^2. PDL1 immunopositivity was scored in both the tumor fraction (by the percentage of positive sarcoma cells) and the lymphocyte fraction (by the count of positive lymphocytes). All immunohistochemical markers were scored for cytomembranous positivity, except for FOXP3 (a transcription factor), which was scored for nuclear positivity.

2.3.5 Statistical Analysis

Data analysis was performed using IBM® SPSS® statistics software (version 26). An Independent Samples Kruskal-Wallis 1-way ANOVA test was used to assess the differences in

scoring between histological types. Categories were compared pairwise, and significance values were adjusted using the Bonferonni correction for multiple comparisons. Multivariable linear regression was used to assess the relative impact of biomarker scores and clinicopathological factors on survival. Survival correlates were evaluated using a Cox proportional-hazards multiple regression analysis to generate hazard ratios and corresponding 95% confidence intervals. Kaplan Meier curves were generated based on cases positive for CD8 and/or FOXP3, and a log rank test was run to determine if there were differences in the survival distribution for the 3 predominant combinations or expression: CD8- and FOXP3-, CD8+ and FOXP3-, or CD8+ and FOXP3+. Statistically significant differences were defined as $p<0.05$, and p-values for multiple comparisons were adjusted by Bon-Ferroni correction.

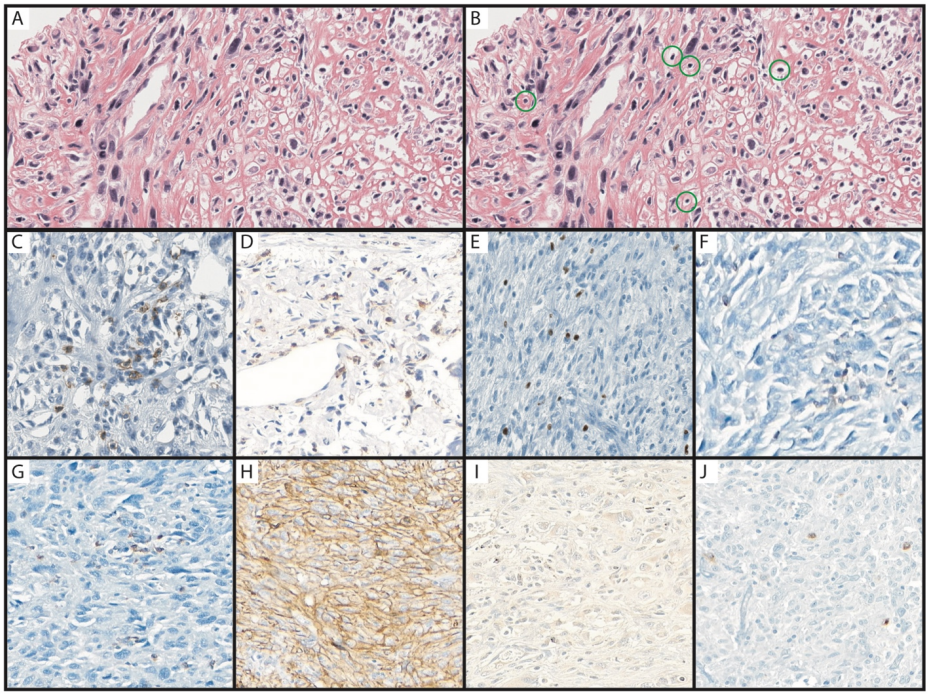


Figure 2.1 20x images of immunohistochemical staining of tumor-infiltrating lymphocyte biomarkers and checkpoints in sarcoma tissue microarray samples.
(A) H&E stain, osteosarcoma; (B) H&E stain, osteosarcoma, representative tumor-infiltrating lymphocytes circled; (C) CD8 (C8/144), dedifferentiated liposarcoma; (D) CD4 (4B12), osteosarcoma; (E) FOXP3 (236A/E7), solitary fibrous tumor; (F) CD56 (123C3.D5), undifferentiated pleomorphic sarcoma; (G) PD-1 (NAT105), leiomyosarcoma; (H) PD-L1 (SP142), undifferentiated pleomorphic sarcoma; (I) LAG3 (17B4), myxofibrosarcoma; (J) TIM3 (D5D5R), gastrointestinal stromal tumor.

2.4 Results

2.4.1 Patient Demographics

In total, specimens from 1072 sarcomas (representing 22 histological types, Table 2.2), 236 benign mesenchymal tumors, 33 carcinomas, and 21 melanomas (Table 2.3). were available for evaluation

Category	Histology	n
Translocation-Associated Sarcomas	Synovial sarcoma	177
	Myxoid liposarcoma	39
	Ewing sarcoma	21
	DFSP	18
	Solitary fibrous tumor	16
	Alveolar rhabdomyosarcoma	10
	Alveolar soft part sarcoma	8
	Low grade fibromyxoid sarcoma	8
	Clear cell sarcoma	7
Mutation and/or Copy-Number-Driven Sarcomas	Osteosarcoma	171
	GIST	149
	MPNST	76
	Chondrosarcoma	71
	Well-differentiated liposarcoma	67
	Dedifferentiated liposarcoma	65
	UPS	58
	Myxofibrosarcoma	35
	Chordoma	28
	Leiomyosarcoma	21
	Embryonal rhabdomyosarcoma	12
	Epithelioid sarcoma	9
	Angiosarcoma	4
Total		1072

Table 2.2 Sarcoma samples on tissue microarrays

Category	Histological type	n
Benign mesenchymal lesion	Leiomyoma	49
	Neurofibroma	42
	Fibromatosis	37
	Enchondroma	30
	Schwannoma	22
	Lipoma	10
	Fibroma (unspecified)	6
	Myofibroma	6
	Osteochondroma	6
	Necrobiosis lipoidica	5
	Nodular fasciitis	5
	Chondroblastoma	4
	Chondromyxoid fibroma	4
	Granuloma annulare	4
	Perineuroma	4
	Fetal rhabdomyoma	2
	Myositis ossificans	2
Carcinoma	Squamous cell carcinoma	15
	Small cell lung cancer	10
	Breast carcinoma	8
Melanoma	Unspecified melanoma	11
	Desmoplastic melanoma	10

Table 2.3 Benign mesenchymal lesions, carcinomas, and melanomas on tissue microarrays

Of the 660 sarcomas for which clinical data was available (Table 2.4), 209 (32%) were classified FNCLCC grade 3, 179(27%) as grade 2, 61(9%) as grade 1, and 215 had no grade specified (32%). The median patient age was 45 (range 0-89), and the median tumor size was 7.0 cm (range 1-42 cm). All of the 660 sarcomas with detailed outcome data were derived from surgical resection tissues. Prior to surgery, 380 (58%) had not received radiation or chemotherapy, while 100 (15%) had received neoadjuvant radiation alone, 36 (6%) had received neoadjuvant chemotherapy alone, and 16 (2%) had received both. Therapy was not known in 128 (19%). Certain sarcoma types were more likely to have been pretreated with neoadjuvant chemotherapy ($p<0.0005$), e.g. osteosarcoma, and/or radiotherapy ($p<0.0005$), e.g. dedifferentiated liposarcoma, Ewing sarcoma, and synovial sarcoma, by Fisher's Exact Test.

Parameter	median	range
Age, years	45.0	1-89
Tumor size, cm	7.0	0.1-40
Parameter	**N**	**%**
Grade		
1	61	9.2
2	179	27.1
3	209	31.7
unknown	211	32.0
Neoadjuvant treatment		
none	380	57.6
radiotherapy	100	15.2
chemotherapy	36	5.5
chemo and radiation	16	2.4
unknown	128	19.4
Adjuvant treatment		
none	296	44.8
radiotherapy	80	12.1
chemotherapy	59	8.9
chemo and radiation	15	2.3
unknown	210	31.8
Local recurrence		
yes	126	19.1
no	534	80.9
Metastasis		
yes	157	23.8
no	503	76.2

Table 2.4 Patient demographics: clinical parameters and survival outcomes available for 665 cases.

2.4.2 Characterization of Tumor-Infiltrating Lymphocytes

To obtain a broad picture of lymphocyte infiltration across our sample set, we first counted tumor-infiltrating lymphocytes (TILs) on H&E-stained tissue cores. We observed wide differences in TIL counts based on sarcoma subtype, with dedifferentiated liposarcomas displaying distinctly higher levels of infiltrates than any other histological type (median 204 TILs/mm², Fig. 2.2A).

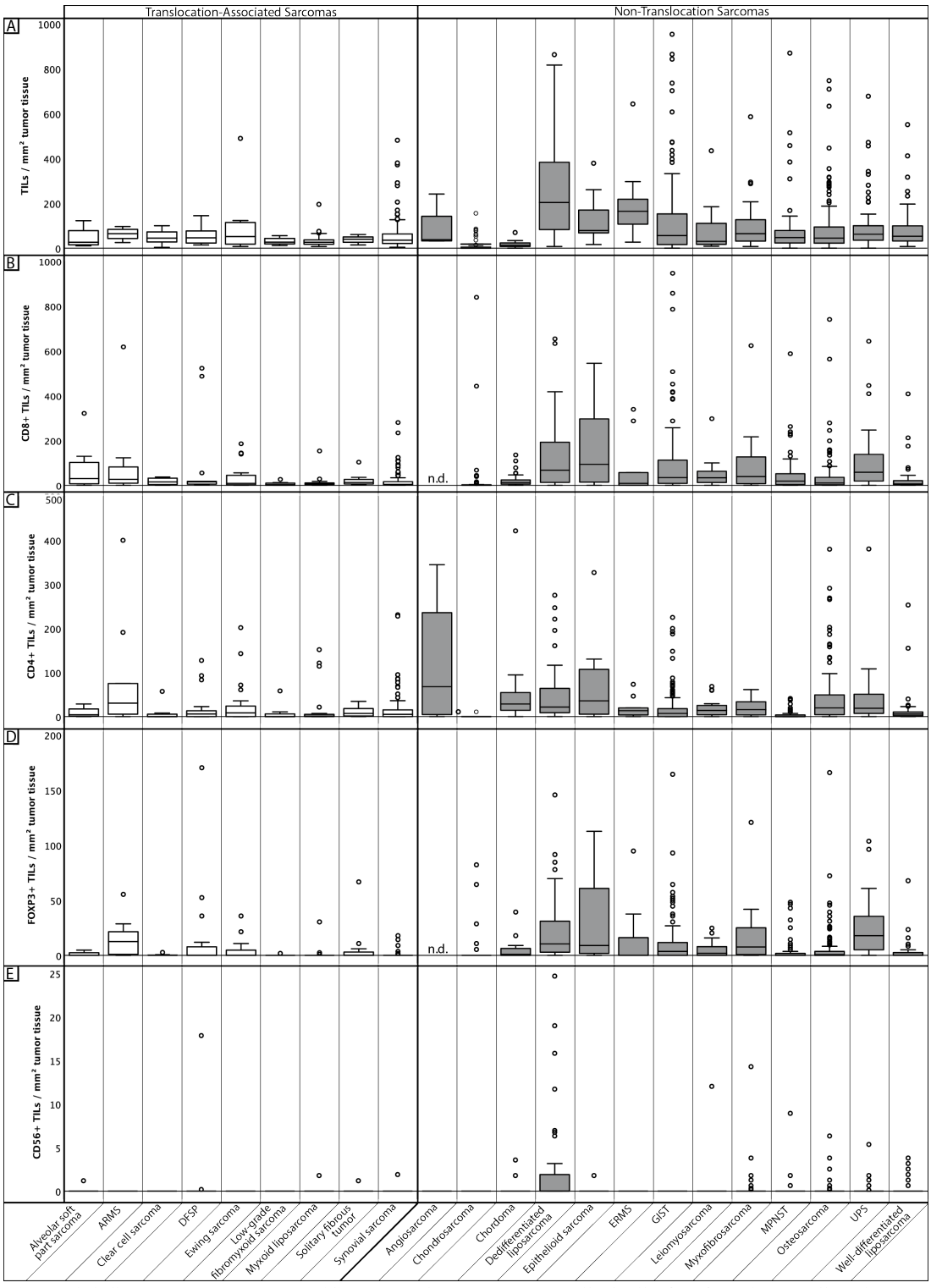


Figure 2.2 Sarcoma subtype-specific scores for TIL counts, CD8, CD4, FOXP3, and CD56.
Boxplots depict comparative counts (per mm^2 tumor tissue) of tumor infiltrating lymphocytes from H&E or immunohistochemically-stained tissue microarrays. Boxes represent the first through third quartiles, horizontal line indicates median, and whiskers indicate range. Extreme outliers are indicated as dots.

We divided sarcoma types into two categories based on their characteristic genomic alterations: 1) translocation-associated sarcomas, and 2) mutation and/or copy number driven (non-translocation) sarcomas (Table 2.2). The non-translocation sarcomas as a group had significantly higher levels of TILs than translocation-associated sarcomas (median 54 TILs/mm² vs. 34 TILs/mm², $p<0.0001$; Fig. 2.3). Notably, chondrosarcoma exhibited the lowest lymphocyte counts overall (median 0 TILs/mm²; Fig. 2.2A), likely accounted for by the avascular nature of cartilage. The levels of TILs in translocation-associated sarcomas were more aligned with those observed in benign mesenchymal neoplasms, while the profiles of the non-translocation sarcomas corresponded more closely with those observed in carcinoma or melanoma (Fig. 2.3).

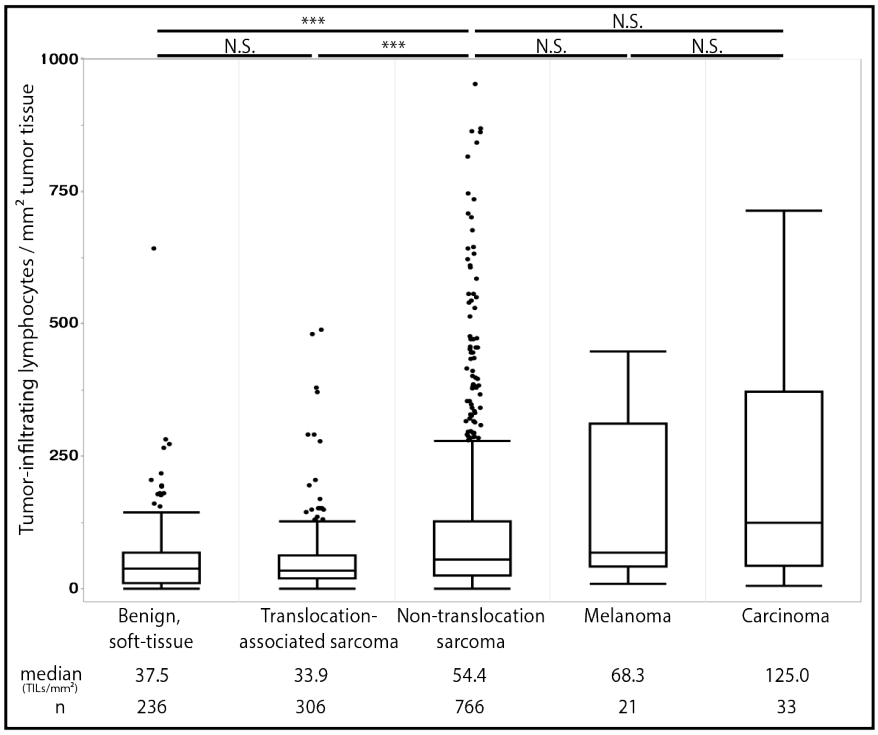


Figure 2.3 Boxplots depicting comparative counts of tumor-infiltrating lymphocytes by subtype category. Scored from H&E-stained tissue microarray cores of benign soft-tissue neoplasms, translocation-associated sarcomas, and mutation and/or copy number driven (non-translocation) sarcomas, melanomas, and carcinomas. Boxes represent the first through third quartiles, horizontal line indicates median, and whiskers indicate range. Extreme outliers are indicated as dots. *** represents $p<0.001$, significance calculated by Independent Samples Kruskal-Wallis 1-way ANOVA.

We sought to sub-categorize the infiltrating lymphocytes by immunohistochemistry, staining for cytotoxic T cells (CD8), helper T cells (CD4), regulatory T cells (FOXP3), and natural killer cells (CD56). Counts for all lymphocyte subsets were again highest among the non-translocation-associated sarcomas (Fig. 2.4), particularly dedifferentiated liposarcoma (Fig. 2.2B-E). CD8+ lymphocytes were the most numerous TIL subset across most sarcoma types, whereas CD56+ lymphocytes were scarce, with a median score of 0/mm^2 across all types (Fig. 2.4, Fig. 2.2B-E).

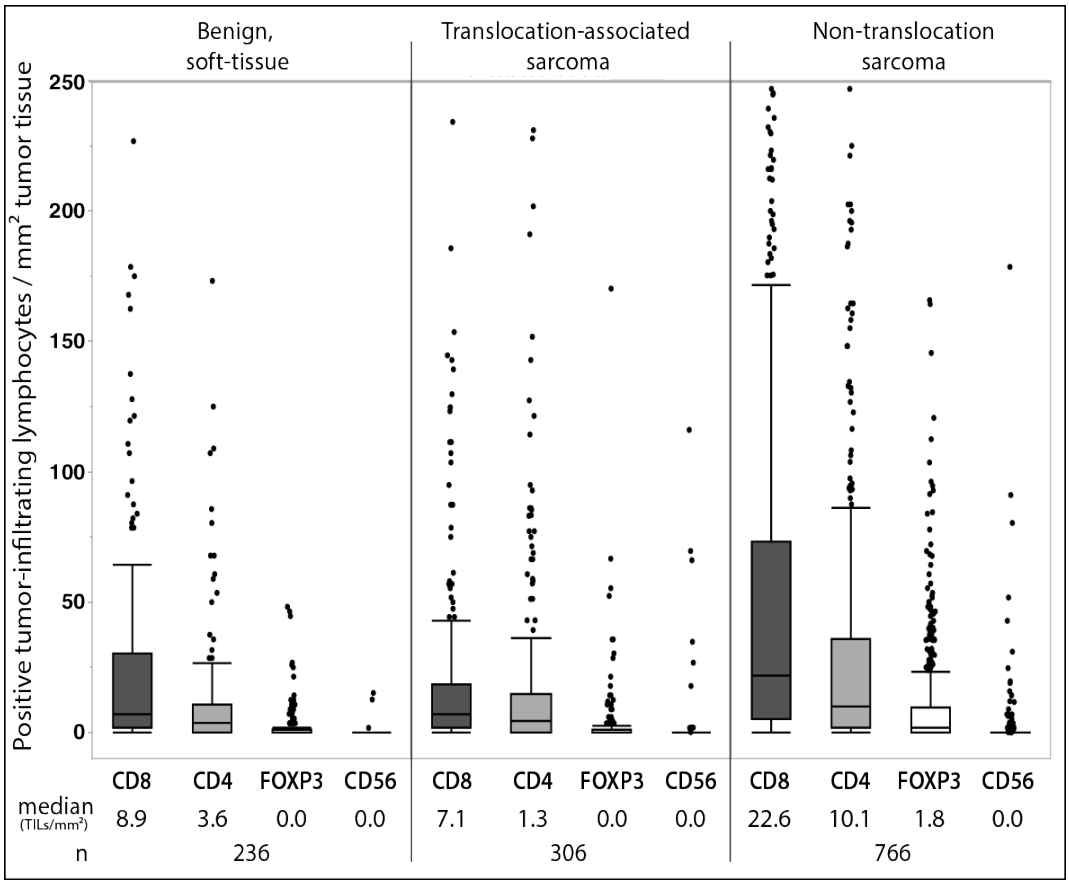


Figure 2.4 Boxplots depicting counts of tumor-infiltrating lymphocytes staining positive for CD8 (cytotoxic T cells), CD4 (helper T cells), FOXP3 (natural killer cells), or CD56 (natural killer cells).
Scored from tissue microarray cores of benign soft-tissue neoplasms, translocation-associated sarcomas, or non-translocation-associated sarcomas. Boxes represent the first through third quartiles, horizontal line indicates median, and whiskers indicate range. Extreme outliers are indicated as dots.

We used serial section staining to examine co-infiltration by multiple lymphocyte subtypes, defining a positive case for each marker as one having at least one positive-staining lymphocyte in any TMA core. Across the non-translocation-associated sarcomas, 50% of cases demonstrated the presence of CD8+ TILs, CD4+ TILs, and FOXP3+ TILs (but not CD56+ TILs, Fig. 2.5), while 17% were positive for CD8 only and 15% were positive for all 4 TIL markers. Cases staining positive for all four markers were mostly osteosarcomas or myxofibrosarcomas. Comparatively, translocation-associated sarcomas were more likely to have no lymphocytic infiltrates (21% of cases) or to be infiltrated by CD8+ TILs, with (25% of cases) or without (24% of cases) CD4+ TILs (Fig. 2.5).

To clarify the impact of tumor grade and of prior exposure to radiation or chemotherapy on the immune infiltration of sarcomas, we ran a multivariate regression analysis for the 660 cases for which clinical data was available. Taking into account grade and neoadjuvant treatment, the distinction of non-translocation- vs. translocation-associated sarcoma was independently predictive of increased tumor-infiltrating lymphocytes per mm^2 of tissue($p=0.002$), as well as increased CD8+ ($p=0.03$) and CD4+ ($p<0.001$) TILs (Table 2.5). Tumor grade did not correlate with lymphocyte infiltration or expression of any TIL subset, except for FOXP3, which showed a positive association (Table 2.5). Prior exposure to radiation was independently predictive of increased CD8+, CD4+, and FOXP3+ TILs ($p=0.006$, $p<0.001$, $p<0.001$, respectively; Table S3), but neoadjuvant chemotherapy was only associated with increased FOXP3+ TILs ($p=0.001$; Table 2.5). CD56 showed no associations with any clinical parameters (Table 2.5).

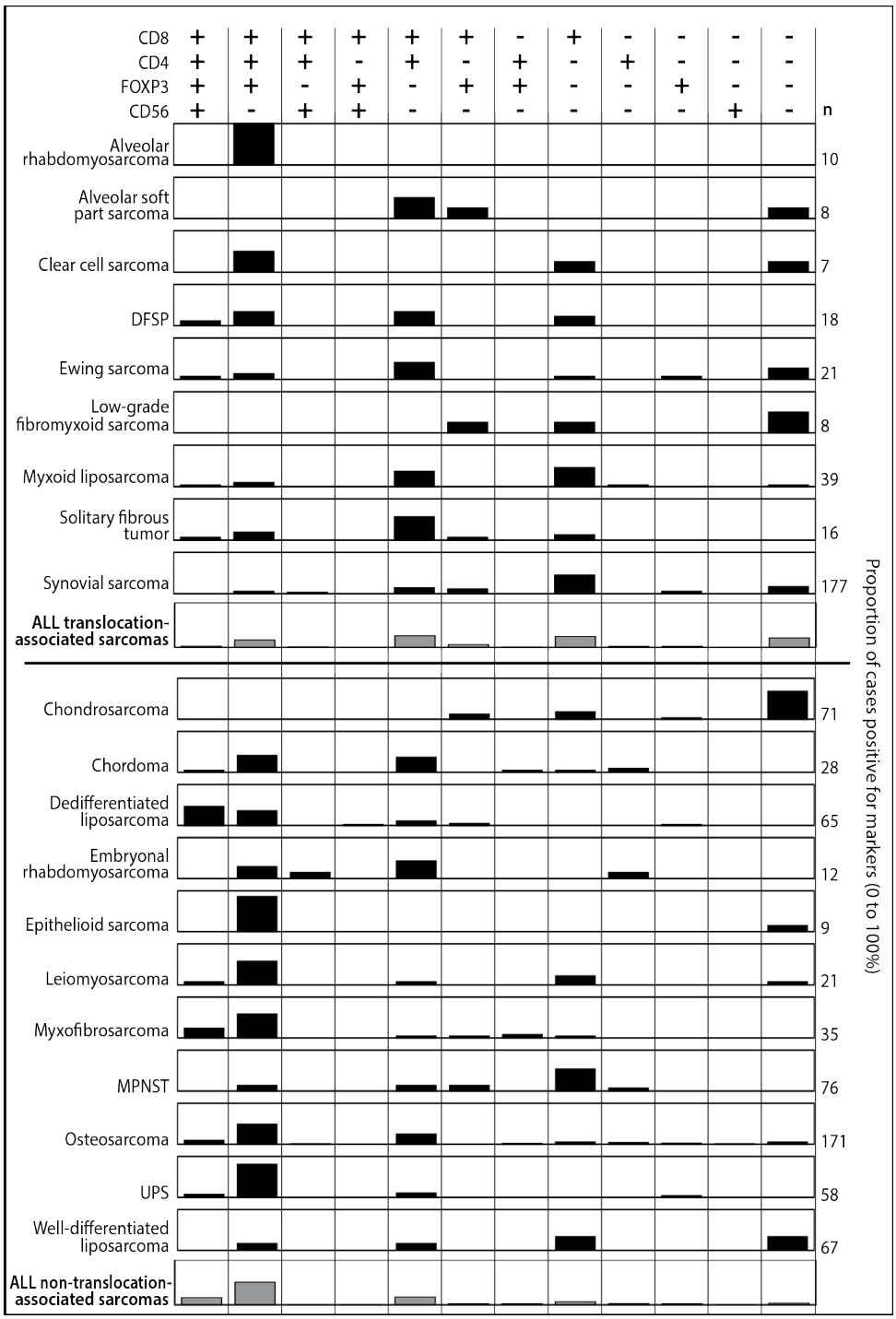


Figure 2.5 Proportion of sarcoma cases positive for one or more of CD8, CD4, FOXP3, and CD56. Scored from immunohistochemically-stained tissue microarray samples. Positive is defined as at least one positive-staining lymphocyte in any tissue microarray core. Panels represent histological type or translocation/non-translocation-associated classification. Angiosarcoma not included here because scores for these four markers were not all available for any of the (n=4) cases due to core dropout.

		Tumor grade			Prior chemotherapy		Prior radiotherapy		Molecular driver	
		1	2	3	+	-	+	-	Translocation-associated	Non-translocation
	n	61	179	209	116	448	52	512	297	361
TIL	median (Q1-Q3)	44 (24-63)	41 (23-88)	46 (25-101)	40 (24-93)	44 (24-94)	72 (37-254)	43 (24-87)	35 (21-61)	56 (27-135)
	cases + (%)	56 (100)	166 (98)	196 (99)	107 (100)	372 (90)	48 (100)	431 (91)	268 (100)	673 (99)
	cases - (%)	0 (0)	3 (2)	2 (1)	0 (0)	45 (10)	0 (0)	45 (9)	1 (0)	98 (2)
	p	0.600			0.120		0.086		**0.002**	
CD8	median (Q1-Q3)	7.1 (2-21)	9.8 (3-41)	17 (6-55)	13 (3-35)	12 (4-41)	43 (10-158)	11 (4-34)	7.1 (2-17)	22 (5-72)
	cases + (%)	52 (87)	139 (84)	176 (90)	100 (90)	334 (82)	46 (98)	388 (83)	246 (87)	609 (90)
	cases - (%)	8 (13)	26 (16)	20 (10)	11 (10)	72 (18)	1 (2)	82 (17)	38 (13)	65 (10)
	p	0.640			0.732		**0.006**		**0.027**	
CD4	median (Q1-Q3)	3.8 (0-24)	4.1 (0-26)	12 (1-42)	22 (3-52)	3.8 (0-23)	29 (5-73)	5.2 (0-28)	2.6 (0-12)	16 (4-44)
	cases + (%)	37 (65)	110 (69)	167 (85)	97 (89)	263 (66)	45 (90)	315 (69)	202 (71)	531 (79)
	cases - (%)	20 (35)	50 (31)	30 (15)	12 (11)	136 (34)	5 (10)	143 (31)	84 (29)	140 (21)
	p	0.760			0.356		**0.00001**		**0.0001**	
FOXP3	median (Q1-Q3)	0.0 (0-2)	0.0 (0-10)	1.8 (0-10)	1.5 (0-6)	0.0 (0-10)	11 (1-46)	0.6 (0-6)	0 (0-1)	1.9 (0-11)
	cases + (%)	25 (43)	74 (53)	124 (71)	61 (60)	169 (47)	26 (67)	204 (49)	48 (29)	393 (62)
	cases - (%)	33 (57)	66 (47)	51 (29)	40 (40)	188 (53)	13 (33)	215 (51)	117 (71)	237 (38)
	p	**0.0001**			**0.0001**		**0.000001**		0.479	
CD56	median (Q1-Q3)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-1)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)
	cases + (%)	1 (2)	23 (14)	28 (14)	14 (13)	37 (9)	10 (20)	41 (9)	12 (0)	76 (11)
	cases - (%)	58 (98)	142 (86)	171 (86)	96 (87)	382 (91)	41 (80)	437 (91)	279 (100)	592 (89)
	p	0.898			0.609		0.993		0.780	
PD-1	median (Q1-Q3)	0.0 (0-0)	0.0 (0-1)	0.0 (0-1)	0.0 (0-2.5)	0.0 (0-0)	0.0 (0-4)	0.0 (0-0)	0.0 (0-0)	0.0 (0-1)
	cases + (%)	12 (20)	42 (26)	54 (27)	28 (25)	82 (20)	16 (32)	94 (20)	37 (13)	139 (20)
	cases - (%)	47 (80)	121 (74)	145 (73)	83 (75)	333 (80)	34 (68)	382 (80)	253 (87)	544 (80)
	p	0.621			0.291		0.093		**0.021**	
PD-L1	median (Q1-Q3)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)	0.0 (0-0)
	cases + (%)	0 (0)	11 (7)	33 (17)	9 (8)	37 (9)	6 (15)	40 (8)	8 (3)	59 (12)
	cases - (%)	54 (100)	144 (93)	160 (83)	97 (92)	369 (91)	33 (85)	433 (92)	277 (97)	440 (88)
	p	0.571			0.798		0.779		0.509	
LAG-3	median (Q1-Q3)	0.0 (0-0)	0.0 (0-3)	0.4 (0-3)	0.3 (0-2)	0.0 (0-2)	3.8 (0-11)	0.0 (0-2)	0.0 (0-0)	0.3 (0-3)
	cases + (%)	14 (23)	78 (48)	111 (57)	51 (49)	148 (39)	24 (53)	175 (40)	33 (18)	315 (45)
	cases - (%)	47 (77)	85 (52)	83 (43)	54 (51)	234 (61)	21 (47)	267 (60)	146 (82)	379 (55)
	p	0.910			0.804		0.069		0.094	
TIM-3	median (Q1-Q3)	0.0 (0-1)	0.0 (0-2)	1.3 (0-4)	1.3 (0-4)	0.0 (0-3)	1.9 (0-7)	0.5 (0-3)	0.0 (0-2)	1.3 (0-4)
	cases + (%)	27 (45)	94 (57)	143 (71)	72 (65)	187 (45)	27 (54)	232 (49)	131 (44)	400 (58)
	cases - (%)	33 (55)	72 (43)	59 (29)	39 (35)	228 (55)	23 (46)	244 (51)	165 (56)	285 (42)
	p	0.986			0.994		0.697		0.318	

Table 2.5 Multivariable linear regression between TIL biomarker scores and tumor grade, neoadjuvant treatment, and tumor molecular classification. Median and quartile scores of positive TILs/mm² (or % positive tumor cells for PD-L1) on tissue microarrays. P values were calculated by multivariate ANOVA regression analysis, with Bon Ferroni correction. Proportions of +/- cases shown for reference, but are not evaluated independently in the multivariable analysis.

2.4.3 Expression of Lymphocytic Immune Checkpoint Biomarkers

We next investigated expression of the targets of immune checkpoint inhibitors: PD-1, PD-L1, LAG-3, and TIM-3. Expression of PD-1 and PD-L1, targets of established checkpoint inhibitor drugs, was low or absent across most sarcoma types (Fig. 2.6, Fig. 2.7A-B). Notably, gastrointestinal stromal tumors showed some of the lowest PD-1 and PD-L1 expression (2% of cases positive) in our sample set (Fig. 2.6, Fig. 2.7A-B). Emerging targets of immune checkpoint inhibitor therapy, LAG3 and TIM3, were both expressed in a significantly higher proportion of cases than PD-1 (Fig. 2.6, Fig. 2.7C-D). While PD-L1 and PD-1 expression were observed in only 10% and 22% of sarcoma cases respectively, LAG-3 and TIM-3 were expressed in 42% and 54% of sarcoma cases, respectively. Positive cases, considered as the presence of at least one positive-staining lymphocyte on a TMA core, were most common among the non-translocation sarcomas, particularly dedifferentiated liposarcoma (77% LAG-3+, 88% TIM-3+), myxofibrosarcoma (68% LAG-3+, 85% TIM-3+), undifferentiated pleomorphic sarcoma (73% LAG-3+, 85% TIM-3+), and leiomyosarcoma (58% LAG-3+, 74% TIM-3+) (Fig. 2.6).

Co-expression of at least 2 immune checkpoints occurred in 29% of sarcomas overall, with 11% of sarcomas positive for all three checkpoint biomarkers, and 16% positive for LAG-3 and TIM-3 but not PD-1 (Fig. 2.8). Of cases with PD-1+ infiltrates, 77% also had LAG-3+ TILs and 84% had TIM-3+ TILs. The large majority of translocation-associated sarcomas (64%) were negative for all three markers, with 9% positive for LAG-3 only, 12% positive for TIM-3 only, and 6% positive for both (but not PD-1). Non-translocation sarcomas demonstrated triple positivity in 16% of cases, with 20% positive for both LAG-3 and TIM-3, 9% positive for LAG-3 only, and 20% positive for TIM-3 only (Fig. 2.8**).** 29% of non-translocation sarcomas were negative for all three markers.

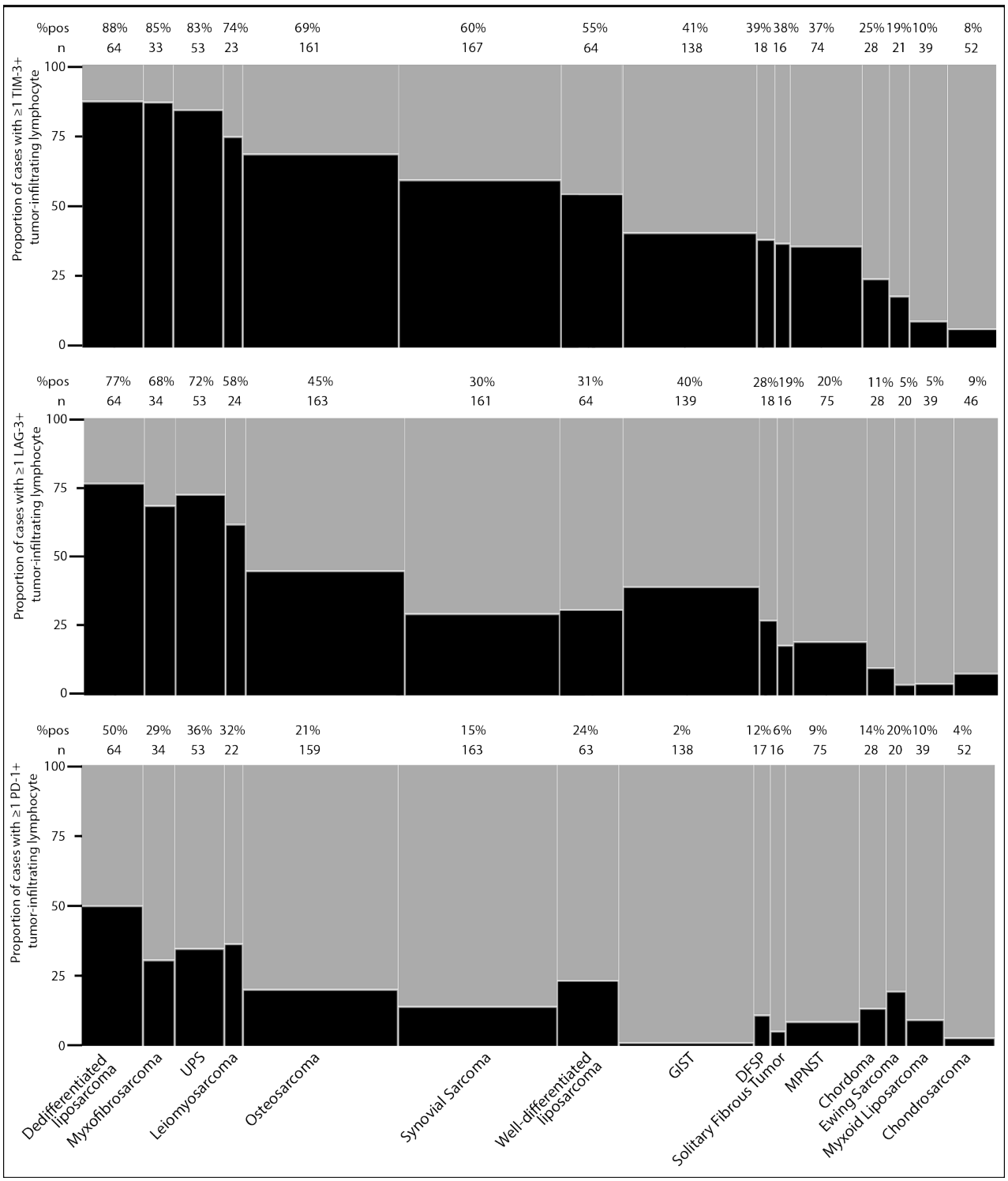


Figure 2.6 Mosaic plots comparing the proportion of cases positive for TIM-3, LAG-3, and PD-across sarcoma subtypes.

Black bars indicate cases that are positive for each marker. A case is marked positive if there is at least 1 positive-staining lymphocyte in any tissue microarray core. Column width is proportional to the number of cases assessed, height to the fraction of positive cases.

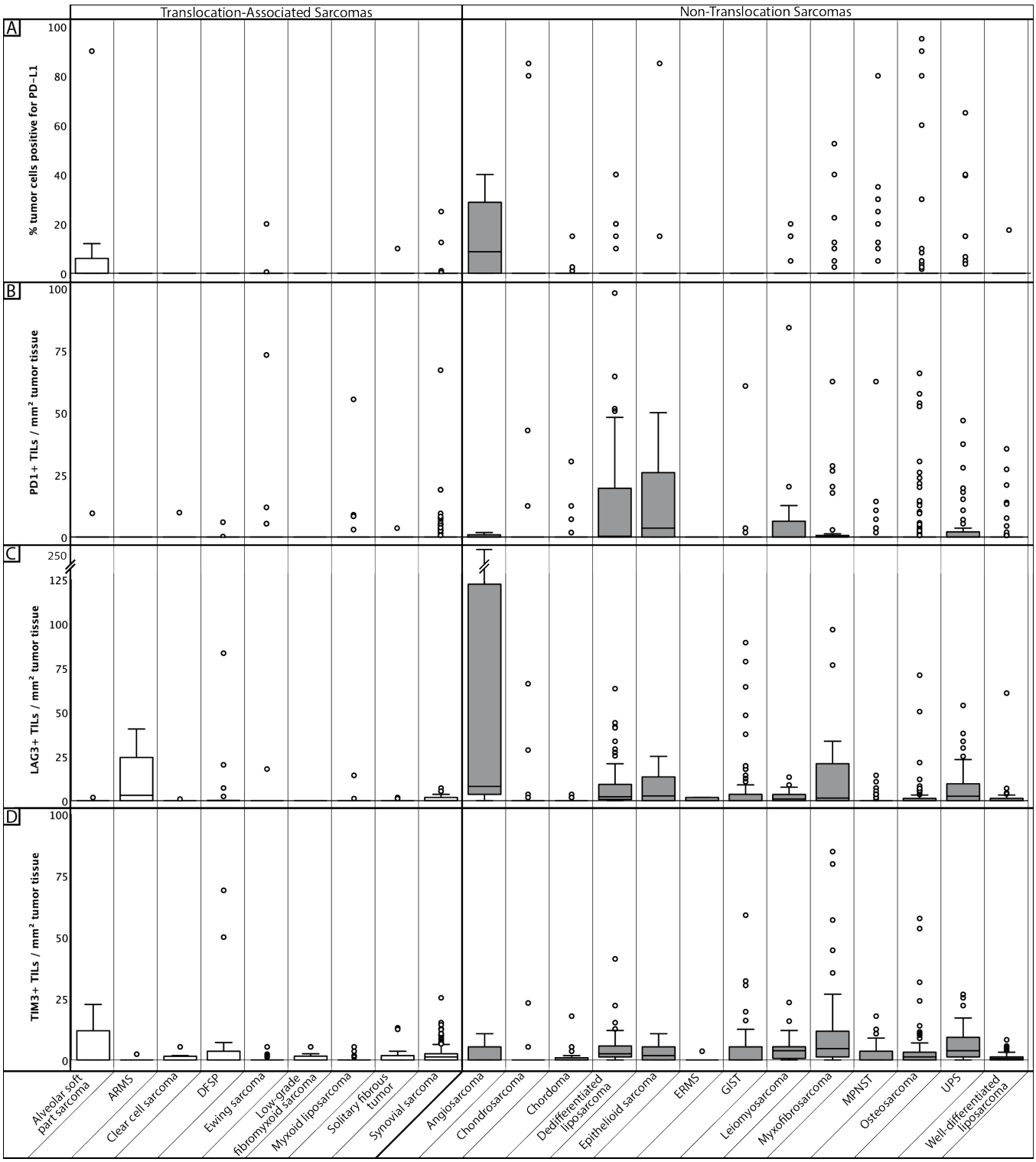


Figure 2.7 Subtype-specific expression of immune checkpoint markers PDL1, PD-1, LAG-3, and TIM-3. Boxplots depict comparative counts (per mm^2 tumor tissue) of tumor infiltrating lymphocytes (or % positive tumor cells, for PD-L1) from immunohistochemically-stained tissue microarrays.

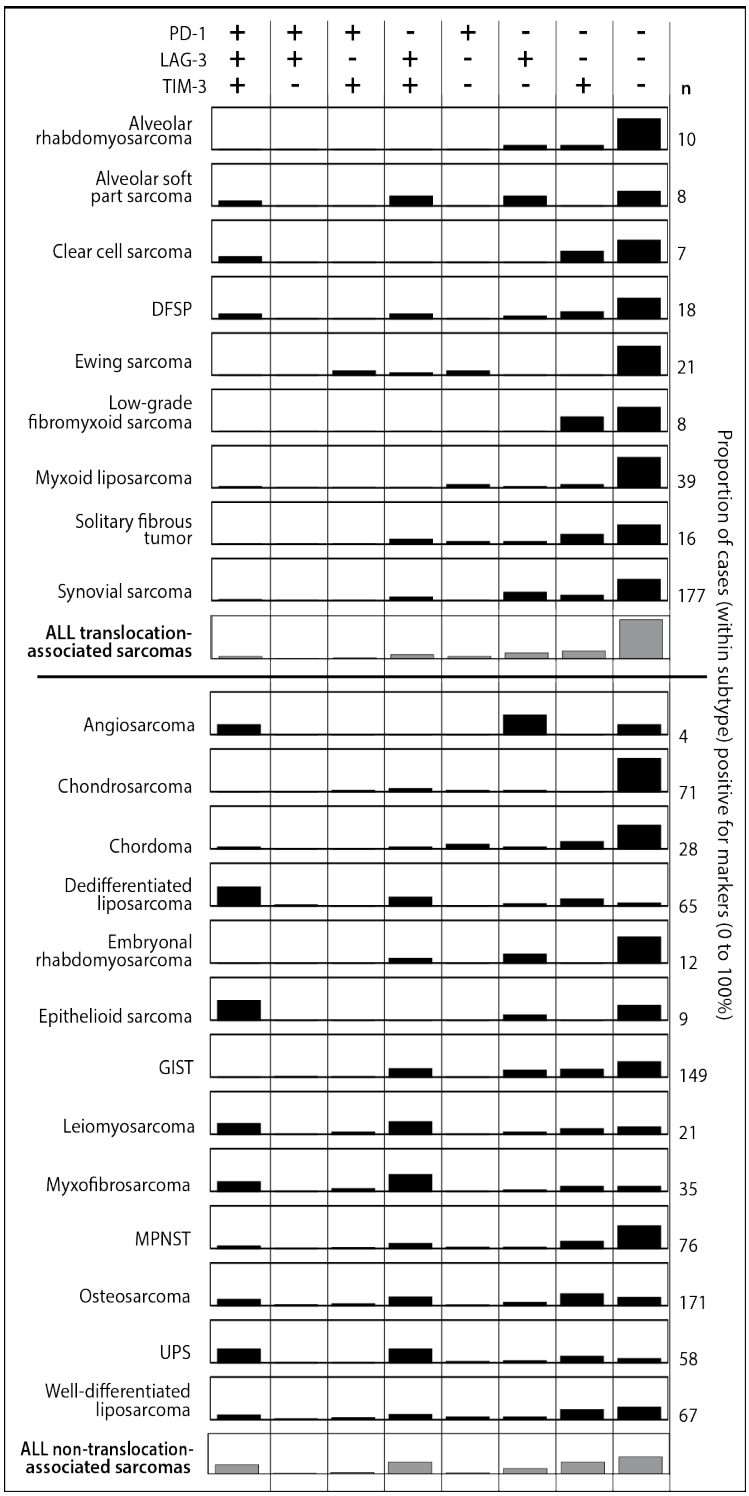


Figure 2.8 Proportion of cases positive for one or more of immune checkpoint biomarkers PD-1, LAG-3, and TIM-3.

Scored from immunohistochemistry on tissue microarray samples. Positive is defined as at least one positive-staining lymphocyte in any tissue microarray core. Panels represent histological type or translocation/non-translocation-associated classification.

By multivariate regression analysis, the distinction of non-translocation vs. translocation sarcoma was only associated with increased expression of PD-1 (p=0.02), but not of the other immune checkpoint biomarkers (Table 2.5). Grade and neoadjuvant therapy did not show a correlation with immune checkpoint biomarker expression (Table 2.5).

2.4.4 Prognostic Implications

We ran a multivariate Cox proportional hazards model including age, grade, histological diagnosis, and scores for TIL and checkpoint markers. Among the non-translocation-associated sarcomas, overall and progression-free survival were marginally better with increasing lymphocytic infiltration (p=0.02 and p=0.01; Fig. 2.9A). Overall survival was worse with increasing numbers of CD56+ TILs (p=0.03) and PD-1+ TILS (p=0.05; Fig. 2.9A). Among the translocation-associated sarcomas, TIL counts and immune checkpoint biomarker expression did not show any significant associations with overall or progression-free survival.

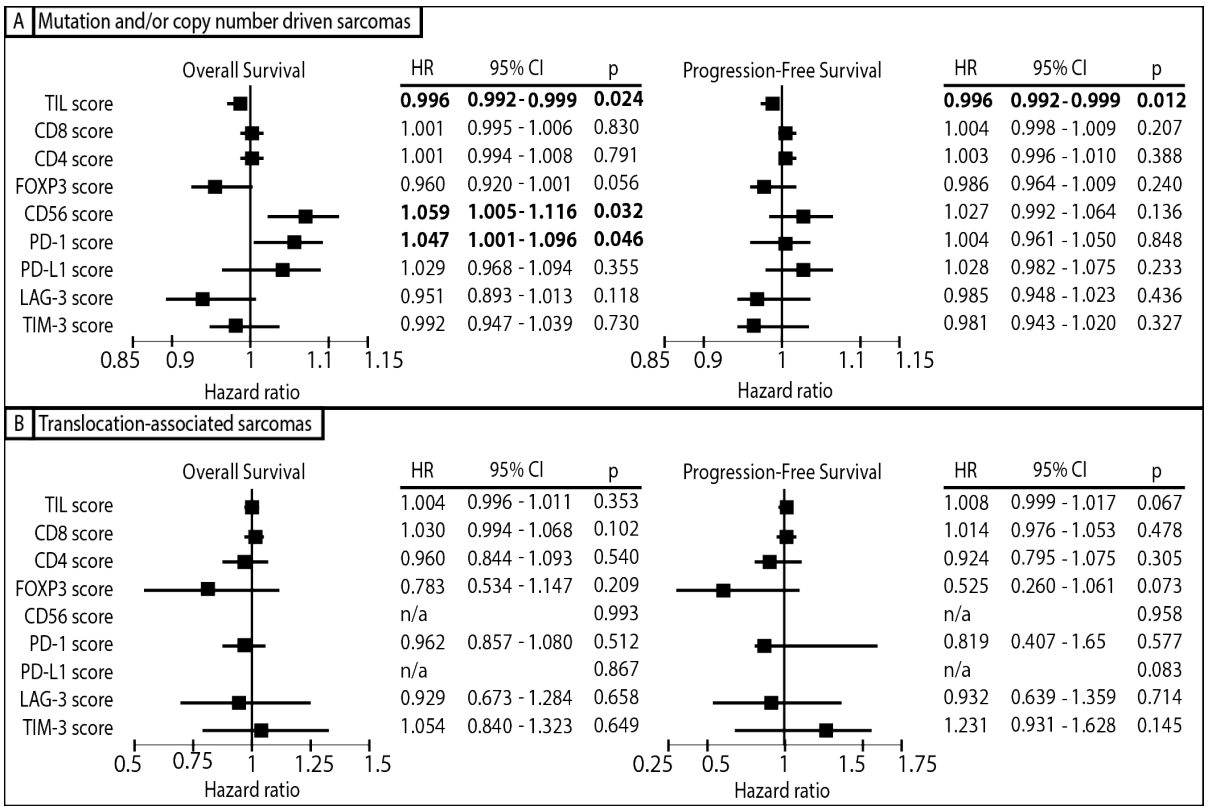


Figure 2.9 Forest plots depicting results of Cox multiple regression analysis of tumor infiltrating lymphocytes and lymphocyte subset staining for overall and progression-survival.
(A) Mutation and/or copy-number driven (non-translocation) sarcomas or (B) Translocation-associated sarcomas. Hazard ratios and p values were determined by Cox proportional-hazards multiple regression analysis, with Bon Ferroni correction. Age, grade, and sarcoma histotype were included in multiple regression analyses, but are not shown in plots.

We generated Kaplan-Meier curves, classified by CD8 and FOXP3 expression, into three groups: 1) no tumor-infiltrating lymphocytes, 2) CD8+ TILs only, 3) CD8+ and FOXP3+ TILs. The pleomorphic sarcoma subtypes as a group, defined here as the non-translocation sarcomas of high genomic complexity (and histologically pleomorphic), including angiosarcoma, dedifferentiated liposarcoma, leiomyosarcoma, myxofibrosarcoma, and undifferentiated pleomorphic sarcoma, showed improved overall survival with increased lymphocytic infiltrates (HR = 4.50, p = 0.034; Fig. 2.10A). In dedifferentiated liposarcoma, for which no cases were TIL-negative, patients positive for both CD8 and FOXP3 had better overall survival than those negative for FOXP3 (HR = 9.46, p = 0.002; Fig. 2.10B), a pattern also observed in malignant

peripheral nerve sheath tumor (HR = 12.66, p = 0.002; Fig. 2.10C). The inverse pattern was observed in myxoid liposarcoma, wherein cases positive for both CD8 and FOXP3 had worse overall survival than those with only CD8 positivity (HR = 32.00, p<0.001; Fig. 2.10D). Translocation-associated sarcomas as a group did not demonstrate any significant associations. The same trends were observed for progression-free survival as overall survival, but statistical evaluations did not reach significance.

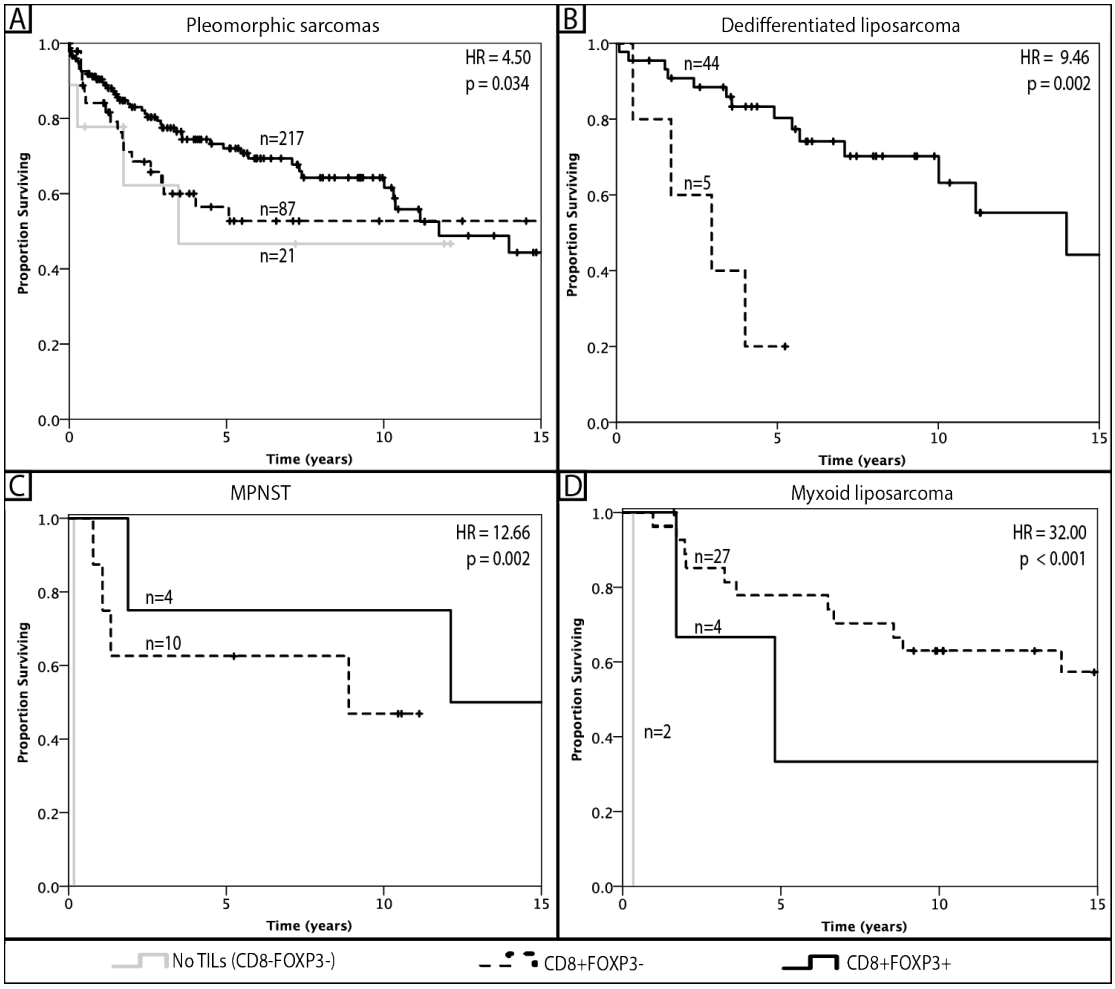


Figure 2.10 Kaplan-Meyer curves for overall survival based on presence of CD8+ and/or FOXP3+ TILs. (A) Pleomorphic sarcomas (angiosarcoma, dedifferentiated liposarcoma, leiomyosarcoma, osteosarcoma, and undifferentiated pleomorphic sarcoma), (B)dedifferentiated liposarcoma, (C) malignant peripheral nerve sheath tumor, (D) myxoid liposarcoma. Curves are stratified according to presence of TIL subsets by immunohistochemical positivity for: CD8 (cytotoxic T cells), and/or FOXP3 (natural killer cells) in tissue microarray cores. Hazard ratios and p-values were calculated by log-rank test.

2.5 Discussion

The effectiveness of systemic treatment for many types of sarcoma remains unsatisfactory, [84,615] and implementation of immunotherapy trials for sarcomas lags behind other, more prevalent diseases. This study provides a systematic characterization of tumor-infiltrating lymphocytes and immune checkpoint biomarker expression across the broadest range of sarcoma types examined to date, bridging the thus far uneven coverage – in terms of both histological types and biomarkers assessed – by studies examining the sarcoma immune microenvironment. [525,526,528,531,534,537,538,543,546,553,556,616-621]

Our analysis indicates that non-translocation sarcomas (mutation and/or copy-number driven) demonstrate greater T-cell immune infiltrates than translocation-associated sarcomas. In particular, dedifferentiated liposarcoma, as well as myxofibrosarcoma, undifferentiated pleomorphic sarcoma, and epithelioid sarcoma, had the most numerous and diverse populations of tumor-infiltrating lymphocytes among the 23 investigated sarcoma types. Our observation that non-translocation sarcomas present an immune microenvironment more like that of melanoma or carcinoma – index indications for immune checkpoint blockade – suggests that these sarcomas are the most suitable candidates for such therapies. This finding is consistent with what has been observed in early clinical trials of immune checkpoint blockade in sarcomas. [545,581,583,600,601]

The driving stimulus for the numerous T-cell infiltrates in non-translocation sarcomas remains unclear. In pan-cancer analyses, overall leukocyte fraction correlated positively with mutational burden, neoantigen counts, and intratumoral heterogeneity across a wide spectrum of malignancy, but negatively with measures of copy number alteration.[622] However, non-translocation sarcomas are largely copy-number driven tumors, with low mutational loads and predicted neoantigens relative to most carcinomas.[622] In fact, within the subset of non-

translocation sarcomas analyzed by The Cancer Genome Atlas (TCGA) (leiomyosarcoma, undifferentiated pleomorphic sarcoma, myxofibrosarcoma, and malignant peripheral nerve sheath tumor),[21] copy number alteration measured as fraction of bases deviating from baseline ploidy correlated negatively with leukocyte fraction (Spearman Rho -0.4193, $p<0.0001$), while no significant correlation with leukocyte fraction or CD8+ T-cells was identified for other measures of DNA damage (e.g. intratumoral heterogeneity, aneuploidy score, mutational burden, or predicted neoantigens).[622] Thus it is likely that other molecular events trigger the influx of lymphocytes in this setting, possibly including dysregulation of cytokines resulting in increased inflammatory infiltrates, or alterations in specific genes contributing to immunogenicity.[622]

Unlike the other non-translocation sarcomas, osteosarcoma and leiomyosarcoma have had disappointing results in clinical trials of checkpoint inhibitors.[581-583,599,623] Our data suggests that these sarcomas are associated with sparse lymphocytic infiltrates, potentially explaining why immunomodulatory therapy has been largely unsuccessful. Our data is largely congruent with findings reported in TCGA study, where leiomyosarcomas showed lower median mRNA expression of CD8 relative to undifferentiated pleomorphic sarcoma/myxofibrosarcoma and dedifferentiated liposarcoma.[21] Further, data from the TCGA pan-cancer analysis indicates that leiomyosarcomas have a lower overall leukocyte fraction, relative to other pleomorphic sarcomas. As expected, translocation-associated sarcomas displayed the lowest overall levels of tumor-infiltrating lymphocytes across our dataset, mirroring the immune microenvironment of benign lesions rather than that of the non-translocation sarcomas. This implies that translocation-associated sarcomas are immunoquiescent, despite high expression of immunogenic cancer testis antigens in many of these sarcomas, *e.g.* synovial sarcoma and myxoid liposarcoma,[572,574,578,579]

and therefore immunostimulatory therapy may be a more appropriate avenue for these sarcomas than would be checkpoint inhibition. Consistent with this idea, translocation-associated sarcomas have had some success with cancer vaccine[624,625] and adoptive T cell strategies.[572-574] Conceivably, some of the other non-translocation sarcoma types with low immunogenicity, such as chordoma, might also do well on similar regimens. Of note, several clinical trials of immune checkpoint inhibitors in carcinoma have reported that tumor mutational burden may be a biomarker of response to PD-L1/CTLA-4 blockade. [626-628] Sarcomas, in contrast, typically have low mutational burden, yet some have responded to immune checkpoint inhibitors, suggesting that different clinical biomarker predictors of response may be needed in this context.

In subtyping TILs, we observed a large predominance of CD8+ (cytotoxic) T cells. This suggests that the immune system is aware of the tumor and poised to activate anti-tumor immunity, but is being rendered inert by one or more immunoregulatory mechanisms. The presence of effector lymphocytes bodes well for the potential success of immune checkpoint blockade in these tumors. Conversely, the near absence of CD56+ TILs suggests that natural killer cell-mediated immune therapies may not be particularly suitable for sarcomas.

We observed low overall expression of PD-1 and PD-L1, a finding contrasting with some studies, [546,555,556,629-632] but agreeing with others. [385,525,633-637] This uncertainty may signify that PD-(L)1 signaling is not as relevant to sarcoma immune evasion as it is in other diseases, perhaps explaining the lackluster results for PD-(L)1 inhibitors in sarcoma clinical trials that have reported to date. [581-583,599-601] Alternatively, any discordance between determination of expression of these markers between our study and others may be explained by 1) the decay of PD-L1 in older tissue blocks, [613,638] as many of the tumor cases used to create our TMAs were >5 years old, and/or 2) the lower sensitivity of the anti-PD-L1 antibody (SP142) used in this study

compared to other clones, such as 22C3. [639,640] Moreover, both tumor cell expression of PD-L1 and lymphocyte expression of PD-1 throughout the tumor may be heterogeneous, which can also account for potential discrepancies in scoring between the TMAs used in different studies. Of note, one of the limitations of immune checkpoint studies in sarcoma is that sarcomas tend to be large, often exceeding 10 cm in size, and may be heterogeneous. Thus, not only TMA cores, but also whole-section staining may underscore PD-L1 expression in large tumors if negative. The implications of this in determining therapeutic eligibility for PD-1 inhibitors is as yet uncertain.

LAG-3 and TIM-3 are emerging immune checkpoints that have yet to be investigated in sarcomas.[364] Expression of both of these biomarkers was observed more frequently across all sarcoma types than either PD-1 or PD-L1. Interestingly, in our sample set, most sarcomas expressing PD-1 also express one or both of LAG-3 and TIM-3, providing another potential explanation for failure of PD-(L)1 inhibitor monotherapy. If tumors are already expressing these alternate immune checkpoint pathways, then lack of response to blockade of a single checkpoint may be expected. The biologic significance of this finding is uncertain; while LAG-3 has been suggested to synergize with PD-1 to mediate T-cell exhaustion and tumor immune escape in some contexts,[410] this has not been shown in sarcoma. We hypothesize that correlative studies on these trials would reveal a compensatory upregulation of these markers following treatment with immune checkpoint monotherapy, as has been shown in other diseases.[412,441,450] Future trials in sarcoma immunotherapy should aim to pursue these alternative immune checkpoints, alone or preferably in combination with other immune checkpoint inhibitors.

In addition to the associations identified between immune infiltration and sarcoma type, we observed that prior exposure to radiation has an independently significant association with a higher quantity of tumor infiltrating lymphocytes, as well as higher expression of immune

checkpoints. This may be a result of radiation-induced immunogenic cell death, wherein dying cells release damage-associated molecular patterns (DAMPs) that attract immune infiltrates.[641,642] This phenomenon has been recently discussed as a potential key mechanism of the abscopal effect, the phenomenon by which localized radiation triggers shrinkage of distal tumor sites and metastases.[643] Our data suggests that the abscopal effect may well be relevant in sarcomas, highlighting the potential to observe a benefit in clinical trials that combine immune checkpoint inhibition with radiotherapy.

Likely due to the small numbers of cases with demonstrable immune infiltration, overall survival among the translocation-associated sarcomas did not associate with any of the immune markers in this study. It is conceivable that any TILs observed in this cohort were simply bystanders, and not involved with anti-tumor immunity. Within our non-translocation sarcoma samples, higher counts of TILs associated with improved overall and progression-free survival, suggesting the presence of an immune response in these histologies has a survival benefit. Higher counts of CD56+ TILs, thought to represent the natural killer subset of lymphocytes, was associated with worse overall survival among the non-translocation sarcomas, which seemingly contradicts the expected role of natural killer lymphocytes in targeting cancer cells. However, CD56+ TILs were rare across our entire sample set, so it may be that the few tumors that did attract natural killer cells were a subset of particularly aggressive sarcomas. PD-1 expression also associated with worse overall survival in this group, presenting an immunoevasive environment that is likely contributing to worse outcomes.

2.6 Conclusions

New systemic therapy options are needed for sarcomas, and immuno-oncology approaches have engendered a great deal of excitement, with many drugs becoming approved for a variety of neoplastic indications. This study contributes to the body of knowledge that may help better select the sarcomas patients most likely to benefit from particular immunotherapy approaches, such as those targeting LAG-3 or TIM-3. Deepening our understanding of the diverse immune microenvironments of sarcomas – including aspects not assessed in this study, such as macrophage and antigen presenting cell infiltration, plasma cells, and immune signaling networks – should be able to inform pragmatic designs of much-needed new clinical trials for the treatment of sarcomas.

Chapter 3: Tumor-Associated Macrophages and Macrophage-Related Immune Checkpoint Expression in Sarcomas

3.1 Abstract

Early trials for immune checkpoint inhibitors in sarcomas have delivered mixed results. Efforts to improve patient outcomes are increasingly looking to combinatorial strategies involving novel immunotherapeutics, including some that target macrophages. To enhance our understanding of the sarcoma immune landscape, this study aims to quantify and characterize tumor-associated macrophage infiltration across sarcoma subtypes, as well as subtype-specific expression of the targetable macrophage-related immune checkpoint CD47/SIRPα. We surveyed immunohistochemical expression of CD68, CD163, CD47, and SIRPα in tissue microarrays representing 1322 sarcoma specimens (spanning 22 subtypes). Non-translocation sarcomas (particularly undifferentiated pleomorphic sarcoma and dedifferentiated liposarcoma) have significantly higher counts of both CD68+ and CD163+ macrophages than do translocation-associated sarcomas. Across nearly all sarcoma subtypes, macrophages outnumbered tumor-infiltrating lymphocytes and CD163+ (M2) macrophages outnumber CD68+ (M1) macrophages. These findings are supported by a new analysis of data from The Cancer Genome Atlas, which shows a correlation between macrophage infiltration and intratumoral heterogeneity, aneuploidy score, focal chromosomal copy number alterations, and homologous recombination defects. CD47 expression is bimodal, with most cases showing either 0% or 100% tumor cell staining, and the highest CD47 scores are observed in chordoma, angiosarcoma, and dedifferentiated liposarcoma. SIRPα scores correlate well with CD47 findings. Given the predominance of macrophage infiltrates over tumor-infiltrating lymphocytes, the bias toward M2

(immunosuppressive) macrophage polarization, and the generally high scores for CD47 and SIRPα, macrophage-focused immunomodulatory agents, such as CD47 or IDO-1 inhibitors, may be particularly worthwhile to pursue in sarcomas, alone or in combination with lymphocyte-focused agents.

3.2 Introduction

Sarcomas are an extremely diverse group of aggressive cancers originating from bone and soft tissues, with over 50 biologically and clinically distinct types described by the World Health Organization.[1] Unfortunately, due to the uncommonness of some subtypes, sarcoma clinical trials have a common practice of somewhat indiscriminately lumping together biologically-disparate sarcoma types in order to accelerate the recruitment of adequate numbers for statistical evaluation. This practice may disguise real clinical responses to investigated agents, due to the confounding presence of sarcoma types with very different oncogenic mechanisms, immune microenvironments, and clinical behaviours. Most sarcomas have few truly effective options for systemic therapy, due to poor responses to conventional chemotherapy and a paucity of applicable targeted therapy options.

Sarcomas were the first cancers to be successfully treated by immunotherapy (Coley's bacterial toxins in 1891),[521] so the promise of modern cancer immunotherapy has been greatly anticipated in the field. Immune checkpoint inhibitors have begun to be investigated in sarcomas,[581-583] but due to low numbers for any individual subtype, it has been difficult to glean any conclusive findings. More biologically-rational trial designs are needed to properly understand the impact of immune checkpoint inhibitors in sarcomas. Furthermore, trials of immune checkpoint inhibitors in other cancers are beginning to reveal high levels of therapeutic resistance by immune escape mechanisms, including activation of other (untargeted) immune

checkpoints.[644] Increasingly, immune checkpoints are being targeted by combinatorial therapeutic inhibition of two or more immune checkpoints, including some macrophage-related checkpoints, such as the CD47/SIRPα "don't eat me" signal. To guide design of such combinatorial studies, particularly in a group as heterogeneous as sarcomas, it is critical to have a detailed, type-specific understanding of the immune microenvironments of these diseases.

Tumor-associated macrophages (TAMs) have numerous roles within a tumor, involving influencing tumor cell proliferation, stromal formation and dissolution, vascularization, and mediating both pro- and anti-neoplastic inflammation.[274] TAMs are conventionally divided into two subgroups: M1 (classically-activated) and M2 (alternatively-activated) macrophages. M1-polarized macrophages are considered to be *pro-inflammatory*, as they have high levels of phagocytic activity and play an important role in amplifying the immune response through secretion of pro-inflammatory cytokines.[275] They are activated primarily by interferon-gamma, (IFNγ)[276,277] and once activated, M1 macrophages have two key functions: 1) direct tumor killing via complement-mediated phagocytosis,[275] and 2) secretion of high levels of pro-inflammatory cytokines and chemokines, which function to recruit more M1 macrophages and to activate the Th1 adaptive immune response.[275,279-282] M2-polarized macrophages are considered to be *anti-inflammatory*, as they are mainly involved in tissue repair and the resolution of inflammation.[275] They are activated primarily by IL-4, IL-10, and IL-13 (alternative activation).[277,283] Once activated, M2 macrophages downregulate expression of the pro-inflammatory cytokine IL-12 and upregulate expression of the anti-inflammatory cytokine IL-10.[284] IL-10 inhibits multiple immune functions: M1 macrophage proliferation/activation, helper T cell proliferation, antigen presentation, and production of pro-inflammatory cytokines.[223] Prior to polarization, macrophages first exist as M0 (uncommitted) macrophages, and polarization is plastic, largely

determined by cytokine stimuli. [282,291] Macrophages can switch polarization in response to external stimuli, such as IFNγ (shown to switch M2 macrophages to M1)[210] and IL-10 (shown to switch M1 macrophages to M2).[294]

High frequencies of TAMs are generally a poor prognostic factor in most cancer types.[272,273] Different subsets of TAMs have been linked to poor prognosis in breast cancer, [645,646] prostate cancer,[647] cervical cancer,[648] and non-small cell lung cancer.[649] While a number of studies have described tumor-infiltrating lymphocytes in sarcomas,[525-540] little is known about the involvement of tumor-associated macrophages. D'Angelo *et al* qualitatively assessed the presence of TAMs in a mixed-subtype study of 50 sarcomas (14 GIST, 5 synovial sarcoma, 4 leiomyosarcoma, and 1-3 cases of 18 other subtypes) and identified macrophages in 45 cases (90%).[525] In a study focusing on the sarcomas present in the Cancer Genome Atlas (TCGA), gene expression signatures[554] were used to identify immune infiltrates for 206 soft-tissue sarcomas (80 leiomyosarcoma, 50 dedifferentiated liposarcoma, 44 UPS, 17 myxofibrosarcoma, 10 synovial sarcoma, and 5 MPNST).[21] Macrophage scores were highest among dedifferentiated liposarcoma, myxofibrosarcoma, and UPS. In leiomyosarcoma, two studies have demonstrated an association between higher density of CD68+ or CD163+ macrophage infiltration and worse clinical outcomes. [547,549] This might provide an explanation for the poor performance of leiomyosarcomas in the lymphocyte-based immune checkpoint inhibitor clinical trials. [581-583,650]

CD47 is a ubiquitously-expressed cell-surface protein that protects viable erythrocytes from phagocytosis, sometimes referred to as the "don't eat me" signal. [455-461] In cancer, CD47 can be over-expressed to evade phagocyte-mediated anti-tumor immunity. Signaling occurs by interaction with SIRPα (signal-regulatory protein alpha), a cell-surface receptor expressed by macrophages and dendritic cells.[462] Activation of SIRPα by CD47 suppresses phagocytosis by

activating SHP1/2 (SH2-domain-containing tyrosine phosphatases 1 and 2), which inhibits myosin-II, preventing contraction with F-actin for macrophage movement and phagocytic action.[463] Anti-CD47 monoclonal antibodies have impressive activity in mouse xenograft models,[471-476,484,485] leading to the opening of a number of clinical trials targeting the CD47/SIRPα axis. In a phase Ib study of 22 non-Hodgkin lymphoma patients treated with CD47 mAb Hu5F9-G4 ± rituximab, an objective response was observed in 11 patients (50%, complete response in 36%) (NCT02953509).[489] In another phase I trial of Hu5F9-G4 in 16 patients with advanced solid tumors, stable disease was seen in 2 patients (12.5%), lasting 8 months and 16 months, respectively (NCT02216409).[485,490] A phase I study of TTI-621 – a recombinant fusion of the CD47-binding domain of SIRPα with the Fc domain of IgG4 – in relapsed/refractory hematologic malignancies observed progression-free survival of 161 in a patient with diffuse large B-cell lymphoma and of 70 days in a patient with follicular lymphoma.[491] Overexpression of CD47 has been observed across most cancers,[468-475] including osteosarcoma,[651] but to date has never been systematically examined across sarcomas.

In this study, we survey a large number of sarcomas (1322 patients, 22 sarcoma types) to determine type-specific expression patterns of M1 and M2 TAMs and of the CD47/SIRPα immune checkpoint, and examine associations with clinical parameters including survival.

3.3 Materials and Methods

3.3.1 Patient Tumor Samples

From the University of British Columbia (Vancouver, BC, Canada), 14 formalin-fixed, paraffin-embedded tissue microarrays (TMAs) were included: TMA 01-003 (synovial sarcoma and differential diagnoses, 82 cases in duplicate)[604]; TMA 03-008 (chondroid tumors, 121 cases in duplicate)[605]; TMA 06-001E (gastrointestinal stromal tumors, 129 cases in duplicate)[606];

TMA 06-007 (myxoid liposarcomas, 69 cases in triplicate)[607]; TMA 09-006 (epithelioid sarcoma and differential diagnoses, 53 cases in duplicate)[608]; TMA 10-004 (28 chordomas, in duplicate); TMA 10-009 (8 alveolar soft part sarcomas, 2 alveolar rhabdomyosarcomas, 2 desmoplastic small round cell tumors, in triplicate)[608]; TMA 12-004 (BCL2-positive tumors, 35 cases in triplicate)[609]; TMA 12-005 (pediatric spindle cell lesions, 134 cases in duplicate)[609]; TMA 12-006 (translocation-associated sarcomas, 10 cases in duplicate)[609]; TMA 12-010 (5 dedifferentiated liposarcomas and 5 undifferentiated pleomorphic sarcomas, in duplicate)[609]; TMA 14-006 (4 myxoid liposarcomas, 3 myxofibrosarcomas, 3 chondrosarcomas, 1 synovial sarcoma, 1 malignant peripheral nerve sheath tumor, in duplicate)[566]; TMA 14-007 (dedifferentiated liposarcomas with well-differentiated areas, both components for 57 cases in duplicate)[610]; and TMA MPNST (malignant peripheral nerve sheath tumor and differential diagnoses, 176 cases in duplicate)[611]. From Mount Sinai Hospital (New York, NY, USA), 4 TMAs were included: MSH-OSa (osteosarcomas, 280 cases in duplicate); MSH-SS (synovial sarcomas, 70 cases in duplicate); MSH-SFT (solitary fibrous tumor, 140 cases in duplicate); and MSH-UPS (74 undifferentiated pleomorphic sarcomas, 52 myxofibrosarcomas, 18 leiomyosarcomas, 13 dedifferentiated liposarcomas, 9 dermatofibrosarcoma protuberans, and differential diagnoses; 210 cases total in duplicate).

3.3.2 Tissue Microarray Preparation

All tissue specimens were derived from surgical resection specimens from Mount Sinai Hospital, NY (MSH TMAs) [612], 20 centres throughout Norway (TMA 06-001), or Vancouver General Hospital, BC (all other TMAs). Cores with a diameter of 1.0 mm (TMA 14-007, all MSH TMAs) or 0.6 mm (all other TMAs) were extracted from representative tumor tissue, as

identified by a bone and soft tissue subspecialty pathologist (TO Nielsen, EG Demicco). TMAs were cut to 4-µm-thick sections, mounted to Fisherbrand™ Superfrost™ Plus charged glass slides (Thermo Fisher Scientific Inc, Waltham, MA), and incubated for 1h at 60°C.

3.3.3 Immunohistochemistry

Immunohistochemical staining was performed on serial TMA sections, which were cut in batches and processed immediately. All antibodies were applied using the Ventana DISCOVERY® ULTRA semi-automated staining system (Ventana Medical Systems Inc, Tucson, AZ), as described previously[610]. Briefly, heat-induced antigen retrieval was performed using the Cell Conditioning 1 (CC1, Ventana) protocol. Slides were incubated with primary antibodies (Table 3.1) in DISCOVERY antibody diluent (Ventana) for 2h at 21°C. For CD68, CD47, and SIRPα, slides were incubated for 16 minutes at 37°C in DISCOVERY Universal secondary antibody (Ventana), and chromogen visualization was performed by DAB map detection (Ventana). For CD163, slides were incubated for 2h at 21°C with the UltraMap anti-mouse secondary antibody (Ventana) and visualized using the UltraMap DAB Kit (Ventana).

Marker	Clone	Details	Vendor	Dilution
CD68	KP1	mouse mAb	Agilent Dako, Carpinteria, CA, USA	1:1000
CD163	10D6	mouse mAb	Leica Biosystems (Novocastra), Wetzlar, Germany	1:500
CD47	B6H12	mouse mAb	Lifespan Biosciences, Seattle, WA, USA	1:50
SIRPα	A-1	mouse mAb against SIRPα/β	Santa Cruz Biotechnology, Dallas, TX, USA	1:50

Table 3.1 Details of primary antibodies used for tumor-associated macrophage immunohistochemistry.

Following immunohistochemistry, all slides were counterstained with hematoxylin and mounted. Digital images of immunostained tissue microarrays were acquired using the Olympus BLISS high-definition virtual microscope and slide scanner (Olympus Life Science Solutions: Bacus Laboratories, Lombard, IL, USA) or the Aperio digital pathology slide scanner (Leica Biosystems, Wetzlar, Germany).

Positive control tissue for all antibodies was normal lymph node, negative control tissue was cerebellum. CD68 and CD163 are validated, clinically-available antibodies, while CD47 and SIRPα are research-grade commercial antibodies that were validated by the manufacturers. We did not perform independent validation studies beyond the use of appropriate tissue controls.

3.3.4 Histological Scoring

Scoring was performed by pathologists experienced in scoring biomarkers in bone and soft tissue tumors (EG Demicco, D Gao). All immunohistochemical markers were scored for cytomembranous positivity. Replicate cores were scored separately, with the pathologist blinded to replicate status and final histological diagnosis. Macrophage biomarkers (CD68, CD163, SIRPα) were scored by counting the number of positive-staining macrophages per TMA core (Fig. 3.2A,B,D), divided by the area of the core to yield a value for macrophages / mm^2, up to 2000/mm^2 (scores above this threshold were marked as 2000/mm^2). CD47 was scored as the percentage of positive-staining sarcoma tumor cells (Fig. 3.2C). CD47 staining intensity was also scored, but excluded from analyses due to mainly uniform intensity across positive samples.

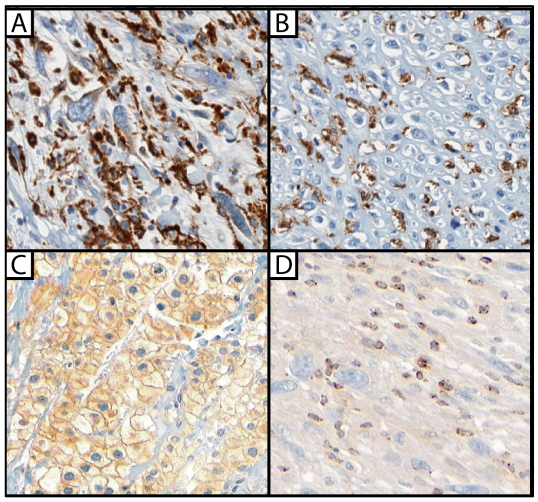


Figure 3.1 Immunohistochemical staining of macrophage and checkpoint biomarkers in sarcoma tissues. (A) CD68 (KP1 antibody), dedifferentiated liposarcoma; (B) CD163 (10D6), osteosarcoma; (C) CD47 (B6H12), chordoma; (D) SIRPα (A-1), dedifferentiated liposarcoma.

3.3.5 TCGA Data Analysis

Data on the relative proportion of macrophages and macrophage subsets (M0, M1, M2), and measures of DNA damage, was extracted from published data (Thorsson *et al.* (2018),[622] Supplemental table 1 – PanImmune Feature Matrix of Immune Characteristics,[652] and analyzed according to the 6 sarcoma types defined in the TGCA sarcoma analysis:[21] dedifferentiated liposarcoma (n=50), myxofibrosarcoma (n=17), undifferentiated pleomorphic sarcoma (n=44), leiomyosarcoma (n=80), malignant peripheral nerve sheath tumor (n=5), and synovial sarcoma (n=10). Data on macrophages and polarized subsets of macrophages are represented as a percent of the total immune infiltrate (leukocyte fraction) in a given case and do not reflect absolute differences between cases. DNA damage measures were defined in Thorsson *et al*[622] as follows: 1) Intratumoral genomic heterogeneity was assessed as subclonal genome fraction – the percent of the tumor genome not represented by the plurality clone; 2) Homologous recombination deficiency (HRD) score summed 3 variables: number of regions of>15 Mb with loss of heterozygosity, breaks between adjacent segments of >10 Mb and allelic imbalances in subtelomeric regions; and 3) Aneuploidy score was defined as the sum total of amplified or deleted chromosome arms. Segments altered represent the total number of altered chromosome segments in a sample. The non-silent mutation rate, and predicted single nucleotide and indel neoantigens (pMHCs) were likewise taken as calculated by Thorsson *et al*.[622]

3.3.6 Statistical Analysis

Data analysis was performed using IBM® SPSS® statistics software (version 26). An Independent Samples Kruskal-Wallis 1-way ANOVA test was used to assess the differences in scoring between histological types. Categories were compared pairwise, and significance values were adjusted using the Bonferonni correction for multiple comparisons. Correlations between

scores for different biomarkers and correlation between TCGA macrophage signatures and DNA damage were assessed using Spearman's correlation coefficient. Multivariable linear regression was used to assess the relative impact of biomarker scores and clinicopathological factors on survival. Survival correlates were evaluated using a Cox proportional-hazards multiple regression analysis to generate hazard ratios and corresponding 95% confidence intervals. Kaplan Meier curves were generated based on cases positive for each marker, and a log rank test was run to determine if there were differences in the survival distributions. Statistically significant differences were defined as $p<0.05$, and p-values for multiple comparisons were adjusted by Bon-Ferroni correction.

3.4 Results

3.4.1 Patient Demographics

Surgical resection specimens from 1322 sarcomas (22 histotypes, Table 3.2) and 236 benign bone or soft-tissue tumors (Table 3.3) were available for evaluation. Clinical data was available for 895 sarcomas (Table 3.2). The mean patient age was 41.6 (range 0-94), and the mean tumor size was 7.8 cm (range 1-42 cm). By FNCLCC criteria (Table 3.2), 235 (26%) were classified as grade 3, 233 (26%) as grade 2, 73 (8%) as grade 1, and 354 had no grade specified (40%). Prior to surgery, 151 patients (17%) had received neoadjuvant chemotherapy, 39 (4%) had received neoadjuvant radiotherapy, 18 (2%) had received both, and 416 patients (47%) had not been treated by chemotherapy or radiotherapy (Table 3.2). Neoadjuvant therapy was not known in 271 (30%) cases. By Fisher's Exact Test, there was (as expected) an association between certain histological types and administration of neoadjuvant chemotherapy, *e.g.* osteosarcoma($p<0.0005$), and/or radiotherapy, *e.g.* dedifferentiated liposarcoma, Ewing sarcoma, alveolar and embryonal rhabdomyosarcoma, and synovial sarcoma ($p<0.0005$).

Sarcoma Type	Total		Clinical		Clinical Parameters	Clinical	
	n	%	n	%		n	%
Translocation-Associated	**404**	**30.6**	**337**	**37.7**	**FNCLCC Grade**		
Synovial sarcoma	144	8.7	131	14.6	1	73	8.2
Solitary fibrous tumor	126	7.6	112	12.5	2	233	26.0
Myxoid liposarcoma	41	2.5	35	4.2	3	235	26.3
DFSP	35	2.1	20	2.2	unknown	354	39.6
Ewing sarcoma	21	1.3	15	1.7	**Neoadjuvant treatment**		
Low-grade fibromyxoid sarcoma	11	0.7	9	1.0	none	416	46.5
Alveolar rhabdomyosarcoma	10	0.6	6	0.7	chemotherapy	151	16.9
Alveolar soft part sarcoma	8	0.5	4	0.4	radiation therapy	39	4.4
Clear cell sarcoma	8	0.5	5	0.6	chemo + radiation	18	2.0
Non-Translocation	**918**	**69.4**	**558**	**62.3**	unknown	271	30.3
Osteosarcoma	268	16.2	243	27.2	**Adjuvant treatment**		
GIST	129	7.8	0	0.0	none	314	35.1
UPS	85	5.1	65	7.3	chemotherapy	115	12.8
Dedifferentiated liposarcoma	81	4.9	59	6.6	radiation therapy	69	7.7
MPNST	81	4.9	20	2.2	chemo + radiation	15	1.7
Chondrosarcoma	71	4.3	65	7.3	unknown	382	42.7
Well-differentiated liposarcoma	67	4.0	6	0.7	**Locoregional recurrence**		
Myxofibrosarcoma	56	3.4	38	4.2	yes	323	36.1
Chordoma	28	1.7	23	2.6	no	411	45.9
Leiomyosarcoma	27	1.6	20	2.2	unknown	161	18.0
Embryonal rhabdomyosarcoma	12	0.7	8	0.9	**Metastasis**		
Epithelioid sarcoma	9	0.5	8	0.9	yes	351	39.2
Angiosarcoma	4	0.2	3	0.3	no	410	45.8
TOTAL	**1322**	**100**	**895**		unknown	134	15.0

Table 3.2 Clinical and histopathological characteristics of sarcoma samples.

"Sarcoma Type" column describes all samples in our dataset ("Total"; N=1322) as well as those for which clinical data is available ("Clinical"; n=895); "Clinical Parameters" column describes only the subset of cases for which clinical data is available.

Histological type	n
Leiomyoma	49
Neurofibroma	42
Fibromatosis	37
Enchondroma	30
Schwannoma	22
Lipoma	10
Fibroma (unspecified)	6
Myofibroma	6
Osteochondroma	6
Necrobiosis lipoidica	5
Nodular fasciitis	5
Chondroblastoma	4
Chondromyxoid fibroma	4
Granuloma annulare	4
Perineuroma	4
Fetal rhabdomyoma	2
Myositis ossificans	2

Table 3.3 Benign mesenchymal lesions on tissue microarrays

3.4.2 Quantification of Tumor-Associated Macrophages

We quantified tumor-associated macrophages using immunohistochemical markers CD68 and CD163. Pleomorphic sarcoma types demonstrated the highest counts of both CD68+ and CD163+ macrophages (Fig. 3.2A-B), particularly undifferentiated pleomorphic sarcoma (median CD68=460/mm^2, CD163=500/mm^2, n=75), dedifferentiated liposarcoma (median CD68=356/mm^2, CD163=474/mm^2, n=76), myxofibrosarcoma (median CD68=373/mm^2, CD163=291/mm^2, n=55), and leiomyosarcoma (median CD68=260/mm^2, CD163=273/mm^2, n=26). Angiosarcomas had the highest counts for both macrophage markers, (CD68=486/mm^2, CD163=1081/mm^2), but these counts were scored from only 4 patients (Fig. 3.2AB).

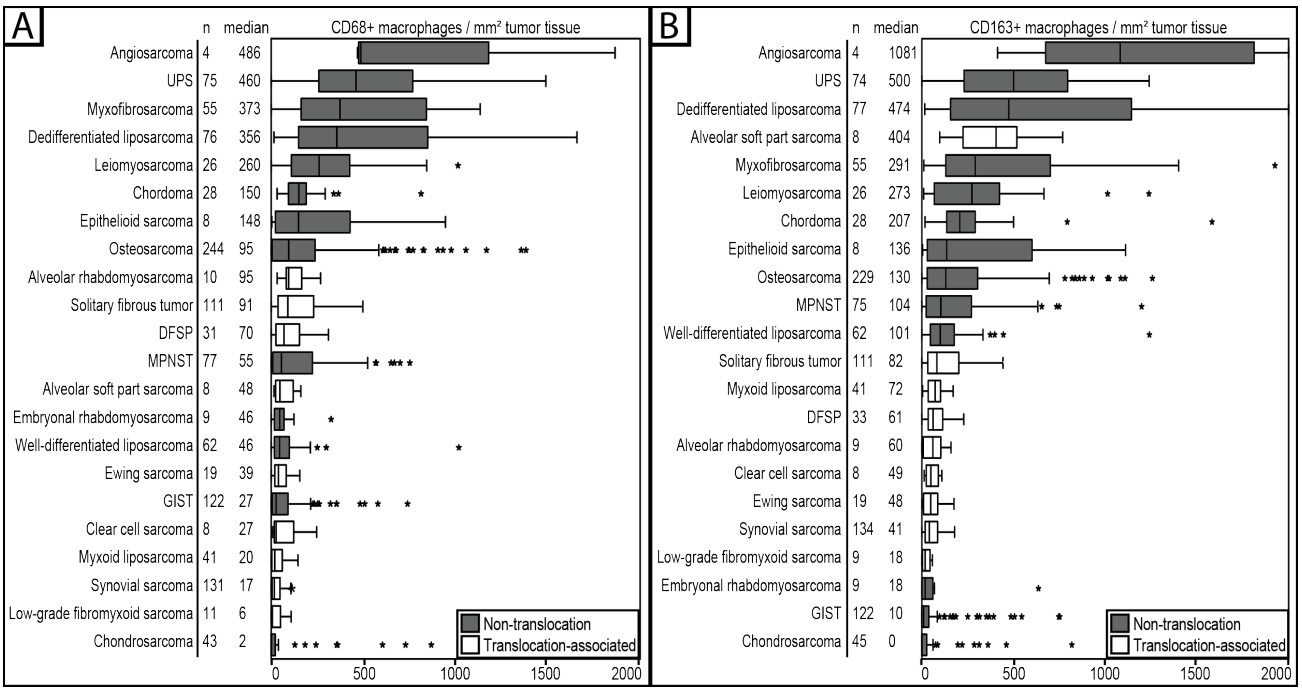


Figure 3.2 Quantification of tumor-associated macrophages across sarcoma subtypes.
(A) Boxplots depicting comparative counts of CD68+ macrophages across sarcoma subtypes. (B) Boxplots depicting comparative counts of CD163+ macrophages across sarcoma subtypes. Boxes represent the first through third quartiles, vertical line indicates median, and whiskers indicate range. Extreme outliers are indicated as dots.

As a group, sarcomas driven by mutations and/or copy-number alterations (non-translocation-associated sarcomas) had significantly higher ($p<0.001$) macrophage counts (median CD68=121.8/mm^2, CD163=150.6/mm^2) than did the translocation-associated sarcomas (median CD68=23.2/mm^2, CD163=52.5/mm^2) or benign mesenchymal tumors (median CD68=17.9/mm^2, CD163=37.3/mm^2) (Fig. 3.3). Translocation-associated sarcomas as a group showed no significant difference in macrophage infiltrate counts when compared to benign mesenchymal tumors; however, alveolar soft part sarcomas had some of the highest counts for CD163+ macrophages, with a median count of 404/mm^2 (Fig. 3.2B). Across the sample set, there was a strong correlation between counts of CD68+ and CD163+ macrophages (Spearman's rho = 0.746, $p<0.001$).

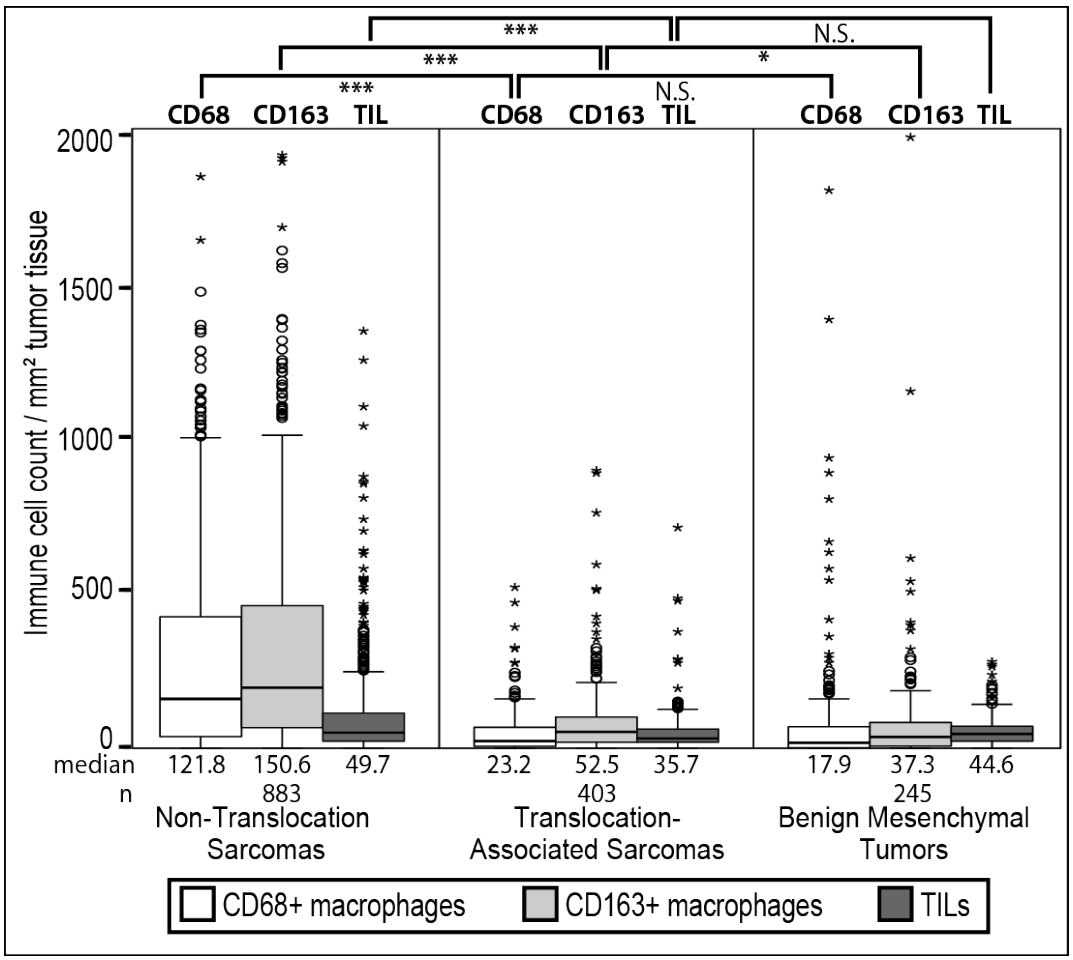


Figure 3.3 Comparison of tumor-associated macrophages to tumor-infiltrating lymphocytes across malignant and benign mesenchymal tumors.
(A) Boxplots depicting counts of CD68+ macrophages (white), CD163+ macrophages (light grey), and tumor-infiltrating lymphocytes (TILs; dark grey). Boxes represent the first through third quartiles, horizontal line indicates median, and whiskers indicate range. Extreme outliers are indicated as dots. *** represents $p<0.001$, significance calculated by Independent Samples Kruskal-Wallis 1-way ANOVA.

Across nearly all sarcoma subtypes investigated, macrophage infiltrates outnumbered tumor-infiltrating lymphocytes (Fig. 3.4). Macrophage predominance was particularly evident among the non-translocation sarcomas, with over ten-fold CD68:TIL ratios for chordoma, angiosarcoma, undifferentiated pleomorphic sarcoma, myxofibrosarcoma, and leiomyosarcoma (Fig. 3.5). Non-translocation sarcomas had a significantly higher CD68:TIL ratio (mean: 7.0, 95% CI: 6.0-7.9) than was observed among the translocation-associated sarcomas (mean: 2.2, 95% CI: 1.6-2.8)($p<0.001$, Fig. 3.5).

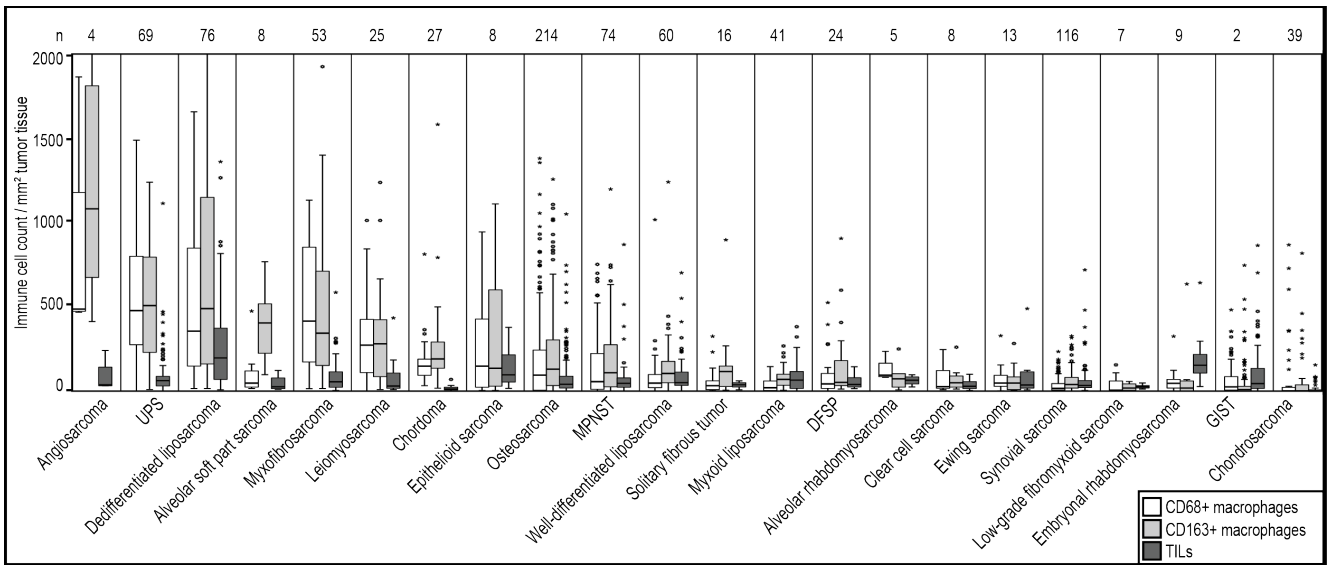


Figure 3.4 Comparison of tumor-associated macrophages to tumor-infiltrating lymphocytes across sarcoma subtypes.

Boxplots depict comparative counts of CD68+ macrophages (white), CD163+ macrophages (light grey), and tumor-infiltrating lymphocytes (TILs; dark grey) across sarcoma subtypes. Boxes represent the first through third quartiles, horizontal line indicates median, and whiskers indicate range. Extreme outliers are indicated as dots.

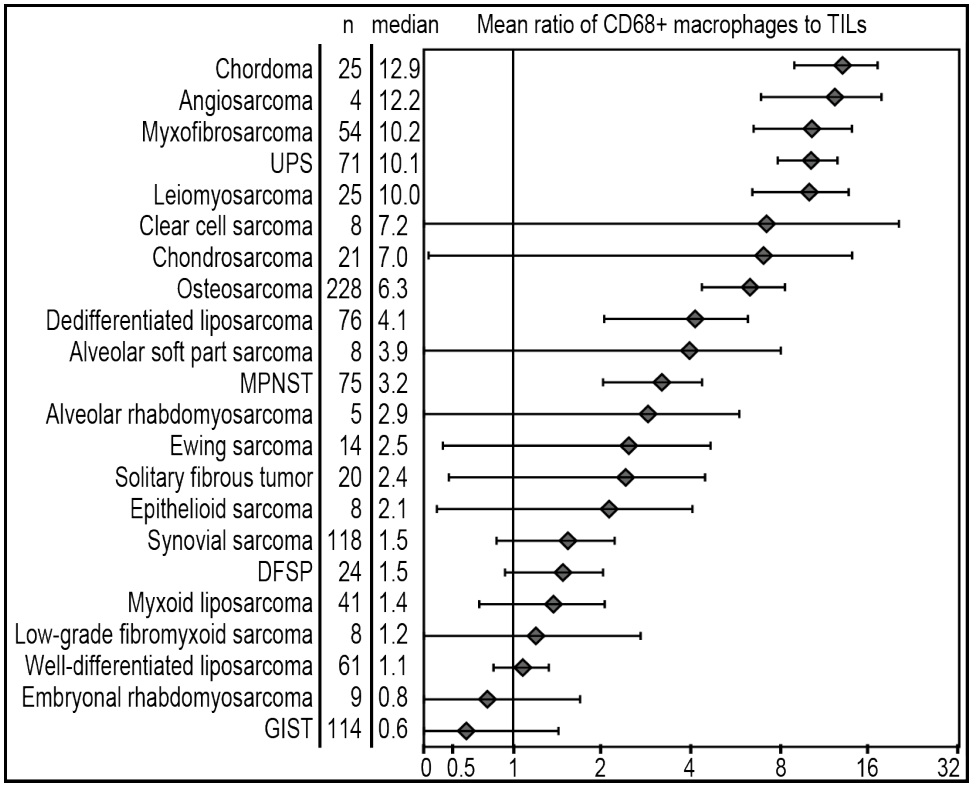


Figure 3.5 Mean ratio of CD68+ macrophages to tumor-infiltrating lymphocytes across sarcoma subtypes.

Ratios based on counts of positive-staining immune cells per mm² tumor tissue, scored from tissue microarray cores. Error bars represent 95% confidence interval of the mean.

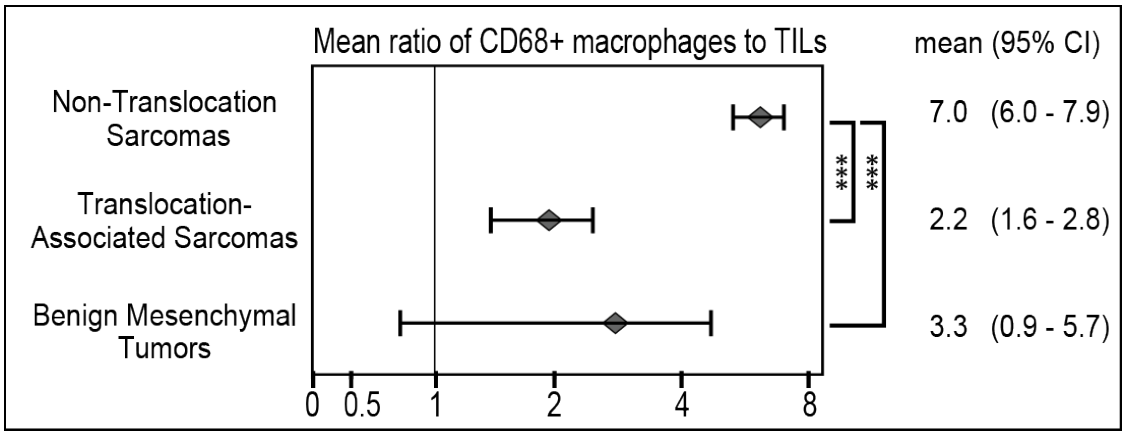


Figure 3.6 Mean ratio of CD68+ macrophages to tumor-infiltrating lymphocytes across malignant and benign mesenchymal tumors.
Ratios based on counts of positive-staining immune cells per mm² tumor tissue, scored from tissue microarray cores. Error bars represent 95% confidence interval of the mean.

We compared our immunohistochemical quantitation of macrophage density with the macrophage signatures calculated from mRNA expression data for sarcomas analyzed in The Cancer Genome Atlas (TCGA). Similar to our own findings at the protein level, the TCGA data showed that the highest macrophage signatures were found in dedifferentiated liposarcoma and undifferentiated pleomorphic sarcoma/myxofibrosarcoma, while leiomyosarcoma had the lowest macrophage signatures out of the 3 non-translocation sarcomas evaluated, and synovial sarcoma, had the lowest overall expression of macrophage RNA signatures.[21] We further investigated the relative proportion of macrophages in sarcoma immune infiltrates – calculated from published gene expression signatures[622] – and in dedifferentiated liposarcoma, myxofibrosarcoma, undifferentiated pleomorphic sarcoma, and malignant peripheral nerve sheath tumor, macrophages were more likely to comprise over 50% of the total immune infiltrates, while in leiomyosarcoma and synovial sarcomas, the median contribution of macrophages to the overall immune infiltrate was less than 50% (Fig. 3.7). These findings support the validity of our immunohistochemical quantitation and relative macrophage:TIL ratios in sarcomas.

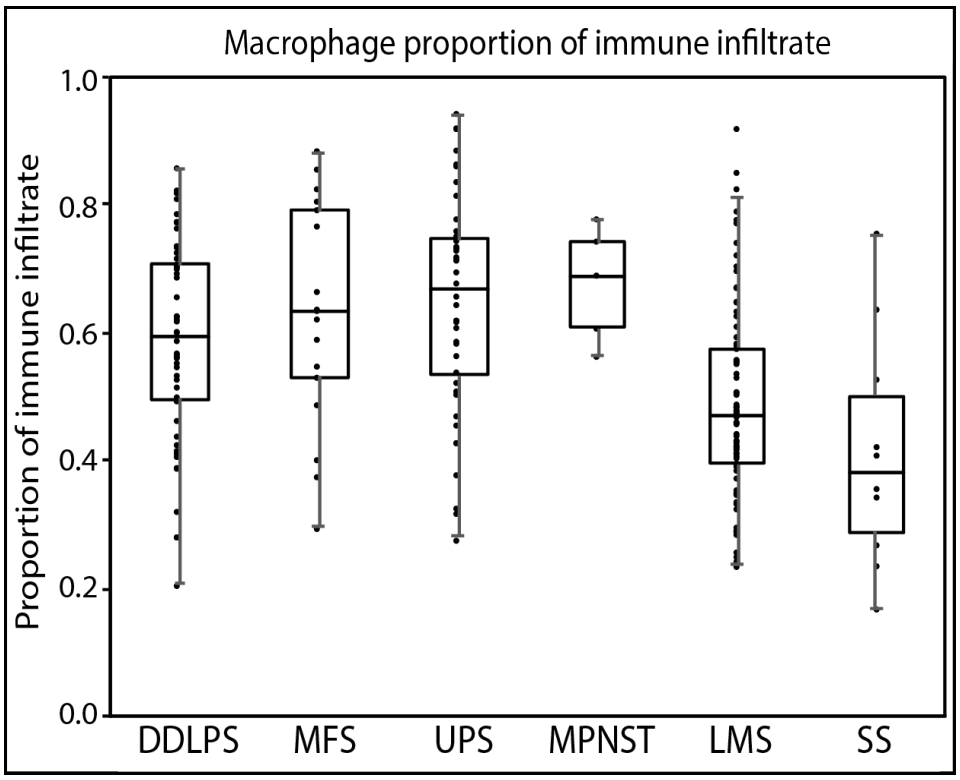


Figure 3.7 Boxplot illustrating proportion of tumor-immune infiltrates represented by macrophages in sarcomas using mRNA expression signatures.
Gene expression signatures were calculated in Thorsson *et al* (2018) using CIBERSORT and normalized to leukocyte fraction to produce the published values shown here. Boxes represent the first through third quartiles, horizontal lines indicate median, and whiskers indicate range. Dots indicate individual tumors.

3.4.3 Macrophage Polarization

Using CD163 as a marker of M2 (anti-inflammatory) macrophages and CD68 as a marker of M1 (pro-inflammatory) macrophages, we observed that sarcomas tend to have a higher proportion of CD163+ macrophages than CD68+ macrophages (Fig. 3.8), particularly alveolar soft part sarcoma (mean CD163/CD68=10.6, n=8), malignant peripheral nerve sheath tumor (median CD163/CD68=6.3, n=68), myxoid liposarcoma (mean CD163/CD68=5.3, n=35), and synovial sarcoma (mean CD163/CD68=5.2, n=117). The CD163:CD68 ratio was relatively balanced in epithelioid sarcoma, undifferentiated pleomorphic sarcoma, myxofibrosarcoma, and both rhabdomyosarcoma subtypes. The only sarcoma type that exhibited strong CD68-predominance was low-grade fibromyxoid sarcoma (mean CD163/CD68=0.3, n=6) (Fig. 3.8).

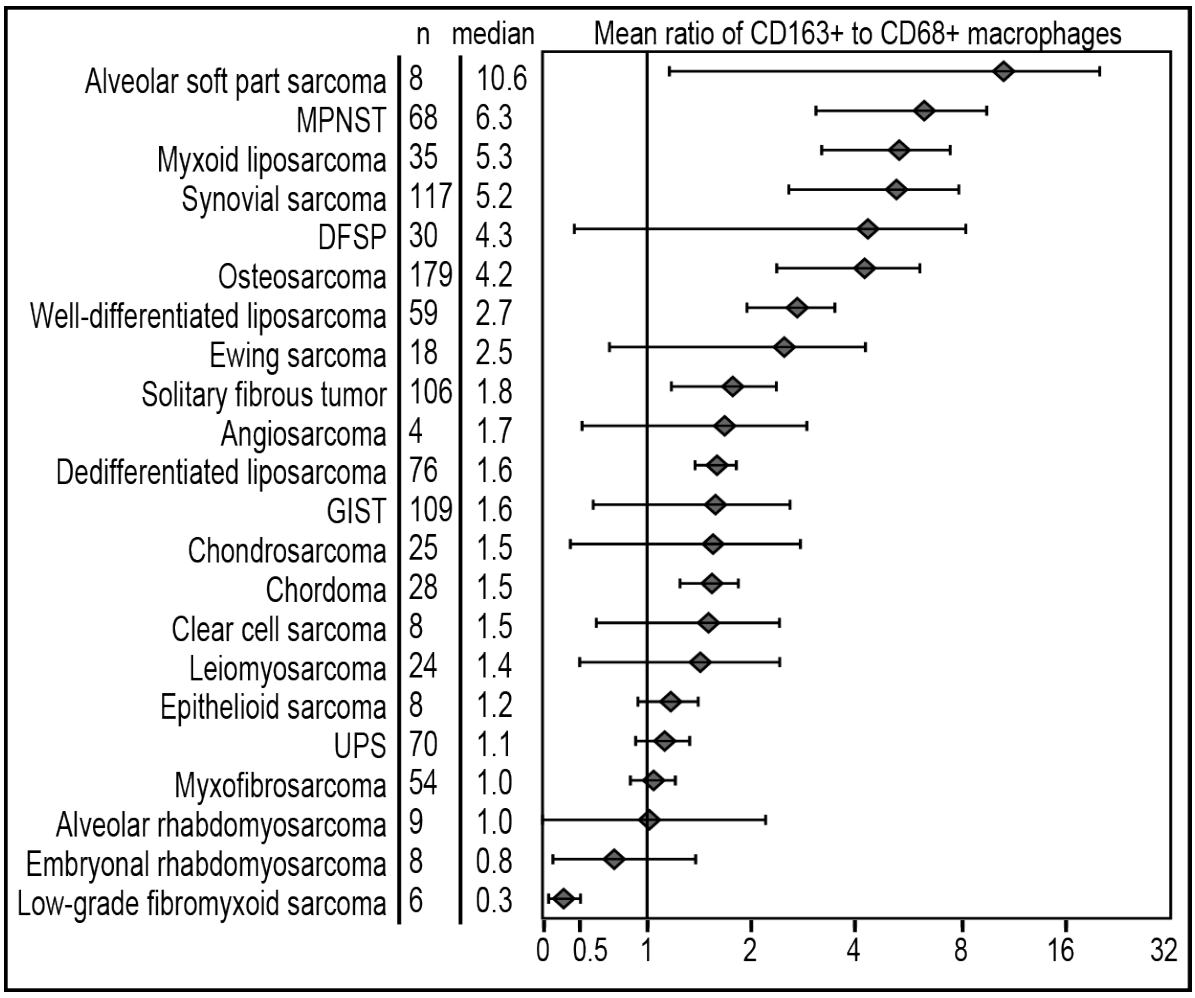


Figure 3.8 Mean ratio of M2 to M1 macrophage across sarcoma subtypes.
Mean ratio of CD163:CD68, based on counts of positive-staining immune cells per mm² tumor tissue, scored from tissue microarray cores. Error bars represent 95% confidence interval of the mean.

Similarly, within the sarcoma types analyzed at the mRNA level by TCGA,[622] M2-signature macrophages represented a larger fraction of the total immune infiltrate than either M0-signature or M1-signature macrophages (Fig. 3.9A-C). The median calculated M2:(M0+M1) ratio was higher in the non-translocation sarcomas, ranging from 2.1 (malignant peripheral nerve sheath tumor) to 8.6 (dedifferentiated liposarcoma), whereas synovial sarcoma tended to have more M0-signature and M1-signature macrophages than M2-signature macrophages (median ratio 0.7).

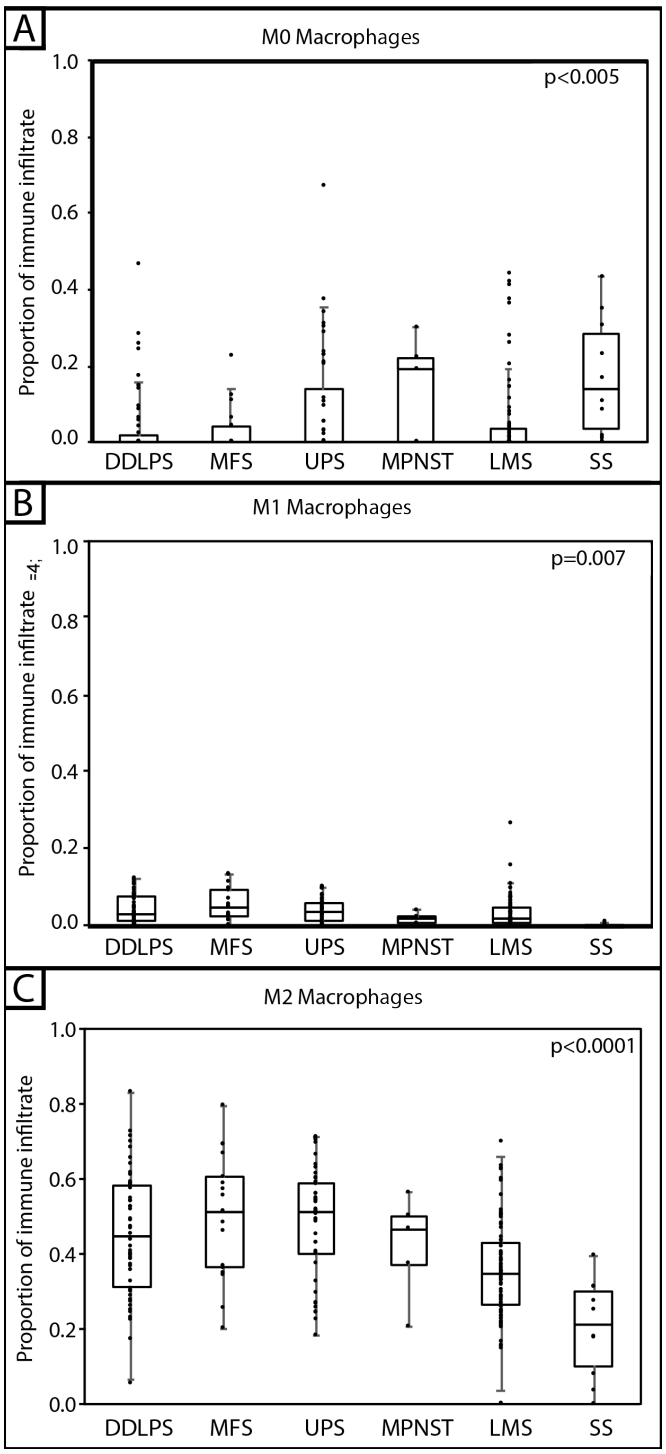


Figure 3.9 Boxplot illustrating proportion of tumor immune infiltrates represented by each subset of macrophages in sarcomas using mRNA expression signatures.

(A) M0-signature macrophages, (B) M1-signature macrophages, (C) M2-signature macrophages. Gene expression signatures were calculated in Thorsson *et al* (2018) using CIBERSORT and normalized to leukocyte fraction to produce the published values shown here. Boxes represent the first through third quartiles, horizontal lines indicate median, and whiskers indicate range. Dots indicate individual tumor specimens. P-values shown are calculated by 1-way ANOVA.

In order to determine if macrophage infiltrates might be related to the extent of genomic alterations in sarcoma, we calculated Spearman rank correlation coefficients between macrophage proportion and measures of genomic complexity in 4 major non-translocation sarcoma types: dedifferentiated liposarcoma (n=50), leiomyosarcoma (n=80), undifferentiated pleomorphic sarcoma (n=44), and myxofibrosarcoma (n=17) using published data.[622] Overall, intratumoral heterogeneity showed a significant positive correlation with increasing proportion of macrophages in the immune microenvironment ($p<0.05$), as did measures representing arm-level chromosomal alterations (aneuploidy score), focal chromosomal copy number alterations (number of chromosomal segments altered), and homologous recombination defects (Fig. 3.10). No changes in relative macrophage proportion were observed based on small nucleotide alterations, such as non-silent mutation rate or number of predicted neoantigens (Fig. 3.10). However, among individual tumor types, only leiomyosarcoma showed strong correlations between macrophage infiltrates and chromosomal alterations (Fig. 3.10).

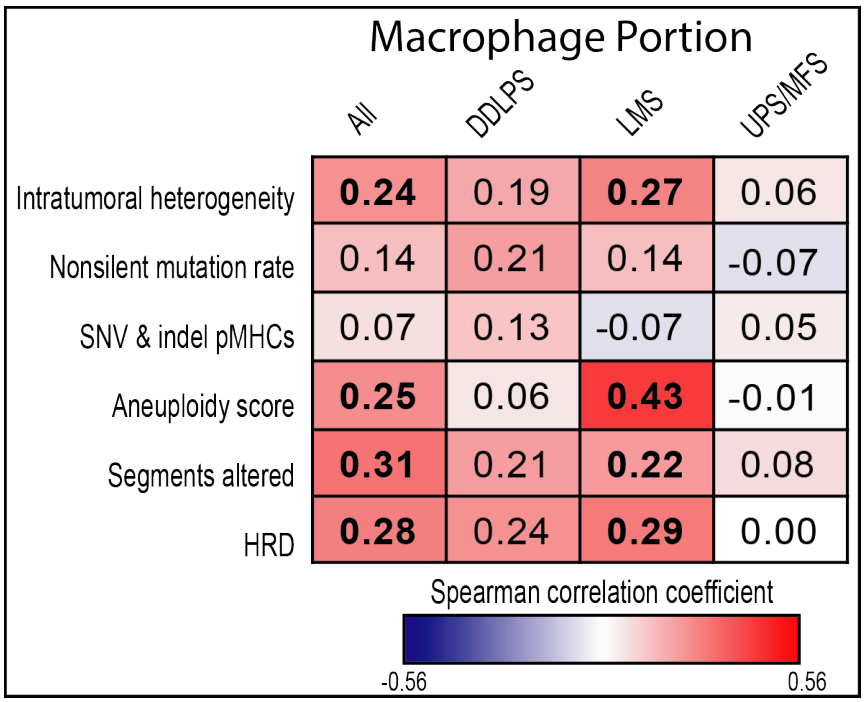


Figure 3.10 Heatmap displaying degree of association between DNA damage measures and the relative proportion of tumor immune infiltrate represented by macrophages.
Macrophage signatures and measures of DNA damage defined and calculated by Thorsson *et al* (2018). Degree of association measured by Spearman rank correlation coefficient. Values in bold indicate statistically significant correlations ($p \leq 0.05$).

When the DNA damage scores were correlated with individual subsets of M0-, M1-, and M2- signature macrophages (Fig. 3.11A-C), it was found that the higher proportion of macrophages in cases with higher intratumoral heterogeneity, increased copy number alterations, and more homologous recombination defects likely consists mainly of M2-signature macrophages (Fig. 3.11C), although leiomyosarcomas also showed increased proportions of M0-signature macrophages in cases with more frequent copy number alterations and homologous recombination defects (Fig 3.11A). While the M1:M2 ratio weakly correlated with measures of DNA damage, these findings did not reach significance in most cases (Fig. 3.11D).

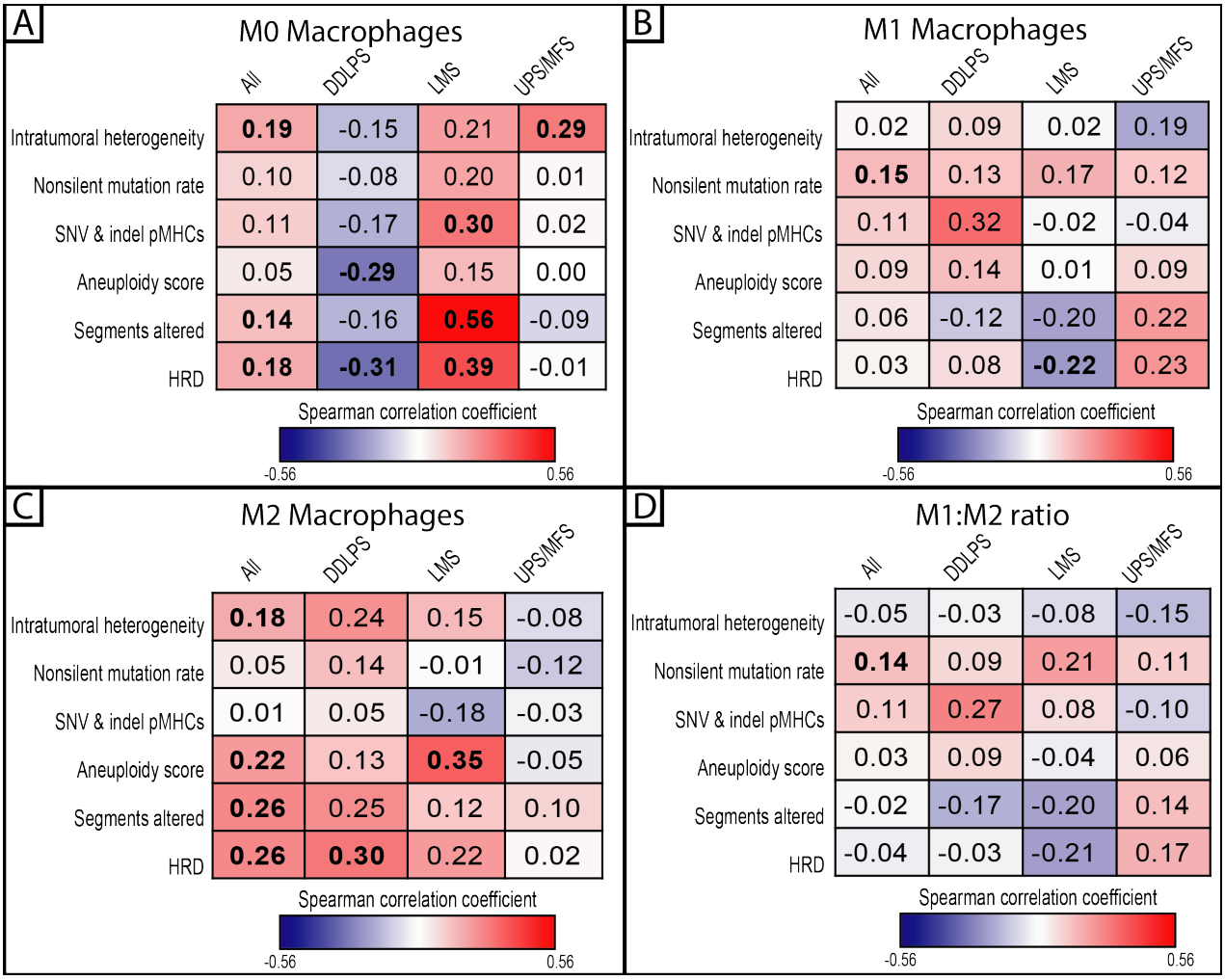


Figure 3.11 Heatmaps displaying degree of association between DNA damage measures and the relative proportion of immune infiltrate represented by macrophage subsets.
M0 macrophages, (B) M1 macrophages, (C) M2 macrophages, (D) M1:M2 ratio. Macrophage signatures and measures of DNA damage defined and calculated by Thorsson *et al* (2018). Degree of association measured by Spearman rank correlation coefficient. Values in bold indicate statistically significant correlations ($p \leq 0.05$).

3.4.4 Macrophage-Related Immune Checkpoint: CD47/SIRPα

We next surveyed our sarcoma tissues for tumor-cell-expressed immune checkpoint CD47 and corresponding macrophage-expressed receptor SIRPα. The distribution of CD47 scores was distinctly bimodal across the full sample set, with most tissue microarray cores staining at either 0% or 100% positive tumor cells (42.1% and 23.2% of total cases, respectively; Fig. 3.12A). Over half (51%) of our dataset expressed at least focal CD47, and subtype-specific

expression varied widely. The "bimodal" pattern of expression persisted in some subtypes (osteosarcoma, chondrosarcoma, gastrointestinal stromal tumors), while in other subtypes, samples skewed in one direction. Sarcoma types with consistently low CD47 expression included undifferentiated pleomorphic sarcoma (0% expression in 88% of cases), Ewing sarcoma and dermatofibrosarcoma protuberans (0% expression in 70% of cases), malignant peripheral nerve sheath tumor and leiomyosarcoma (0% expression in 69% of cases), and myxofibrosarcoma (0% expression in 65% of cases)(Fig.3.12A). Notable high expressors of CD47 included chordoma and angiosarcoma, which demonstrated CD47 staining in all cases, with 100% of cells expressing CD47 in 82% of chordomas and 75% of angiosarcomas, and all remaining cases still showing some cells expressing this antigen (Figure 3.12A). Dedifferentiated liposarcoma and epithelioid sarcoma also exhibited high proportions of CD47 staining, with all tumor cells expressing CD47 in 48% and 63% of samples, respectively (Fig. 3.12A).

Tumor-associated macrophage expression of receptor SIRPα corresponded well with CD47 expression in some sarcoma types, but not in others (overall Spearman's rho = 0.252, $p<0.001$). Overall, 31% of cases had at least one SIRPα+ macrophage in any tissue microarray core. The sarcoma types most commonly infiltrated by SIRPα+ macrophages were angiosarcoma (75% of cases), dedifferentiated liposarcoma (72%), chordoma (71%), well-differentiated liposarcoma (63%), and myxoid liposarcoma (53%) (Fig. 3.12B). SIRPα infiltration was least common in low-grade fibromyxoid sarcoma (0% of cases), chondrosarcoma (3%), epithelioid sarcoma (13%), and MPNST and synovial sarcoma (both 14%) (Fig. 3.12B).

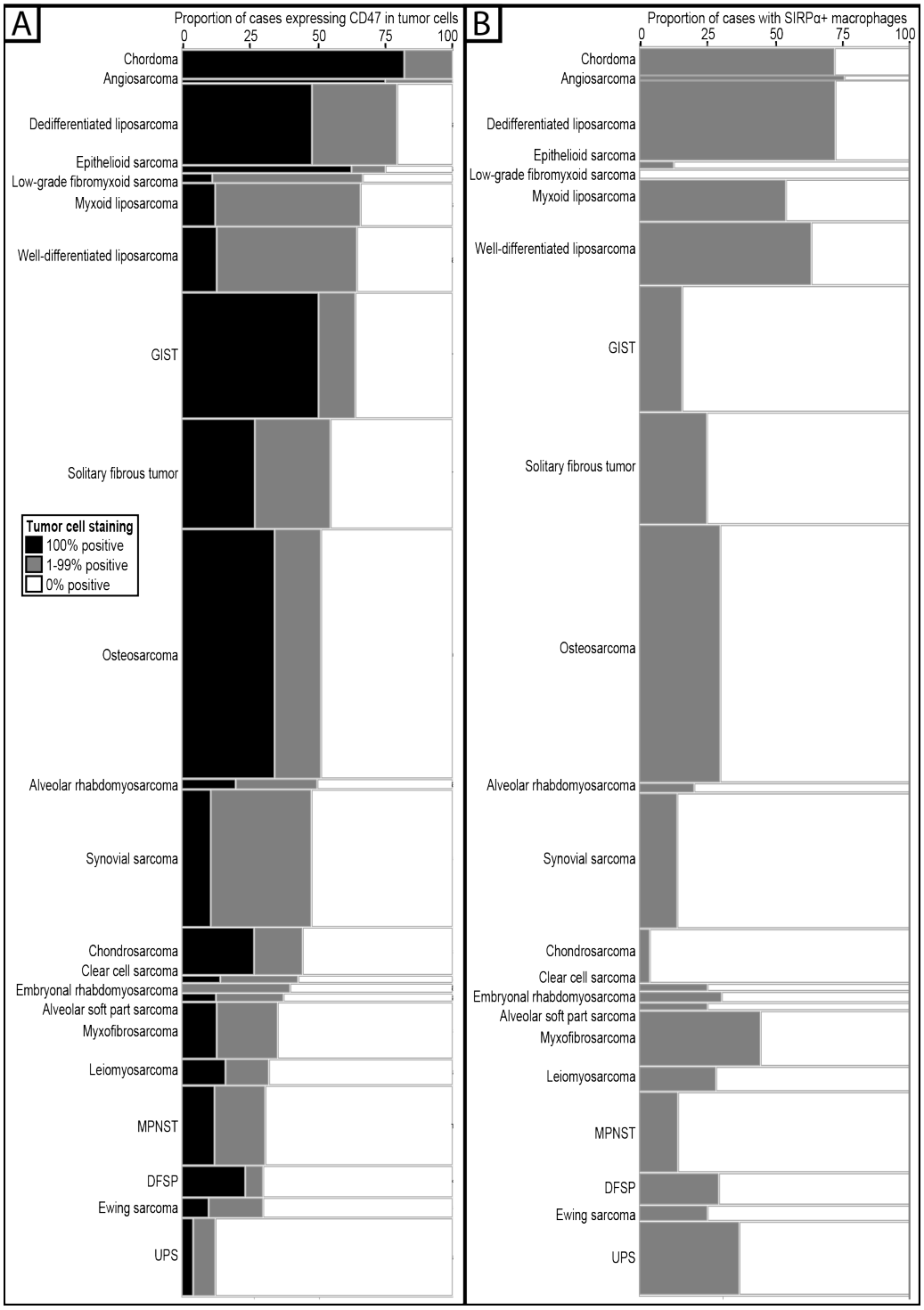


Figure 3.12 Mosaic plot depicting the proportion of sarcoma cases expressing CD47 and SIRPα.
(A) CD47 scored by % of tumor cells positive by immunohistochemistry. Cases categorized by: 0% (white), 1-99% (grey), or 100% (black) of tumor cells expressing CD47. (C) SIRPα scored by number of positive-staining macrophages by immunohistochemistry. Cases with ≥1 SIRPα+ macrophage in any tissue microarray core are marked as positive (grey). Row height proportional to the # of cases assessed.

Mutually high scores for both CD47 and SIRPα expression were observed in dedifferentiated liposarcoma (Spearman's rho = 0.569, p<0.001), well-differentiated liposarcoma (Spearman's rho = 0.610, p<0.001), myxoid liposarcoma (Spearman's rho = 0.351, p=0.03), as well as chordoma and angiosarcoma (*n* too small; non-significant)(Fig. 3.12; Fig. 3.13). Concurrently low scores for both CD47 and SIRPα expression were observed in MPNST (Spearman's rho = 0.453, p<0.001)(Fig. 3.12; Fig. 3.13). While epithelioid sarcoma and low-grade fibromyxoid sarcoma had comparatively frequent expression of CD47 on tumor cells, SIRPα+ macrophage infiltration was low or absent (Figure 4 B-C; Figure S2). Contrastingly, while undifferentiated pleomorphic sarcoma had the lowest proportion of cases positive for CD47 (12%), SIRPα expression was seen at a higher than average frequency (37% of cases)(Fig. 3.12; Fig. 3.13).

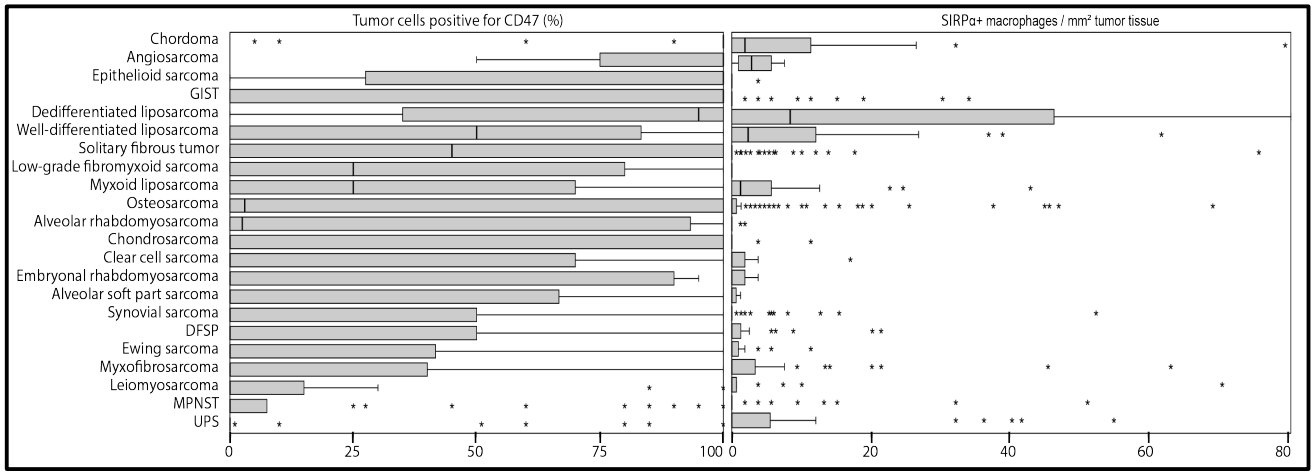


Figure 3.13 Scores for macrophage-associated immune checkpoint CD47/SIRPα across sarcoma subtypes. Boxplots depict the comparative range of scores by sarcoma type for CD47 (% of positive tumor cells) and SIRPα (positive macrophages per mm^2 tumor tissue). Boxes represent the first through third quartiles, vertical lines indicate median, and whiskers indicate range. Extreme outliers are indicated as dots.

3.4.5

3.4.6 Clinical Correlates

In multivariable linear regression analysis taking into account tumor grade and neoadjuvant treatment, those sarcoma types not associated with a pathognomonic translocation had significantly higher counts of both CD68+ macrophages ($p=0.006$) and CD163+ macrophages ($p=0.008$), as well as SIRPα+ macrophages ($p=0.003$)(Table 3.4). Neoadjuvant radiation therapy was significantly associated with higher counts of CD163+ macrophages ($p=0.037$) and SIRPα+ macrophages ($p<0.001$) and with higher tumor cell expression of CD47 ($p=0.005$)(Table 3.4). Neither tumor grade nor neoadjuvant chemotherapy showed significant correlation with any of the macrophage biomarkers investigated.

		Tumor grade			Prior chemotherapy		Prior radiotherapy		Molecular driver	
		1	2	3	+	-	+	-	Translocation -associated	Non-translocation
	n	71	213	223	159	419	54	524	337	558
CD68	median (Q1-Q3)	32 (2-142)	80 (15-283)	203 (35-516)	158 (29-293)	75 (11-396)	323 (77-598)	83 (13-321)	22 (5-48)	214 (55-516)
	cases + (% col)	53 (77%)	191 (90%)	206 (92%)	138 (90%)	356 (86%)	54 (100%)	440 (86%)	284 (92%)	422 (%)
	cases - (% col)	16 (23%)	22 (10%)	17 (8%)	15 (10%)	57 (14%)	0 (0%)	72 (14%)	24 (8%)	68 (%)
	p	0.442			0.279		0.114		**0.006**	
CD163	median (Q1-Q3)	85 (20-146)	114 (36-349)	256 (63-588)	213 (48-404)	123 (37-416)	492 (114-911)	120 (37-369)	57 (18-113)	259 (86-578)
	cases + (% col)	67 (99%)	198 (93%)	213 (98%)	138 (91%)	378 (92%)	52 (96%)	464 (91%)	298 (96%)	446 (%)
	cases - (% col)	1 (1%)	15(7%)	4 (2%)	13 (9%)	34 (8%)	2 (4%)	45 (9%)	13(4%)	42 (%)
	p	0.353			0.072		**0.037**		**0.008**	
CD47	median (Q1-Q3)	12 (0-88)	25 (0-79)	2 (0-88)	35 (0-100)	5 (0-75)	60 (25-92)	2 (0-80)	10 (0-85)	10 (0-85)
	cases + (% col)	35 (53%)	108 (51%)	107 (49%)	74 (48%)	214 (52%)	34 (63%)	254 (50%)	148 (49%)	247 (%)
	cases - (% col)	31 (47%)	103 (49%)	113 (51%)	79 (52%)	196 (48%)	20 (37%)	255 (50%)	152 (51%)	248 (%)
	p	0.452			0.521		**0.005**		0.808	
SIRPα	median (Q1-Q3)	0 (0-1)	0 (0-4)	0 (0-6)	0 (0-3)	0 (0-5)	7 (0-43)	0 (0-3)	0 (0-0)	0 (0-6)
	cases + (% col)	19 (27%)	69 (33%)	98 (44%)	56 (35%)	145 (35%)	28 (52%)	173 (33%)	72 (24%)	188 (%)
	cases - (% col)	52 (73%)	143 (67%)	123 (56%)	103 (65%)	274 (65%)	26 (48%)	351 (67%)	231 (76%)	325 (%)
	p	0.180			0.084		**0.000003**		**0.0003**	

Table 3.4 Multivariable linear regression between tumor-associated macrophage biomarker scores and tumor grade, neoadjuvant treatment, and tumor molecular classification.

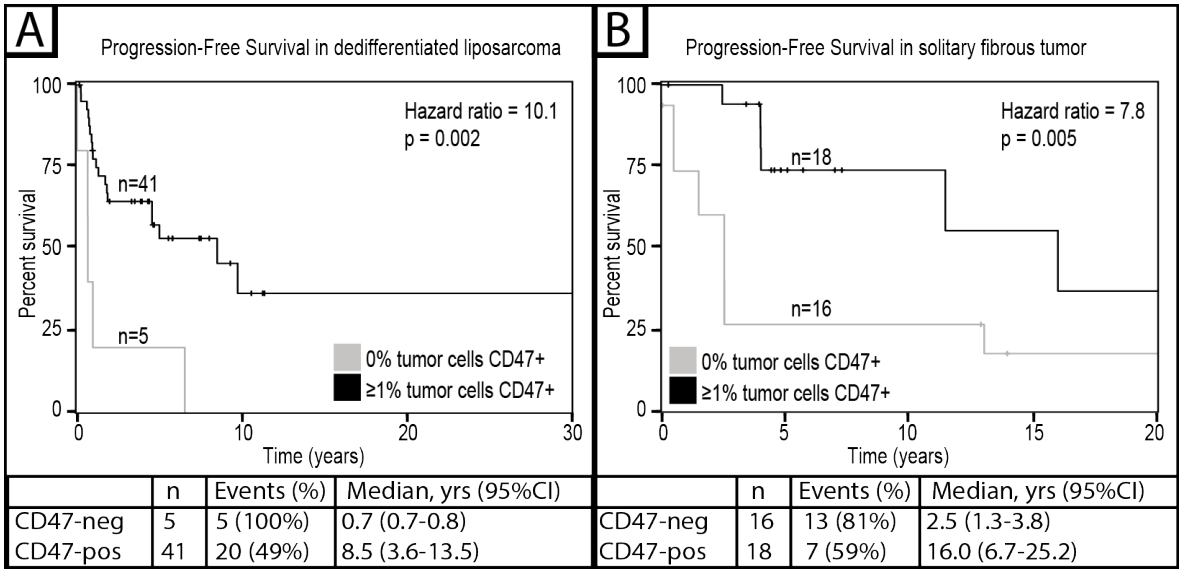
Median and quartile scores of positive macrophages/mm² (or % positive tumor cells for CD47) on tissue microarrays. P values were calculated by multivariate ANOVA regression analysis, with Bon Ferroni correction. Proportions of +/- cases shown for reference, but are not evaluated independently in the multivariable analysis.

Multivariate Cox regression analysis was conducted for the full sample set, taking into account patient age, tumor grade, histological diagnosis, and immunohistochemical scores for CD68, CD163, CD47, and SIRPα. For overall survival, only age ($p<0.001$) and grade ($p=0.03$) were significantly associated with outcomes among the non-translocation sarcomas (Table 3.5). Overall survival among translocation-associated sarcomas was only significantly associated with tumor grade ($p=0.05$; Table 3.5). For progression-free survival, grade ($p=0.05$) and higher CD163 ($p=0.05$) scores were associated with worse outcomes (Table 3.5) among non-translocation sarcomas. Progression-free survival among translocation-associated sarcomas was significantly associated with age ($p=0.007$) and grade ($p=0.003$; Table 3.5).

	Non-Translocation Sarcomas				**Translocation-Associated Sarcomas**			
	Hazard ratio	95%CI min	95%CI max	p	Hazard ratio	95%CI min	95%CI max	p
Overall Survival								
Age	**1.027**	**1.014**	**1.041**	**0.00006**	1.008	0.991	1.025	0.35
Grade	**0.203**	**0.047**	**0.87**	**0.03**	**0.271**	**0.074**	**0.992**	**0.05**
CD68 score	0.999	0.998	1.001	0.48	0.997	0.986	1.008	0.57
CD163 score	1.000	0.999	1.001	0.92	1.002	0.996	1.008	0.54
CD47 score	0.993	0.98	1.006	0.27	0.999	0.976	1.023	0.95
SIRPα score	1.002	0.995	1.008	0.62	1.005	0.995	1.014	0.36
Progression-Free Survival								
Age	1.009	0.999	1.019	0.06	**1.031**	**1.008**	**1.054**	**0.007**
Grade	**0.392**	**0.154**	**1.002**	**0.05**	**0.07**	**0.012**	**0.397**	**0.003**
CD68 score	1.001	1.000	1.001	0.22	1.003	0.992	1.015	0.61
CD163 score	**0.999**	**0.999**	**1.000**	**0.05**	0.998	0.991	1.004	0.47
CD47 score	0.994	0.983	1.004	0.22	1.008	0.987	1.029	0.46
SIRPα score	0.996	0.991	1.000	0.07	1.002	0.991	1.013	0.73

Table 3.5 Cox proportional-hazards multiple regression analysis of tumor-associated macrophage biomarkers for overall and progression-free survival in sarcomas.
Immunohistochemical scores are based on the mean scores of duplicate tissue microarray cores. CD47 was scored by percentage of positive tumor cell staining; CD68, CD163, and SIRPα were scored by the count of positive macrophages per mm^2 tumor tissue. Hazard ratios and p-values were calculated by cox proportional-hazards multiple regression, with Bon Ferroni correction.

Using a Kaplan-Meier log rank test to assess the sarcoma types with an adequate number of events for analysis (Table 3.6), we found that sarcoma type-specific overall and progression-free survival had no significant associations with CD68+ or CD163+ macrophage infiltration. CD47 positivity (≥1% tumor cell staining) was associated with better progression-free survival in dedifferentiated liposarcoma (Fig. 3.14A; HR=10.1, p=0.002) and solitary fibrous tumor (Fig. 3.14B; HR=7.8, p=0.005), with CD47 expression associated with an increase in median PFS from 0.7 years to 8.5 years and from 2.5 years to 16.0 years, respectively. The same trend was observed in undifferentiated pleomorphic sarcoma (HR=4.4, p=0.035) and myxofibrosarcoma (HR=4.0, p=0.045).



	n	Events (%)	Median, yrs (95%CI)
CD47-neg	5	5 (100%)	0.7 (0.7-0.8)
CD47-pos	41	20 (49%)	8.5 (3.6-13.5)

	n	Events (%)	Median, yrs (95%CI)
CD47-neg	16	13 (81%)	2.5 (1.3-3.8)
CD47-pos	18	7 (59%)	16.0 (6.7-25.2)

Figure 3.14 Kaplan-Meyer curves for progression-free survival based on CD47 positivity.
(A) Dedifferentiated liposarcoma, (B) Solitary fibrous tumor. CD47 positivity defined as ≥1% tumor cell staining. Scored from immunohistochemistry on tissue microarray. Hazard ratios and p-values were calculated by log-rank test.

3.5 Discussion

With largely negative results emerging from lymphocyte-related immune checkpoint blockade trials for sarcomas,[581-583] studies are turning to combinatorial regimens with novel immune checkpoint inhibitors, including some that are macrophage-related. Selecting good candidates for these trials is challenging without a detailed understanding of the immune microenvironments that may differ across the diverse types of sarcomas. This study provides a systematic characterization of tumor-associated macrophages and macrophage-related immune checkpoint biomarker expression. To our knowledge, this is the largest study to date to characterize tumor-associated macrophages in sarcomas, and the first description of CD47 and SIRPα expression across sarcoma types.

Our study depicts a widely variable, but highly sarcoma-type-specific expression of both CD68+ and CD163+ tumor-associated macrophages. TAMs were most frequently observed in angiosarcoma, UPS, dedifferentiated liposarcoma, and myxofibrosarcoma, whereas they are most sparse in synovial sarcoma, myxoid liposarcoma, clear cell sarcoma, and low-grade fibromyxoid sarcoma. This suggests that pleomorphic sarcoma types are inducing higher levels of phagocytic inflammation than translocation-associated sarcomas, perhaps due to greater mutational/neoantigen burdens, the specific cytokine/chemokine profile of the tumor cells, or higher levels of necrosis. [653-657] These levels of TAMs far exceeded the levels of TILs we previously published from the same tissue set. While comparing different cell types, stained at different times, with antibodies of different sensitivities may not be the most precise way possible to assess relative infiltration, results do suggest that macrophages dominate the immune microenvironment of sarcomas more than do lymphocytes. No studies in sarcomas have directly compared TIL and TAM infiltrates, but even going back as far as a seminal 1986 *New England*

Journal of Medicine review, malignant tumors were described a "wounds that do not heal," with a common feature of macrophage predominance.[658] In a spontaneous breast cancer mouse model, it has been found that TAMs outnumber CD103+ dendritic cells, considered to be the main APC population presenting antigens to T cells in tumors.[659,660] By engaging non-effective APCs, T cells might be prevented from CD103+ dendritic-cell-mediated activation. A recent study in a mouse mammary carcinoma (transplanted and cell-line) xenograft found that interaction with macrophages impedes CD8 T cell migration, preventing them from reaching tumor cells and limiting the efficacy of PD-1 inhibitors.[661]

Among macrophages, we found that CD163+ macrophages were the predominant phenotype, which agrees with findings in melanoma,[662] non-small cell lung cancer,[663] and colorectal carcinoma.[664,665] The presence of large numbers of M2 TAMs suggests a highly immunosuppressive tumor microenvironment, but due to the plasticity of TAM polarization, it may represent anti-tumor immunity that is suppressed but poised for reactivation. [666] In our study, higher expression of CD163 was associated with worse progression-free survival. RNA expression data from TCGA strongly supported our immunohistochemistry-based conclusions; from that analysis, we found that intratumoral heterogeneity, aneuploidy score, focal chromosomal copy number alterations, and homologous recombination defects all correlated with having a higher proportion of the immune infiltrate be macrophages. A possible explanation for this association could be that DNA damage can upregulate the expression of damage-associated molecular patterns (DAMPs), which promote inflammation, particularly from macrophages.[667] Indeed, DAMPs have been suggested to contribute to the recruitment of M2-polarized macrophages.[668,669] Another possible explanation may be that rapidly-growing tumors are more likely to accumulate DNA damage, but also more likely to outgrow their blood supply,

leaving behind necrotic tissue, which also recruits macrophages. Similarly, in our cohort, we observed an increased macrophage presence in following radiotherapy, which may be due to tumor cell radiation-induced damage, leading to recruitment of "healing" M2 macrophages. CD163 predominance might also be explained by high expression of the CD47-SIRPα "don't eat me" signal, which was frequently expressed in our sample set. SIRPα signaling has been shown to modulate macrophage polarization in mice, suggesting that activation of SIRPα receptor shifts macrophage polarization toward M2. Both CD47 and SIRPα were observed frequently in our sample set (with a positive correlation), though we saw differential expression depending on sarcoma type.[670] All of our cases of chordoma (n=28) and angiosarcoma (n=4) expressed CD47, with most showing 100% tumor cell expression. Undifferentiated pleomorphic sarcomas expressed CD47 the least frequently, but SIRPα expression was mid-range. In general, our CD47 staining was bimodal, indicating all-or-nothing signaling that might prove useful as a predictive marker of response to CD47 therapies.

Our findings are limited by small numbers of cases for some subtypes and by our use of tissue microarray specimens, which do not take into account intratumoral heterogeneity or immune infiltrates near the tumor margins; however, tissue microarrays may represent diagnostic core needle biopsy specimens reasonably well,[671] and we have measured infiltrates by cells/mm^2 to reflect this. Furthermore, there is some disagreement regarding the immunohistochemical identification of M1 versus M2 macrophages. Some studies choose to define CD68 as a general macrophage marker, back-calculating M1 macrophages as M1 = CD68 - CD163. However, other studies have shown that CD68 tends to underestimate the total macrophage count.[672] The evaluation of M1 or M2 macrophages is further complicated by the nature of macrophage polarization, given that these phenotypes exist as a spectrum rather than a dichotomy. In our

sample set, CD68 scores were lower than CD163 scores, suggesting that CD68 was not staining all macrophages. We therefore defined CD68 as generally staining M1 macrophages, while CD163 represented M2 macrophages. The strong correlation between our immunohistochemical studies and our more robust mRNA signature studies enhances our confidence that this method of M1 vs. M2 distinction is satisfactory for the conclusions drawn herein. To clarify this distinction, follow-up studies could include multiplex staining, expression of iNOS and ARG-1 as respective M1 and M2 biomarkers, and/or flow cytometry studies.[673,674]

The observation that CD47+ cases (>1%) had better survival compared to cases negative for CD47 in dedifferentiated liposarcoma and solitary fibrous tumor suggests that CD47 positivity might offer some survival benefit for some sarcoma types, possibly as a reflection of overall increased immune activation. This trend differs from what has been observed in most other cancer types; [476,675-679] However, this association was in a relatively small number of cases, using a speculative cut-off for CD47 positivity, and as such, it requires validation in a larger cohort. Regardless of prognostic value, therapeutic agents targeting the CD47/SIRPα axis seem a worthwhile pursuit for several sarcoma subtypes. These agents could be tested in clinical trials alone or in combination with inhibitors of the macrophage-metabolic immune checkpoint IDO-1, given their possibly synergistic effects on macrophages. Drugs targeting IDO-1 – an intracellular enzyme of macrophages and dendritic cells that catalyzes the rate-limiting step of tryptophan metabolism to kynurenine – are currently under development for numerous cancer types.[557]

3.6 Conclusions

In conclusion, herein we present the largest study to date of tumor-associated macrophage biomarkers in sarcomas. We find that there is a very high density of tumor-associated macrophages relative to tumor-infiltrating lymphocytes, and that these are predominantly of M2 polarization. We also observe high levels of expression of the macrophage-related immune checkpoint CD47 – particularly among angiosarcoma, chordoma, dedifferentiated liposarcoma, and epithelioid sarcoma – which may indicate that these sarcoma subtypes could be the best candidates for emerging anti-CD47 therapies.

Chapter 4: T-cell Receptor Repertoire in Sarcomas

4.1 Abstract

Identifying the patients who will have a lasting response to new immune checkpoint inhibitors remains a major challenge for this emerging modality of therapy. This is especially applicable in the field of sarcomas, which has very limited pre-clinical or clinical information to help direct therapeutic decisions for immunotherapy. Numerous pathological and molecular tumor characteristics are being investigated worldwide in a search for a reliable and practical predictive biomarker for response to immune checkpoint blockade. One biomarker that has drawn particular interest of late – due in part to its direct relevance to anti-tumoral immunity – is the evaluation of the complete repertoire of rearranged T cell receptors (TCRs) in a given tumor or sample of the peripheral blood. A number of early studies have shown that a highly targeted (clonal) population of intratumoral T cells (or alternatively, a rich diversity of peripheral T cells) can predict response to immune checkpoint inhibition. In this study, we perform a correlative analysis of clinical trial specimens from 25 sarcoma patients before and after treatment with the combination immune checkpoint therapy of tremelimumab (anti-CTLA-4) and durvalumab (anti-PD-1). Using targeted sequencing of the V and J segments of the T cell receptor β chain, we demonstrate that T cells within genomically-complex sarcoma subtypes (characterized by copy-number alterations or complex mutational profiles) tend to have more diverse (less clonal) and richer (more unique TCR rearrangements) T cell receptor repertoires than the genomically-simple translocation-associated sarcomas. Furthermore, the TCR repertoire clonality in peripheral blood samples increases in most cases following initiation of therapy, suggesting clonal expansion of specific antitumoral T cell populations. This study awaits the completion of the trial endpoint to assess any relationship between TCR repertoire and response to therapy.

4.2 Introduction

The discovery of immune checkpoints and the subsequent clinical success of immune checkpoint inhibitors has generated much excitement around this rapidly expanding class of therapies. In sarcoma clinical trials, outcomes have been heterogeneous and subtype-dependent,[581-583] raising a critical need for predictive biomarkers. The ability to deliver immune checkpoint therapies to only the patients most likely to benefit will help improve the margin of response, minimize needless toxicities, and inform rational combination therapies from an ever-growing list of checkpoint inhibitors.[492] Immune checkpoint inhibitor therapy not only carries toxicities, but also represents an expensive treatment regimen, so within a public healthcare system like we have in Canada, biomarkers to differentiate responders from non-responders are of special relevance.

Recent trials of immune checkpoint inhibitors have made efforts to identify predictive biomarkers. However, conventional immunohistochemical biomarkers have proven challenging, as studies fail to agree about expression cut-points[493] and the most obvious biomarkers do not reliably predict response (*e.g.*, many responses have been documented to PD-1 inhibitors by tumors negative for PD-L1).[493] An emerging biomarker that is very different from immunohistochemical markers is the diversity of the T-cell receptors (TCR) among tumor-infiltrating lymphocytes (TILs). This is easily assayed in extracts prepared from surgically-resected tumors or peripheral blood lymphocytes obtained by a simple blood sample. Evaluation of TCR repertoire involves quantitative sequencing of the variable (antigen recognition) regions of the T cell receptors on the T lymphocytes (usually the conventional α/β T cells) in a given sample. T cell repertoire diversity is commonly quantified using the Shannon entropy metric, which has 2 complementary components: 1) “richness,” the number of unique

sequences, and 2) “clonality” or “evenness,” the distribution of unique sequences, wherein low clonality/high evenness indicates equal numbers of all clones, whereas high clonality/low evenness indicates a skewed population, wherein a few clones predominate. [511,512] Two studies in melanomas treated with PD-1 inhibitors found that the pre-treatment TCR repertoire was more clonal (less diverse) among those who responded to therapy than in those who had progressive disease. [499,513] A similar study in non-small cell lung cancer treated with nivolumab also identified higher TCR clonality among responders, but in this study, TCR repertoire was assessed after initiating therapy.[514] In two studies of melanoma, comparison of the pre-treated TCR repertoire with that obtained after PD-1-inhibitor therapy showed a 10-fold increase in clonal TCR sequences (reflecting the clonal expansion of those T cells) in the responding group only, suggesting a tumor-specific response to therapy. [499,515] However, other studies in melanoma have failed to identify any correlation between TCR repertoire and response to immune checkpoint blockade. [510,516]

Assessment of peripheral T-cell populations is appealing, due to the ease of specimen collection. In a pilot study in metastatic melanoma, peripheral blood TCR repertoire was assessed prior to ipilimumab treatment, and both richness and evenness (*i.e.,* low clonality) were significantly associated with objective response (but not overall survival).[517] In urothelial cancer, baseline peripheral TCR evenness was associated with improved overall and progression-free survival on atezolizumab, though intratumoral TCR clonality did not show survival associations.[518] Similarly, a study in metastatic pancreatic cancer found that peripheral TCR evenness was associated with survival in patients treated with a CTLA-4 inhibitor, but not in those treated with a PD-1 inhibitor.[519]

Studies characterizing the TCR repertoire in sarcomas have been few and limited to descriptive analyses within a subset of tumor types. Pollack *et al.*[553] conducted immunosequencing of the TCR-Vβ region of 65 sarcoma tumor specimens, including myxoid liposarcoma, well/de-differentiated liposarcoma, leiomyosarcoma, UPS, and synovial sarcoma. TCR clonality was higher (more oligoclonal) among leiomyosarcomas and UPS than among well/de-differentiated liposarcomas, myxoid liposarcomas, or synovial sarcomas.[553] Furthermore, higher TCR clonality correlated with higher expression of both PD-1 and PD-L1.[553]

In this study, we employ the CapTCR-Seq method[680] to characterize the TCR β-chain repertoire of clinical trial specimens for 25 sarcoma patients treated with CTLA-4 inhibitor tremilimumab plus PD-1 inhibitor durvalumab. We have available for assessment pre-treatment tumor tissue, pre-treatment peripheral blood, on-treatment (4 week) peripheral blood, and, for 4 patients, post-treatment tumor tissue. While the clinical trial is still underway and has not yet matured to assess its primary endpoint (overall response at 48 months), the present analysis comparatively characterizes baseline TCR richness and clonality across the tumor specimens on the trial, as well as the impact of immune checkpoint inhibitors on the TCR repertoire in the peripheral blood samples taken before and after initiation of therapy.

4.3 Materials and Methods

4.3.1 Clinical Trial Details

An immune checkpoint inhibitor protocol was developed for the Australian Cancer Molecular Screening and Therapeutics (MoST) Program, a framework for multiple parallel signal-seeking clinical studies of novel therapies for patients with high-mortality cancers and unmet clinical need (ACTRN12616000908437).[681] 64 patients with “rare and neglected cancers” were recruited to this signal-seeking, phase II clinical trial of combination immune checkpoint

blockade, including 25 with sarcomas (listed in Table 4.1). Patients were treated with 1500 mg IV durvalumab (PD-1 inhibitor) + 75mg IV tremelimumab (CTLA-4 inhibitor) every 4 weeks, up to 4 cycles (16 weeks), followed by 1500 mg IV durvalumab every 4 weeks (starting on week 16) for up to 9 cycles. The primary endpoint for this trial is objective response rate (by RECIST 1.1) at 48 months, with secondary endpoints of overall and progression-free survival at 48 months.

Category	Sarcoma type	Subtype
Non-Translocation Sarcomas	Chondrosarcoma	Unspecified
	Chordoma	n/a
	Epithelioid hemangioendothelioma	n/a
	Epithelioid sarcoma	n/a
	Leiomyosarcoma	Soft-tissue
	Leiomyosarcoma	Soft-tissue
	Leiomyosarcoma	Soft-tissue
	Leiomyosarcoma	Soft-tissue
	Leiomyosarcoma	Uterine
	Leiomyosarcoma	Uterine
	Leiomyosarcoma	Uterine
	Leiomyosarcoma	Uterine
	Liposarcoma	Dedifferentiated
	Liposarcoma	Dedifferentiated
	Pleomorphic sarcoma	n/a
	Pleomorphic sarcoma	n/a
Translocation-Associated Sarcomas	Clear cell sarcoma	n/a
	Ewing sarcoma	n/a
	Ewing sarcoma	n/a
	Rhabdomyosarcoma	Alveolar
	Solitary fibrous tumor	n/a
	Synovial sarcoma	Unspecified
Sarcomas of Intermediate Malignant Potential	Angiomatoid fibrous histiocytoma	n/a
	Myoepithelioma	n/a
	Liposarcoma	Well-differentiated

Table 4.1 Sarcoma subtypes of patients on the Australian clinical trial of durvalumab ± tremelimumab.

Tumors were surgically resected prior to initiating checkpoint inhibitor therapy. Surgical resection specimens were fixed in formalin and embedded in paraffin. Two peripheral blood samples were collected: 1) prior to initiating checkpoint inhibitor therapy, and 2) after the first 4 weeks on treatment. For four patients, post-treatment tumor tissue was also available for analysis

(1 soft-tissue leiomyosarcoma, 1 dedifferentiated liposarcoma, 1 alveolar rhabdomyosarcoma, and 1 angiomatoid fibrous histiocytoma).

4.3.2 DNA Isolation

DNA was isolated as described by Mulder *et al.*[680] Briefly, DNA from blood samples was isolated using a Gentra Puregene kit (Qiagen, Hilden, Germany), and DNA from tissue samples was isolated using a Qiagen Allprep (Qiagen) kit, both according to the manufacturer's instructions. For tumor tissue samples, FFPE (formalin-fixed, paraffin-embedded) tissue was de-paraffinized by xylene vortexing and centrifugation steps. Peripheral blood samples were pelleted by centrifugation. De-paraffinized tissue pellets were dried using a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in QIAamp buffer ATL (Qiagen). Cell lysis was performed by 48-hour incubation at 65°C with proteinase K. DNA was purified by ethanol and resuspended in Tris and EDTA buffer (Qiagen).

4.3.3 Hybrid Capture and Library Construction

DNA hybridization with TCR β-chain V and J locus probes was performed as described by Mulder *et al.*[680] Briefly, DNA was sheared using a focused-ultrasonicator (Covaris, Inc., Woburn, MA, USA) and ligated with BIOO Scientific NEXTFlex sequencing library adapters, then amplified by PCR. Illumina DNA libraries were generated from fragmented DNA using the KAPA HyperPrep Kit (Sigma-Aldrich, St. Louis, MO, USA). Library fragment size distribution was determined using the Agilent TapeStation D1000 kit and quantified by fluorometry using the Invitrogen Qubit. Probe hybridization was performed overnight following the Roche SeqCap (Roche Life Science, Penzberg, Germany) conditions with xGen-blocking oligos (IDT, Coralville, IA, USA) and human Cot-1 blocking DNA (Invitrogen, Carlsbad, CA, USA). After

hybridization, libraries were amplified by PCR, purified, and sequenced using Illumina NextSeq 500 (Illumina, San Diego, CA, USA).

4.3.4 Capture Analysis and Statistics

The MiXCR software package (version 2.1.1)[682] was run on the libraries using the default settings for RepSeq-enriched libraries, in combination with the R package tcR (R statistical environment version 3.3.1).[683] *Richness* for each sample was calculated by the number of unique rearrangements in that sample. *Diversity* was calculated based on the Shannon–Wiener index (Shannon entropy), which is a function of both the relative richness (number of unique rearrangements present) and relative *clonality* (abundance of each rearrangement) of a given sample.[684] Shannon entropy was normalized for this dataset (to the range 0-1) by dividing Shannon entropy by the natural logarithm of the total number of unique rearrangements in the dataset [Normalized Shannon Entropy = Shannon Entropy/ln(42489)], as done by Tumeh *et al*.[499]The Kruskal-Wallis Independent Samples pairwise comparison test was used to assess differences in the distribution of richness and Shannon entropy between translocation/non-translocation/intermediate malignant potential sarcoma subtypes. The related-samples Wilcoxon signed rank test was used to compare TCR richness and Shannon entropy between pre-treatment and 4-weeks-post-treatment peripheral blood samples.

4.4 Results

4.4.1 Clinical Trial Outcomes

While the primary endpoint for this trial (48 months) has not yet been reached, preliminary outcomes at 6 months showed 45% progression-free survival (no local recurrence or metastasis) and 5 partial responses (3 liposarcoma and 2 leiomyosarcomas).[685] Median event-free

survival was 32 weeks for adult sarcoma patients versus 7 weeks for pediatric sarcoma patients (HR=0.172, p<0.0001).[685]

4.4.2 TCR Repertoire Richness

We determined the richness of the T cell receptor (β chain) repertoire in pre-treated tumor and peripheral blood, as well as blood 4 weeks after initiating checkpoint inhibitor treatment, by the number of unique TCR-β rearrangements present (Fig. 4.1). In total, we identified 42,489 unique TCR-β rearrangements across our dataset. In general, blood samples had higher TCR richness than tumor samples (Fig. 4.1). The highest baseline intratumoral TCR richness was seen in a patient with epithelioid sarcoma (1073 unique rearrangements), whereas the lowest was seen in a patient with Ewing sarcoma (0 TCR rearrangements found in the sampled tumor)(Fig. 4.1). In the baseline peripheral blood samples TCR repertoire richness was highest in chondrosarcoma (2156 unique sequences), dedifferentiated liposarcoma (1750 unique sequences), and two cases of uterine leiomyosarcoma (2464 and 1745 unique sequences)(Fig. 4.1). The patient with dedifferentiated liposarcoma maintained a comparatively rich peripheral TCR repertoire at 4 weeks on treatment (1477 unique sequences), whereas the other three patients showed a drop in richness, all to <400 unique TCR-β rearrangements (Fig. 4.1).

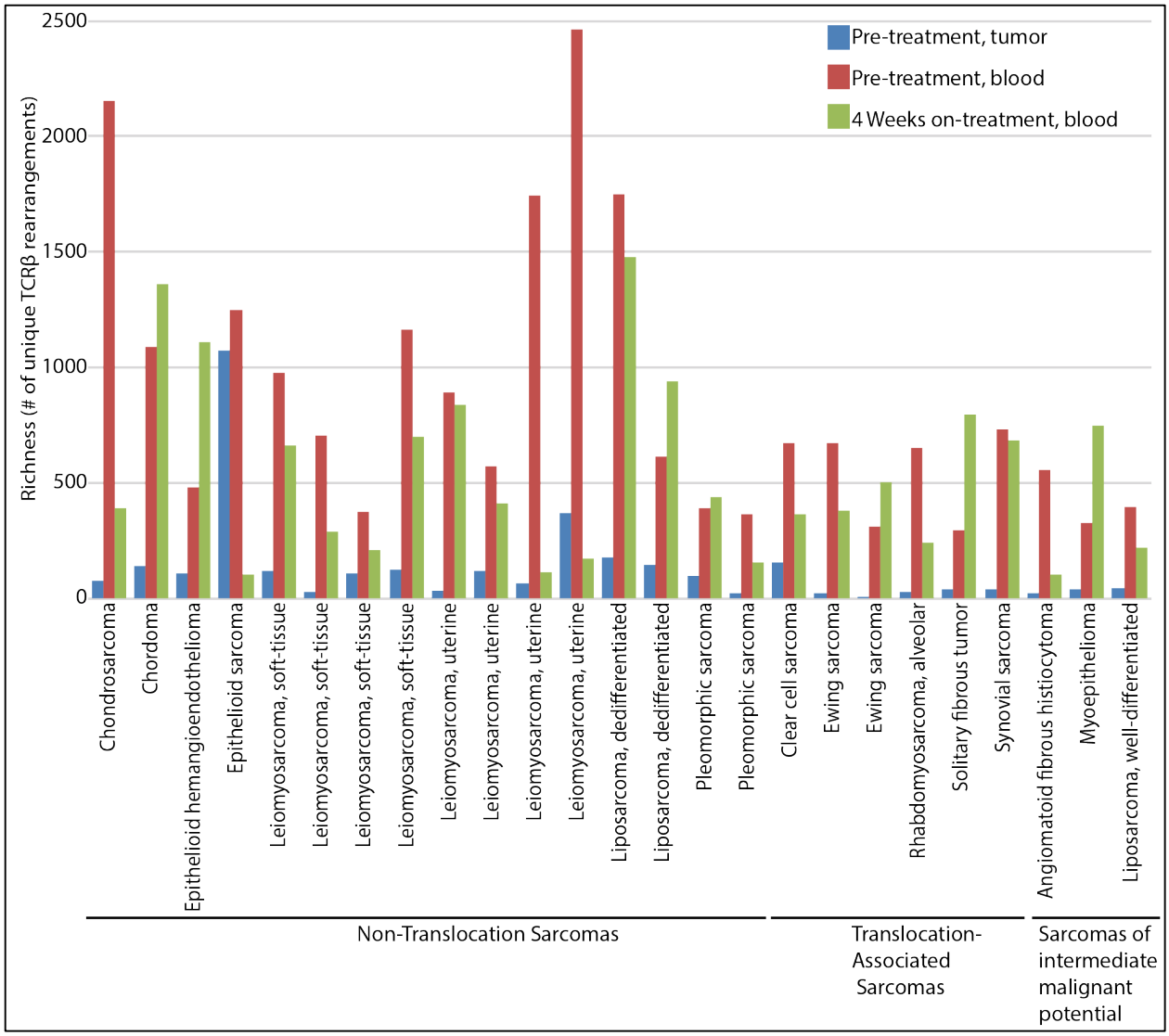


Figure 4.1 Richness of TCR-β rearrangements in sarcomas at baseline (tumor and blood) and after 4 weeks of checkpoint inhibitor therapy (blood).

While none of the sarcoma subtypes had sufficient numbers to draw any statistically significant conclusions about TCR clonality differences between individual diagnostic entities, grouping sarcoma types based on their underlying molecular alterations (as was done in Chapters 2 and 3) demonstrated that translocation-associated sarcomas had significantly lower baseline intratumoral TCR richness than non-translocation sarcomas (p=0.03)(Fig. 4.2).

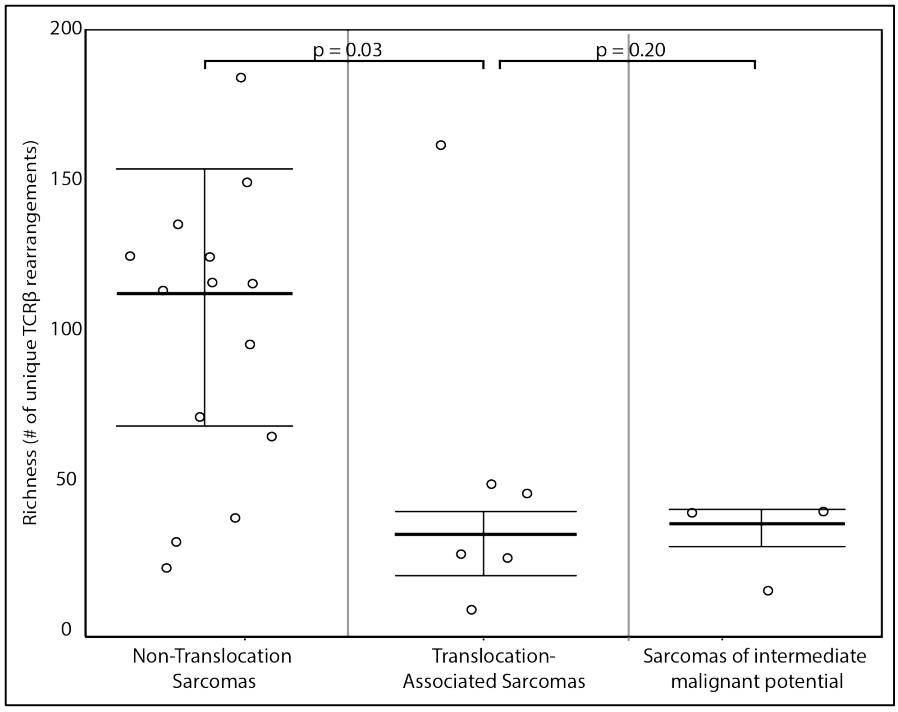


Figure 4.2 Comparison of baseline intratumoral richness of TCR-β rearrangements in sarcomas. Bars indicate median and quartiles. P values evaluated by the Mann-Whitney independent samples comparison test.

Comparison of baseline and on-treatment peripheral blood demonstrated an overall trend of reduced TCR repertoire richness (p=0.05; Fig. 4.3); however, this was not true for every case. Figure 4.4 demonstrates individual changes in TCR repertoire richness after 4 weeks on checkpoint inhibitor therapy. Six cases demonstrated an increase in peripheral TCR richness at 4 weeks: 1 chordoma, 1 epithelioid hemangioma, 1 dedifferentiated liposarcoma, 1 solitary fibrous tumor, 1 myoepithelioma, and 1 Ewing sarcoma (Fig. 4.4).

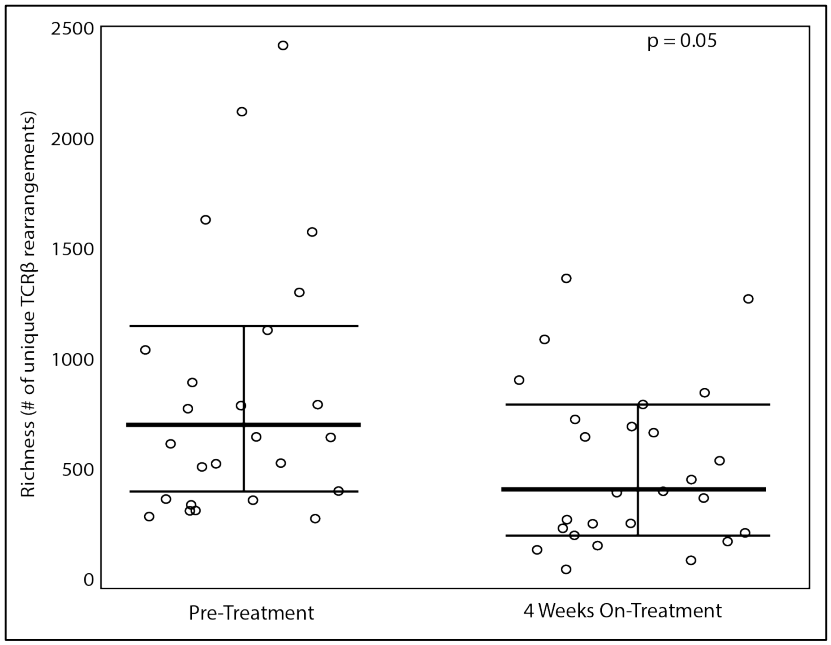


Figure 4.3 Overall comparison of baseline and on-treatment richness of TCR-β in peripheral blood.
Bars indicate median and quartiles. P values evaluated by the related-samples Wilcoxon signed rank test.

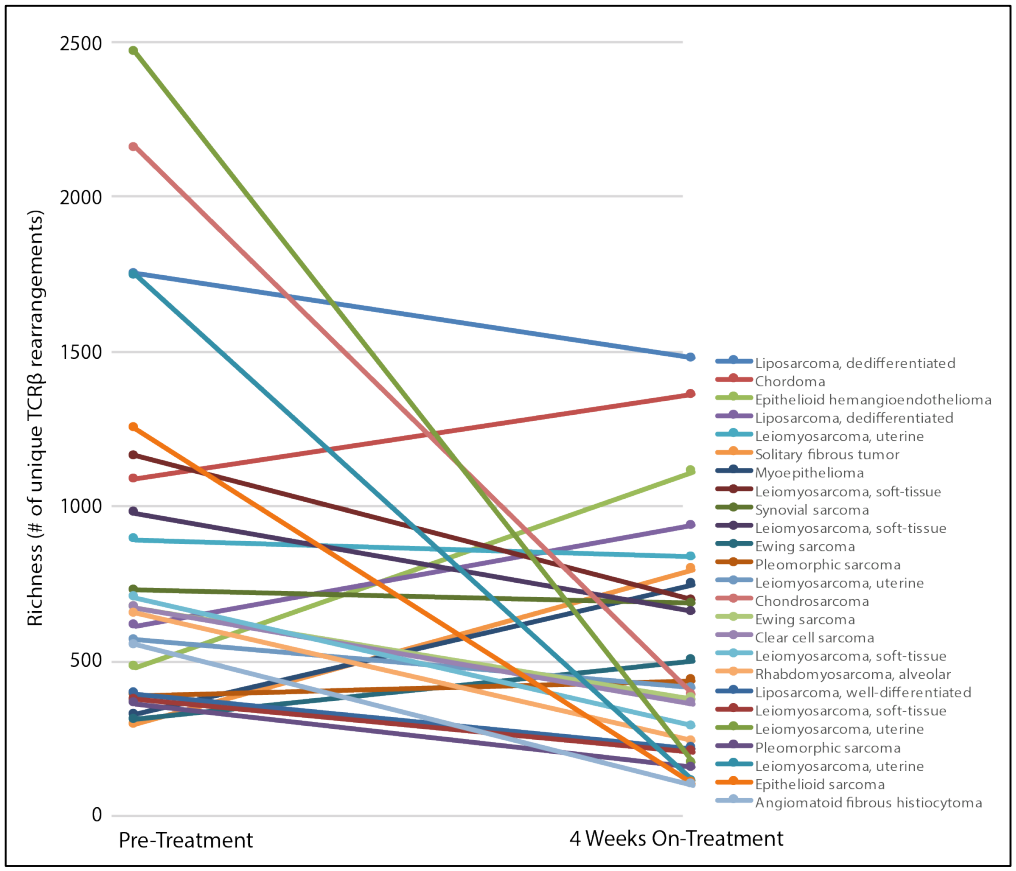


Figure 4.4 Individual comparison of baseline and on-treatment richness of TCR-β in peripheral blood.

For the four patients who had evaluable post-treatment tumor, intratumoral TCR-β richness remained stable for 2 patients (soft-tissue leiomyosarcoma and alveolar rhabdomyosarcoma) and increased for 2 patients (dedifferentiated liposarcoma and angiomatoid fibrous histiocytoma)(Fig. 4.5).

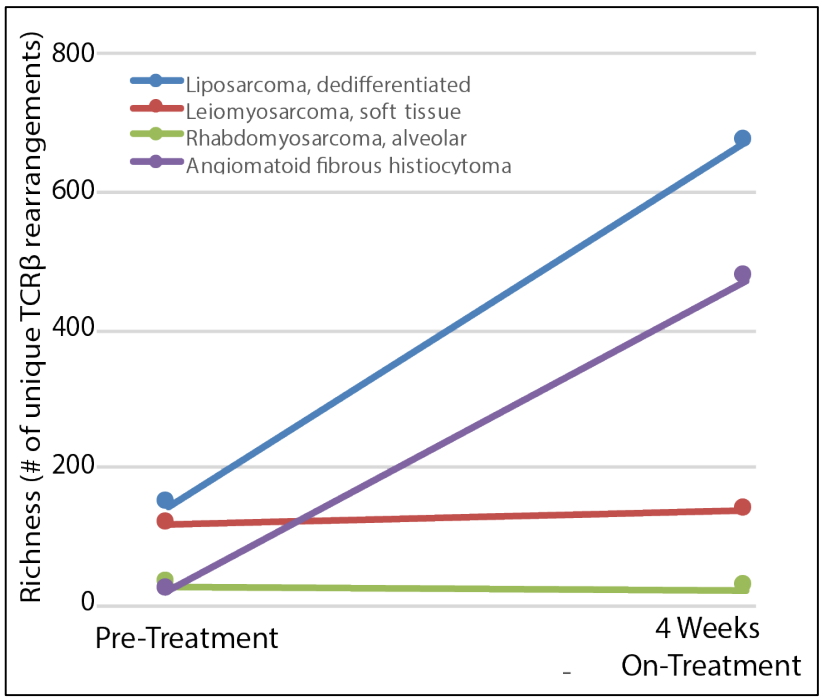


Figure 4.5 Individual comparison of baseline and on-treatment richness of TCR-β in tumor tissue.

4.4.3 TCR Repertoire Clonality

As might be expected, the baseline tumor specimens were much more clonal than peripheral blood samples, as is clearly depicted in Figure 4.6. Among tumors, monoclonal expansion of the most common clone exceeded 50% of reads in 3 leiomyosarcomas (2 uterine, 1 soft-tissue), 1 alveolar rhabdomyosarcoma, 1 Ewing sarcoma, 1 synovial sarcoma, 1 solitary fibrous tumor, and 1 angiomatoid fibrous histiocytoma (Fig. 4.6).

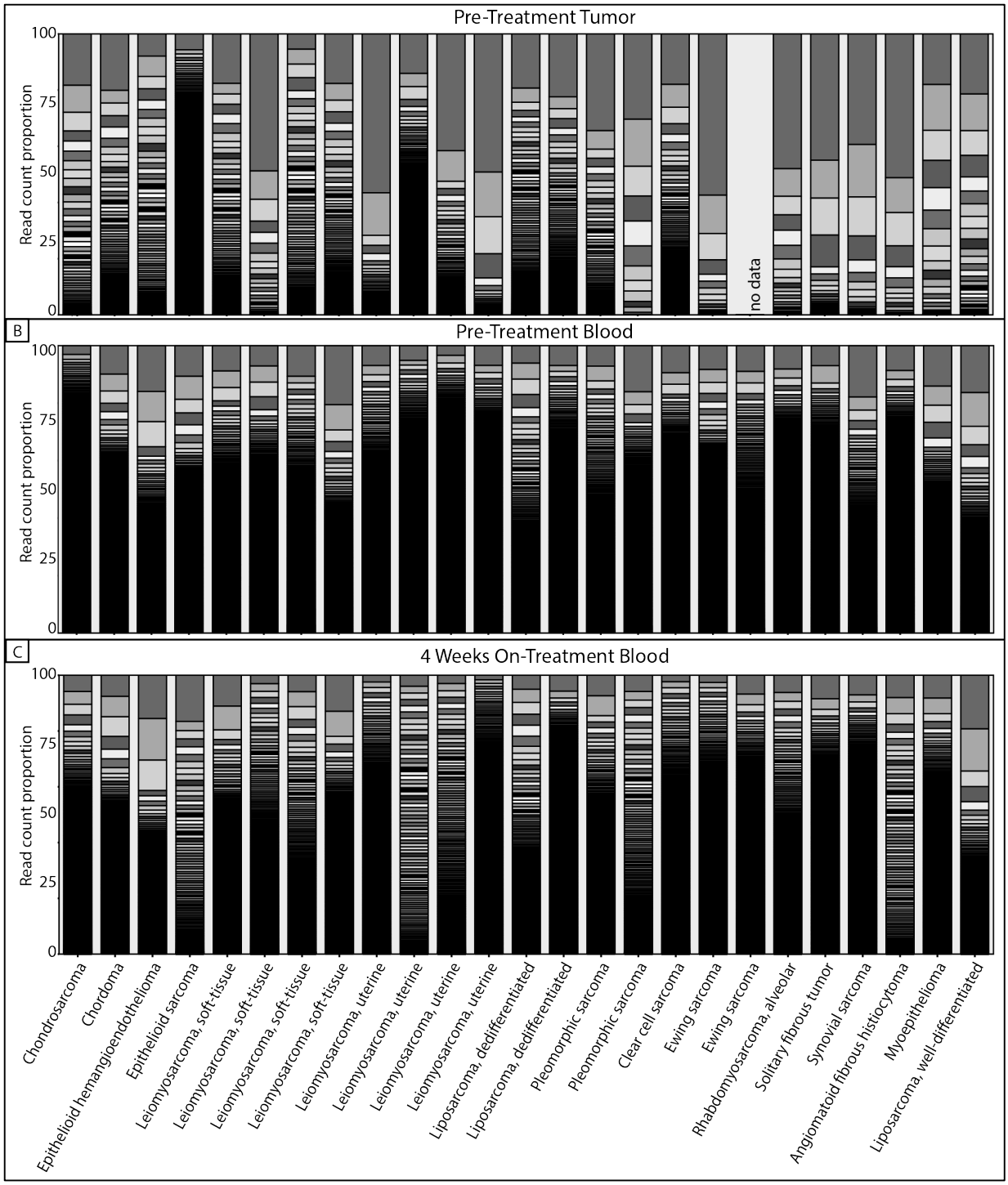


Figure 4.6 Clonality of TCR-β rearrangements in sarcomas at baseline (tumor and blood) and after 4 weeks of checkpoint inhibitor therapy (blood).

Each column represents an individual sample, and boxes within each column represent unique TCR-β rearrangements. The height of each box is proportional to the frequency of that clone within the sample. Greyscale colors are used to differentiate between adjacent clones but do not identify specific clones. The most common clone population is shown at the top (note that black at the bottoms of the columns does not represent a dominant clone, but rather the amalgamated black line delineating rare clones).

As a group, the translocation-associated sarcomas appear to be more clonal than the non-translocation sarcomas (Fig. 4.6). To quantify this difference, we used a normalized Shannon entropy calculation to estimate the diversity of the TCR-β repertoire in each sample.[499] TCR diversity was significantly higher among the non-translocation sarcomas than among the translocation-associated sarcomas (p=0.04; Fig. 4.7), which indicates a more homogeneous/less clonal population (higher entropy).

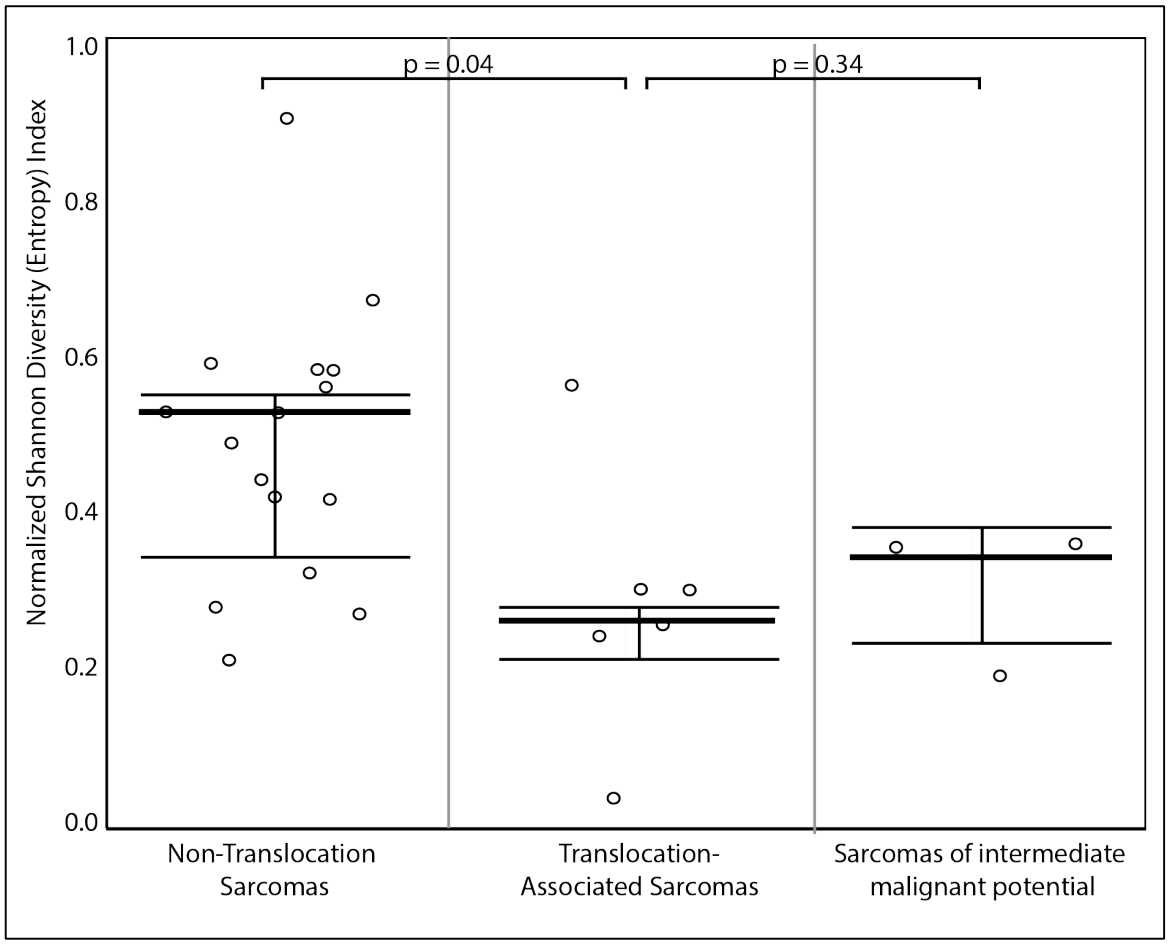


Figure 4.7 Comparison of baseline intratumoral diversity of TCR-β rearrangements in sarcomas. Bars indicate median and quartiles. P values evaluated by Mann-Whitney independent samples comparison test.

Using normalized Shannon entropy to compare peripheral blood TCR populations, we observed an overall trend towards a decrease in TCR diversity 4 weeks after initiating checkpoint inhibitor therapy (p=0.05; Fig. 4.8). However, on an individual basis, five patients had a clear increase in TCR diversity: 1 epithelioid hemangioendothelioma, 1 dedifferentiated liposarcoma, 1 Ewing sarcoma, 1 solitary fibrous tumor, and 1 myoepithelioma (Fig. 4.9). The patients with the most notable decrease in diversity in peripheral TCR repertoire included 1 chondrosarcoma, 1 epithelioid sarcoma, 2 uterine leiomyosarcomas, and 1 angiomatoid fibrous histiocytoma (Fig. 4.9).

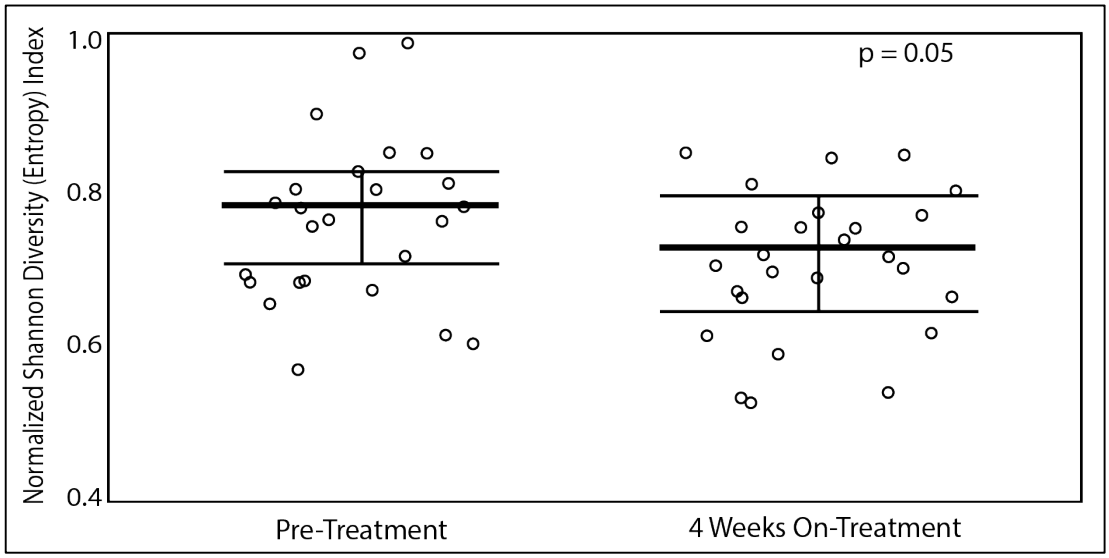


Figure 4.8 Overall comparison of baseline and on-treatment diversity of TCR-β in peripheral blood.
Bars indicate median and quartiles. P values evaluated by the related-samples Wilcoxon signed rank test.

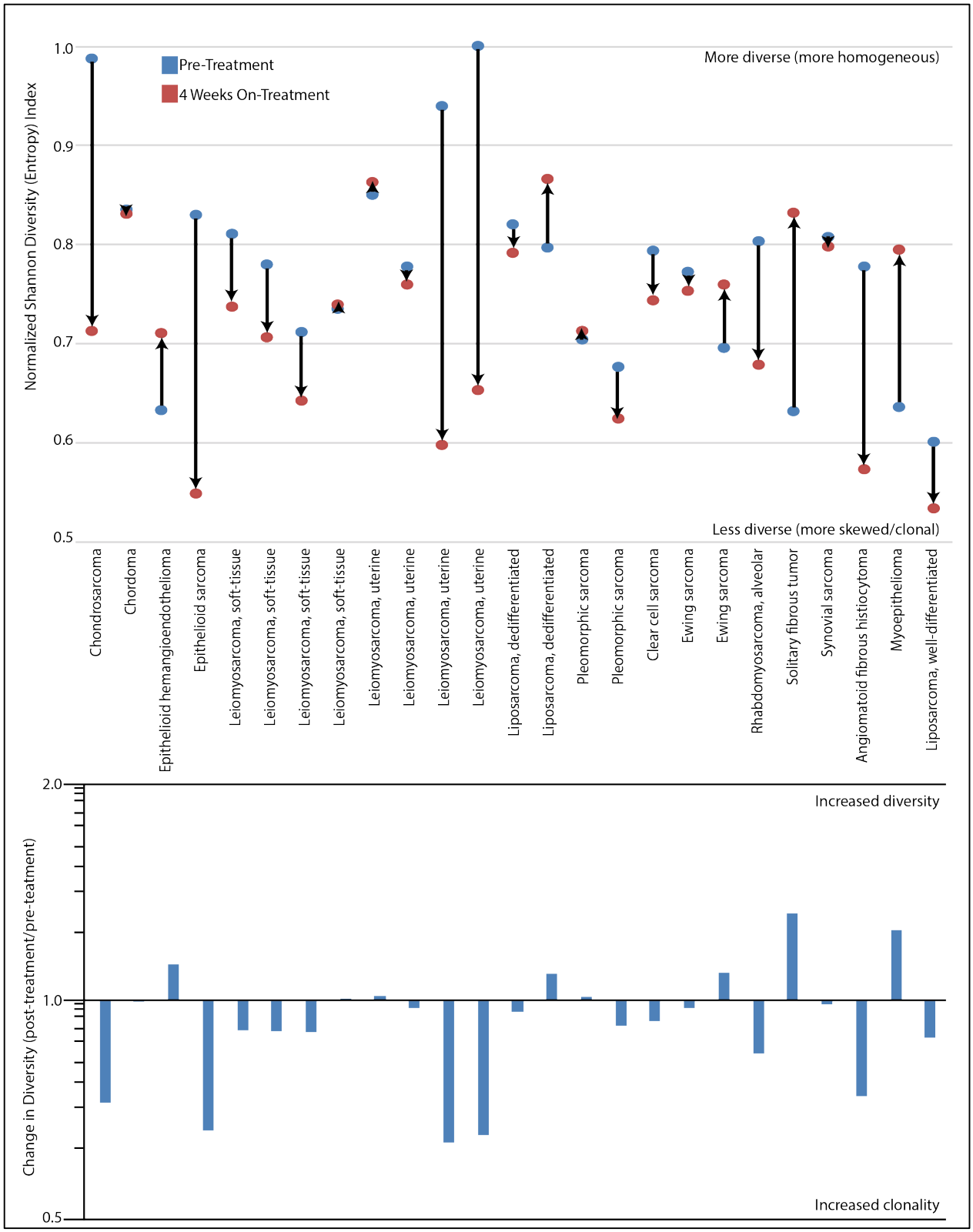


Figure 4.9 Individual changes in peripheral blood TCR-β repertoire diversity after 4 weeks of immune checkpoint inhibitor therapy.

All 4 patients with post-treatment tumor specimens saw an increase in intratumoral TCR diversity, particularly the patient with angiomatoid fibrous histiocytoma, who had a 3.1-fold change in Shannon entropy (Fig.4.10).

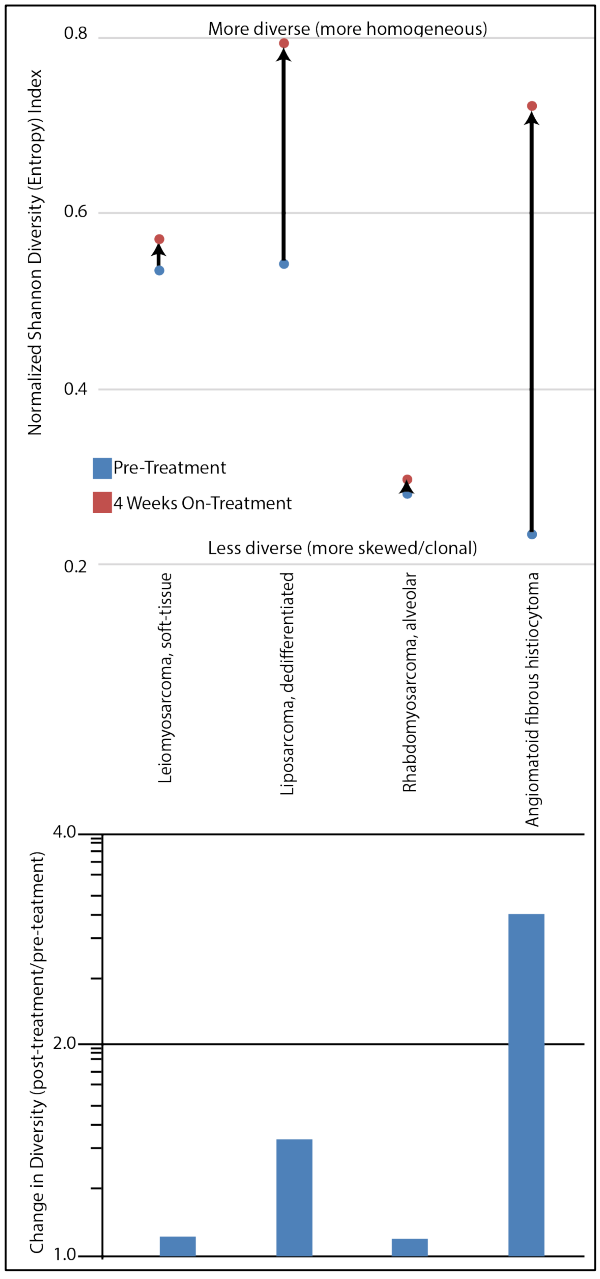


Figure 4.10 Individual changes in intratumoral TCR-β repertoire diversity after 4 weeks of immune checkpoint inhibitor therapy.

4.5 Discussion

Previous studies have demonstrated that patients with a more clonal intratumoral TCR repertoire tend to have better responses to immune checkpoint blockade.[499,513,514] While the final outcomes for this trial are not yet complete for use in this correlative study, conclusions can still be drawn about the nature of the T cell receptor repertoire in these sarcoma patients.

Contrasting the general trend observed by Pollack *et al* of higher TCR clonality among leiomyosarcomas and UPS than among more genomically-simple sarcoma subtypes,[553] we observed higher richness and diversity – and correspondingly, lower clonality – among the genomically-complex (non-translocation) subtypes of this trial. While grouping together individual single cases of biologically disparate sarcoma types under such a large umbrella is not a reliable way to qualify the TCR repertoire of sarcomas, this trend does fit with our previously described data of a more highly infiltrated environment among genomically-complex sarcomas. Greater TCR diversity and richness could very likely be attributable to greater tumor infiltration by T cells. Previous studies have shown that a more clonal population of intratumoral T cells can predict response to immune checkpoint inhibition; [499,513,514] however, in the case of translocation-associated sarcomas, we do not expect that the increased clonality of T cells will predict clinical benefit of immune checkpoint inhibitors relative to the non-translocation sarcomas. Paired with our observation of low overall lymphocytic infiltration in translocation-associated sarcomas, the observation of a less rich, less diverse population of TCR rearrangements among these sarcomas paints a picture of a quiet immune microenvironment, with perhaps just a few tumor antigens (such as CT antigens like NY-ESO-1) attracting and expanding clonal populations of tumor-infiltrating lymphocytes. We predict that the tumors that are both highly infiltrated and highly

clonal (relative to other highly infiltrated tumors) will have the most clinical benefit from immune checkpoint inhibition.

Similar studies in other cancers have found that comparison of baseline and post-treatment TCR repertoires showed an increase in the clonality of the TCR repertoire following treatment, specifically among responders.[499,515] Though we cannot yet separate our patients into responders and progressors, we observed an overall trend of decreased richness and diversity – and therefore increased clonality – 4 weeks after initiating immune checkpoint blockade therapy. While we have not conducted any neoantigen prediction techniques from our sequencing data, this increase in peripheral TCR clonality could very likely be due to expansion of anti-tumoral T cells as a result of effector immune cell reactivation following immune checkpoint inhibition. Among the patients for which we had post-treatment tumor specimens available, all saw an increase in diversity (and therefore a decrease in clonality) following treatment, particularly the cases of dedifferentiated liposarcoma and angiomatoid fibrous histiocytoma, which also saw an increase in TCR repertoire richness. This may again be due to overall increased infiltrates following treatment, or could indicate a poor response to therapy.

The findings of this study are limited by small numbers and mostly single-case representation of individual subtypes (aside from leiomyosarcoma). While this particular trial design is not powered to identify subtype-specific patterns or the validity of a predictive biomarker, it provides a useful look at the variability of the T cell repertoire across sarcomas at a baseline, as well as – for the first time in sarcomas – changes in the blood following treatment with immune checkpoint inhibitors. In the future, integration of this data with the other correlative science investigations run on these trial specimens (immunohistochemistry, tumor

mutational burden) and with the clinical outcomes data will help to clarify some of the discrepancies observed between this study and similar studies.

4.6 Conclusions

The sarcoma subtypes have diverse immune microenvironments, as described in the other chapters of this thesis. This heterogeneity also extends to the repertoire of T cell receptors expressed by tumor-infiltrating and peripheral T cells in sarcoma patients. In this chapter, I found that the TCR repertoire is richer and more diverse among the non-translocation sarcomas than the translocation-associated sarcomas, potentially attributable to increased T cell infiltration. Furthermore, following immune checkpoint blockade therapy, we observe an overall increase in peripheral TCR clonality, likely indicating some level of clonal expansion of tumor-targeting T cells. Alongside the other correlative and outcomes data for this clinical trial of tremelimumab and durvalumab, these findings will provide some much-needed insight into the immune microenvironment of sarcomas and how it changes with immunomodulatory therapy.

Chapter 5: Conclusions

5.1 Summary of Findings

These studies profiled the immune response associated with a broad spectrum of human sarcomas.

Chapter 2, our characterization of tumor-infiltrating lymphocytes (TILs), showed that genomically-complex sarcomas (including those that are mutation and/or copy-number driven) demonstrate higher counts of TILs than translocation-associated sarcomas. These TILs were predominately CD8+, with decreasing prevalence of CD4+, FOXP3+, and CD56+ TILs. Expression of PD-(L)1 was low compared to two other targetable lymphocyte-expressed immune checkpoints LAG-3 and TIM-3, and was most often co-expressed with one or more other checkpoint. Prior exposure to radiation was independently associated with increased TIL infiltration and higher expression of immune checkpoints. Higher TIL counts were associated with improved overall and progression-free survival, whereas expression of PD-1 was associated with worse overall survival.

Chapter 3, our characterization of tumor-associated macrophages (TAMs), also showed higher counts of TAMs among genomically-complex sarcomas relative to translocation-associated sarcomas, and these TAM counts far exceeded the TIL counts described in chapter 2. CD163+ macrophages were the predominant phenotype and were associated with worse progression-free survival. Analysis of TCGA data supported our results, and we found that measures of DNA damage correlated with higher TAM scores (but not TIL scores). Finally, expression of the macrophage-associated, targetable immune checkpoint CD47/SIRPα was more frequent than any of the immune checkpoints characterized in chapter 2.

Chapter 4, our characterization of T cell receptor (TCR) repertoires within tumors, demonstrated increased TCR richness and diversity among the genomically-complex (*i.e.,* non-translocation-driven) sarcomas, which generally translates to decreased clonality. Following treatment with CTLA-4 and PD-1 inhibitors, we observed an overall trend of decreased richness and diversity – and therefore increased clonality – in peripheral blood TCR repertoires. Contrastingly, in our limited number of post-treatment sarcoma tumor specimens, we observed higher richness and diversity – and therefore lower clonality – compared to baseline tumor specimens.

Collectively, these chapters illustrate many new details about the immunologically-quiet translocation-associated sarcomas and the immunologically-active non-translocation sarcomas, infiltrated by cytotoxic T cells of diverse antigenic targets, which are held in check by multiple immune checkpoints and overshadowed by a much larger population of immunosuppressive macrophages. While the specific dynamics of interplay between the sarcoma tumor cells and the numerous immunological players cannot be definitively determined by these studies alone, we can surmise that there exists an immune microenvironment that is "aware" of the presence of the genomically-complex sarcomas, but is held in check through a combination of co-expressed immune checkpoints and a multitude of anti-inflammatory cytokines typically secreted by M2 macrophages. Of course, generalized conclusions about the immune microenvironment of all sarcomas – or even all non-translocation sarcomas – does not do justice to the complexities of the diverse sarcoma subtypes, and a detailed understanding of the nuanced immune microenvironment specific to each sarcoma subtype is one of the true values of this doctoral work. Subtype-specific scores for all immune infiltrates, immune checkpoints, and TCR repertoire metrics are indicated in Table 5.1 and summarized in Figure 5.1.

Subtype	n	TILs	Cytotoxic T cells	Helper T cells	Tregs	NK cells	Lymphocyte-related checkpoints				M1 TAMs	M2 TAMs	Macrophage-related checkpoint		TCR Richness	TCR Diversity
		Median TILs/mm^2	Median CD8+ TILs/mm^2	Median CD4+ TILs/mm^2	Median FOXP3+ TILs/mm^2	Median CD56+ TILs/mm^2	% PD-1 positive	% PD-L1 positive	% LAG-3 positive	% TIM-3 positive	Median CD68+ TAMs/mm^2	Median CD163+ TAMs/mm^2	% CD47 positive	% SIRPα positive	Mean # unique TCR sequences	Mean Shannon entropy
Non-Translocation Sarcomas																
Angiosarcoma	4	43	36	9	1	0	33	0	100	33	486	1081	100	75	-	-
Chondrosarcoma	52	3	0	0	0	0	4	2	9	8	2	0	44	4	76	0.46
Chordoma	28	12	13	29	1	0	14	4	11	25	150	207	100	71	142	0.52
DDLPS	76	231	37	29	10	0	50	48	77	88	356	474	79	72	163	0.55
ERMS	9	179	14	16	0	0	0	17	33	17	46	18	40	30	-	-
Epithelioid sarcoma	8	114	154	61	11	0	57	13	29	57	148	136	75	13	1073	0.86
GIST	122	51	22	12	9	0	2	6	40	41	27	10	64	16	-	-
Leiomyosarcoma	26	74	27	8	2	0	32	47	58	74	260	273	32	28	122	0.43
MPNST	77	48	18	0	0	0	9	22	20	37	55	104	33	14	-	-
Myxofibrosarcoma	55	65	52	16	10	0	29	67	68	85	373	291	35	44	-	-
Osteosarcoma	244	45	10	19	1	0	21	43	45	69	95	130	51	30	-	-
UPS	75	52	45	22	19	0	36	71	72	83	460	500	12	37	62	0.36
WDLPS	63	33	1	0	0	0	24	17	31	55	46	101	66	63	45	0.38
Translocation-Associated Sarcomas																
ARMS	10	81	28	57	42	0	0	0	17	0	95	60	50	20	30	0.28
ASPS	8	26	10	3	0	0	0	0	25	25	48	404	38	25	-	-
Clear cell sarcoma	8	72	15	1	0	0	20	0	20	60	27	49	43	25	158	0.54
DFSP	31	48	15	4	0	0	12	10	28	39	70	61	30	29	-	-
Ewing sarcoma	21	52	5	4	0	0	20	0	5	19	39	48	30	25	11	0.11
Low-grade FMS	11	24	4	0	0	0	0	0	0	43	6	18	67	0	-	-
Myxoid LPS	41	26	7	1	0	0	10	0	5	10	20	72	66	54	-	-
SFT	111	40	13	7	0	0	6	0	19	38	91	82	55	25	39	0.26
Synovial sarcoma	163	33	4	0	0	0	15	11	30	60	17	41	48	14	42	0.26
Angiosarcoma	10	81	28	57	42	0	0	0	17	0	95	60	50	20	30	0.28

Table 5.1 Summary of subtype-specific immune cell marker scores for all markers studied in chapters 2-4.

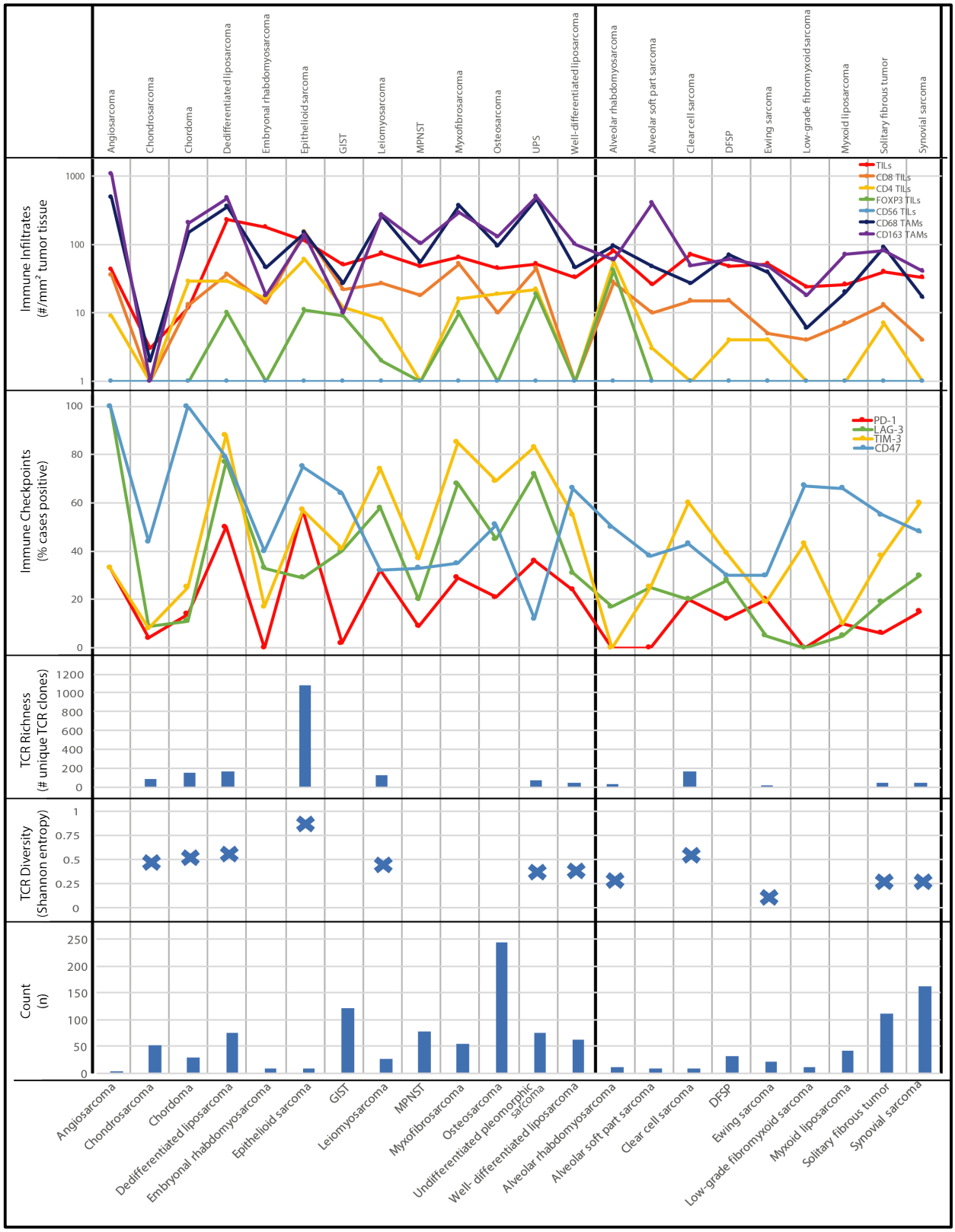


Figure 5.1 Summary of subtype-specific scores for all markers in chapters 2-4.

5.2 Implications

The significance of the work in the chapters of this thesis is twofold. First, my work provides a broad analysis of the sarcoma subtypes to help improve our understanding of the differences among these diseases at an immunological level. Too often, sarcoma subtypes are lumped together by tissue type in translational and clinical research, when in fact these subtypes are fundamentally different in their molecular alterations, pathogenesis, tumor microenvironments, and clinical behaviors. My work provides evidence to clearly distinguish the sarcoma subtypes in a novel way, by demonstrating important differences in their immune microenvironments. Clearly, the sarcoma subtypes trigger differing types of immune surveillance and furthermore, their strategies for immune evasion – both immunoselective and/or immunosubversive – differ considerably from subtype to subtype. One particularly obvious example of this is the case of liposarcomas, which even today are quite commonly (an incorrectly) grouped as a single entity in published reports. However, as demonstrated in every chapter of this thesis, the distinct subtypes of liposarcoma – dedifferentiated, well-differentiated, and (importantly) myxoid – have widely different immunological profiles. Dedifferentiated liposarcoma emerges as the most frequently and diversely infiltrated subtype in this dataset, with among the highest levels of expression for every immune checkpoint; contrastingly, myxoid liposarcoma has a characteristic translocation-associated-sarcoma-type immune microenvironment, devoid of most immune infiltrates and with few cases expressing any of the tested immune checkpoint biomarkers. Further, we know from other studies that myxoid liposarcoma (but not other liposarcoma subtypes) highly expresses CT antigen NY-ESO-1.[566-568] Together, these differences point to entirely different models of immune evasion for the

liposarcoma subtypes, and as such, they (like the other sarcoma subdivisions) should be approached as independent entities when considering immunotherapy.

The second, more translational significance of this work is its value for the design of immunotherapy clinical trials for sarcomas. So far, most of the clinical trials for immune checkpoint inhibitors for sarcomas have been open to any of numerous sarcoma types, with little background data to inform their design.[581-583] While the search is ongoing for a reliable predictive biomarker for immune checkpoint inhibitor therapy, most studies in other cancers agree that some type of immune infiltration is somewhat of a prerequisite.[497-500] My work comparatively demonstrates the extent of an immunological presence across most major sarcoma subtypes, with a central purpose of helping to inform the pragmatic design of immunotherapy clinical trials. Those subtypes that have a demonstrated immune presence should be prioritized for inclusion in clinical trials of immune checkpoint inhibitors, such as dedifferentiated liposarcoma, embryonal rhabdomyosarcoma, epithelioid sarcoma, myxofibrosarcoma, and UPS. Those with high macrophage counts but low TILs, such as angiosarcoma and chordoma, might be better suited to agents targeting macrophage-associated immune checkpoints. Contrastingly, the more immunologically quiet translocation-associated sarcomas are likely better suited to immunostimulatory strategies, such as cancer vaccines or CAR-T cell therapy. In addition to subtype-level information, my work highlights important immune checkpoints – LAG-3, TIM-3, and CD47/SIRPα – that are all expressed more commonly in sarcomas than PD-(L)1 and should be prioritized for sarcoma immunotherapy trials, likely in combination with other immune checkpoint inhibitors.

5.3 Limitations

In all three chapters of this thesis, I use the distinction of translocation-associated versus non-translation sarcomas. However, this is an imperfect way to subdivide sarcoma types, as numerous non-translocation sarcomas are comparatively genomically-simple (GIST, well-differentiated liposarcoma, chordoma) and in theory could behave more like the translocation-associated sarcomas than the copy-number-driven complex sarcomas. As discussed in section 1.1.2, division of sarcomas into "simple" and "complex" is still controversial, and even among the 5 principal investigators of the ImmunoSarc international consortium behind this project, a consensus could not be reached on exactly which entities should be assigned to each category. As such, we made our subdivisions based on presence/absence of a definitive pathognomonic translocation, keeping in mind that the "non-translocation" classification encompasses highly diverse subtypes, so conclusions drawn about this group do not necessarily apply to all subtypes included therein. Furthermore, like many sarcoma studies, our sample set has fairly uneven coverage of sarcoma subtypes, with a few subtypes (osteosarcoma, GIST, synovial sarcoma, solitary fibrous tumor) making up comparatively large proportions of the database. As such, it is important to interpret findings with attention to the sample size for each subtype. The numbers become yet smaller when considering the cases for which clinical data and outcomes are available. Therefore, the prognostic significance of the markers characterized in this data set may be discrepant with others' findings due to incomplete clinical data and/or lack of power.

A major resource for this work is the tissue microarray (TMA) database of archived tissues from Vancouver General Hospital and Mount Sinai Hospital. While tissue microarrays allow for high-throughput profiling of hundreds of specimens in parallel, our TMA cores show a tumor area of only 0.28mm^2-3.14mm^2, which is very likely not representative of the entire (1cm

- 42cm clinical tumor size range in this dataset) tumor. Some studies have shown that in a subset of tumors – so-called altered-excluded tumors – the immune infiltrate resides only in the tumor's invasive margin,[501] which would not be sampled by TMA cores, which are usually extracted from the centre of the tumor (after pathology review to circle areas containing representative viable sarcoma cells. Use of whole-section specimens might resolve this issue; however, it would be incredibly time-consuming (and likely prohibitively expensive) to stain and score the entire set of cases in this study on whole-section for the many markers under investigation. Such labor-intensive staining might not be as consistent, as we would not be able to stain the entire sample set at once for each marker, introducing batch effects that are not an issue with TMAs (the autostained methodology can cover the entire sarcoma TMA series in a single run, but would require >50 runs if whole sections were used) Furthermore, when considering clinical use of biomarkers, sarcoma pathologists are most often working from core-needle biopsy specimens, whose tissue coverage and relatively random sampling origin are quite comparable to that of a tissue microarray core.[671]

Immunohistochemistry is a useful methodology in that it identifies proteins, the functional end-products of gene expression. However, variable antibody sensitivity and specificity, as well as necessarily subjective/semiquantitative scoring methods can affect the reliability of the results. While all efforts were made to use the most validated and accepted antibody clones for each antigen under investigation, comparing scores from 2 different antibodies staining 2 different targets does not conclusively determine the relative abundance of the targets in question. For selection of antibody clones for immune checkpoint biomarkers, this is a controversial area where there are not yet any generally accepted options, so we selected the clones that performed best on our staining optimization protocols with positive and negative

controls. Furthermore, while singleplex serial staining allows an approximate assessment of co-expression of multiple markers, it is necessary to perform multiplex staining on the exact same tissue section to be able to make definitive conclusions concerning cell types defined by expression of more than one marker. This is particularly true for biomarkers that could be expressed on the same cells, such as CD4 and FOXP3, or CD68 and CD163.

Finally, in chapter 4, we use tissues from a single-arm, signal-seeking clinical trial from an Australian clinical trials group. This study was not designed to power any subtype-specific analysis and was skewed towards leiomyosarcoma, shown in most studies to respond poorly to immune checkpoint inhibition. [581-583,599] The trial design was focused on including rare cancers that are usually neglected for clinical trials, and as such, it may not be ideal for the type of analysis that is the focus of this thesis. However, this dataset still has importance as the very first clinical trial to examine TCR repertoires in sarcomas before and after treatment with immune checkpoint inhibitors.

5.4 Future Directions

The experiments presented in the chapters of this thesis constitute primarily discovery-based exploratory analyses. As such, they should be validated both biologically and methodologically. In some cases, these findings could also be validated in relevant genetic animal models that reliably recapitulate human disease in immunocompetent backgrounds. Repetition of immunohistochemical staining in a new tissue database of sarcomas using the conditions and cutoffs established in this work would help validate subtype-specific findings. French collaborators on the ImmunoSarc international consortium behind this project have access to a large European tissue database, Conticabase (12, 262 specimens),[686] on which suitable validation can be performed. Key findings might be validated on whole-section tissues

and/or by multiplex immunohistochemistry. Furthermore, exploration of these targets through other methodologies, such as RNA expression assays or flow cytometry, would help resolve some of the limitations associated with immunohistochemical studies.

While this thesis provides a fairly comprehensive look at the immune microenvironment of sarcomas, larger than any published to date, it is still by no means a complete evaluation. Several sarcoma types are underrepresented in our dataset, particularly compared to their relative prevalence, including leiomyosarcomas, Ewing sarcomas, and rhabdomyosarcomas. We also observed higher counts of lymphocytes than were accounted for by CD8, CD4, FOXP3, and CD56 staining. A more exhaustive evaluation of tumor-infiltrating lymphocytes would also look at B cells, plasma cells, and gamma/delta T cells. Other possible immune infiltrates that could be examined include tissue-resident dendritic cells, neutrophils, basophils, and mast cells. Further, there are a number of emerging immune checkpoints[364] that could potentially be highly relevant in sarcomas, such as natural killer cell immune checkpoints (KIR, NKG2A), macrophage-related immune checkpoint IDO1,[557] and alternate lymphocyte-related immune checkpoints (CTLA-4, BTLA, CD40, VISTA, CD137, GITR, CD27, ICOS, TIGIT, B7-H3). Finally, the peripheral immune response has been explored in other cancers, but never in sarcomas.

Data interpretations throughout the chapters of this thesis often use the distinction of translocation-associated versus non-translocation sarcomas as a surrogate marker of genomic complexity. However, a more quantitative, unequivocal method to assess the relationship between the various immune parameters and genomic complexity would be to determine the tumor mutational burden for our sample set. Numerous studies in other cancers have demonstrated that tumor mutational burden is a strong predictor of response to immune checkpoint inhibitors. [505-510] Furthermore, particularly in sarcomas, full characterization of

genomic modifications, specifically copy number alterations, might be particularly informative with regards to subtype-specific differences in immune infiltration or checkpoint response.

Our analysis of the TCR repertoire of patients on the Australianan MoST clinical trial is as yet incomplete, pending completion of trial and determination of outcomes at 48 months. Once the trial is over, this data will be further analyzed to characterize differences between responders and non-responders. Furthermore, investigators of this clinical trial carried out multiple other correlative studies that will be related to our findings about the TCR repertoire, including data they are generating on TIL counts, PD-(L)1 immunohistochemistry, investigation of cytokines in the blood pre- and post- treatment, and evaluation of tumor mutational burden. Ultimately, the major future direction of this work is in influencing the design of immunotherapy clinical trials. Cohorts of future clinical trials should focus on TIL-infiltrated subtypes, such as dedifferentiated liposarcoma (excluding other liposarcoma subtypes), myxofibrosarcoma, undifferentiated pleomorphic sarcoma, epithelioid sarcoma, and alveolar and embryonal rhabdomyosarcomas. PD-(L)1 monotherapy strategies should be abandoned in favour of combinatorial therapy with novel immune checkpoints LAG-3 and/or TIM-3 that are more commonly expressed in sarcomas. Importantly, tumor-associated macrophages may be particularly relevant in sarcomas, and as such, macrophage-associated immune checkpoints such as CD47/SIRPα should be investigated, particularly for angiosarcoma and chordoma. Far too many sarcoma patients remain without systemic options when their cancer metastasizes. With the recent rapid expansion of immunotherapeutic options and protocols in other cancers, it proceeds that the pragmatic, informed design of immunotherapy clinical trials is of imminent importance for the sarcoma subtypes highlighted herein that have demonstrated clear evidence of immune evasion.

References

1. Fletcher, C. D. M. *WHO Classification of Tumours of Soft Tissue and Bone*. (World Health Organization, 2013).
2. Genadry, K. C., Pietrobono, S., Rota, R. & Linardic, C. M. Soft Tissue Sarcoma Cancer Stem Cells: An Overview. *Front Oncol* **8,** 475 (2018).
3. Xiao, W., Mohseny, A. B., Hogendoorn, P. C. W. & Cleton Jansen, A. M. Mesenchymal stem cell transformation and sarcoma genesis. *Clin Sarcoma Res* **3,** 10 (2013).
4. Mohseny, A. B. *et al.* Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. *The Journal of Pathology* **219,** 294–305 (2009).
5. Yang, J., Ren, Z., Du, X., Hao, M. & Zhou, W. The role of mesenchymal stem/progenitor cells in sarcoma: update and dispute. *Stem Cell Investig* **1,** 18 (2014).
6. Li, F. P. & Fraumeni, J. F. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? *Ann. Intern. Med.* **71,** 747–752 (1969).
7. Ognjanovic, S., Olivier, M., Bergemann, T. L. & Hainaut, P. Sarcomas in TP53 germline mutation carriers: a review of the IARC TP53 database. *Cancer* **118,** 1387–1396 (2012).
8. Kleinerman, R. A., Schonfeld, S. J. & Tucker, M. A. Sarcomas in hereditary retinoblastoma. *Clin Sarcoma Res* **2,** 15 (2012).
9. Brems, H., Beert, E., de Ravel, T. & Legius, E. Mechanisms in the pathogenesis of malignant tumours in neurofibromatosis type 1. *Lancet Oncol.* **10,** 508–515 (2009).
10. Penel, N. *et al.* Frequency of certain established risk factors in soft tissue sarcomas in adults: a prospective descriptive study of 658 cases. *Sarcoma* **2008,** 459386–6 (2008).
11. Mitchell, G. *et al.* High frequency of germline TP53 mutations in a prospective adult-onset sarcoma cohort. *PLoS ONE* **8,** e69026 (2013).
12. Thomas, D. M. & Ballinger, M. L. Etiologic, environmental and inherited risk factors in sarcomas. *J Surg Oncol* **111,** 490–495 (2015).
13. Burningham, Z., Hashibe, M., Spector, L. & Schiffman, J. D. The epidemiology of sarcoma. *Clin Sarcoma Res* **2,** 14 (2012).
14. Hui, J. Y. C. Epidemiology and Etiology of Sarcomas. *Surg. Clin. North Am.* **96,** 901–914 (2016).
15. Canadian Cancer Registry. at <https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=1310011101&pickMembers%5B0%5D=2.1&pickMembers%5B1%5D=3.1&pickMembers%5B2%5D=4.24>
16. Ferrari, A. *et al.* Soft tissue sarcoma across the age spectrum: a population-based study from the Surveillance Epidemiology and End Results database. *Pediatric Blood & Cancer* **57,** 943–949 (2011).
17. de Alava, E. Molecular pathology in sarcomas. *Clin Transl Oncol* **9,** 130–144 (2007).
18. Jain, S., Xu, R., Prieto, V. G. & Lee, P. Molecular classification of soft tissue sarcomas and its clinical applications. *Int J Clin Exp Pathol* **3,** 416–428 (2010).
19. Taylor, B. S. *et al.* Advances in sarcoma genomics and new therapeutic targets. *Nat. Rev. Cancer* **11,** 541–557 (2011).
20. Lim, J., Poulin, N. M. & Nielsen, T. O. New Strategies in Sarcoma: Linking Genomic and Immunotherapy Approaches to Molecular Subtype. *Clinical Cancer Research* **21,** 4753–4759 (2015).

21. Abeshouse, A. *et al.* Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. *Cell* **171,** 950–965.e28 (2017).
22. Brohl, A. S. *et al.* The genomic landscape of the Ewing Sarcoma family of tumors reveals recurrent STAG2 mutation. *PLoS Genet.* **10,** e1004475 (2014).
23. Crompton, B. D. *et al.* The genomic landscape of pediatric Ewing sarcoma. *Cancer Discovery* **4,** 1326–1341 (2014).
24. Tirode, F. *et al.* Genomic landscape of Ewing sarcoma defines an aggressive subtype with co-association of STAG2 and TP53 mutations. *Cancer Discovery* **4,** 1342–1353 (2014).
25. Helman, L. J. & Meltzer, P. Mechanisms of sarcoma development. *Nat. Rev. Cancer* **3,** 685–694 (2003).
26. Nakano, K. & Takahashi, S. Translocation-Related Sarcomas. *Int J Mol Sci* **19,** 3784 (2018).
27. Barr, F. G. *et al.* Rearrangement of the PAX3 paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. *Nature Genetics* **3,** 113–117 (1993).
28. Galili, N. *et al.* Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. *Nature Genetics* **5,** 230–235 (1993).
29. Davis, R. J., D'Cruz, C. M., Lovell, M. A., Biegel, J. A. & Barr, F. G. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. *Clinical Cancer Research* **54,** 2869–2872 (1994).
30. Ladanyi, M. *et al.* The der(17)t(X;17)(p11;q25) of human alveolar soft part sarcoma fuses the TFE3 transcription factor gene to ASPL, a novel gene at 17q25. *Oncogene* **20,** 48–57 (2001).
31. Panagopoulos, I. *et al.* Molecular genetic characterization of the EWS/ATF1 fusion gene in clear cell sarcoma of tendons and aponeuroses. *International Journal of Cancer* **99,** 560–567 (2002).
32. Antonescu, C. R., Nafa, K., Segal, N. H., Dal Cin, P. & Ladanyi, M. EWS-CREB1: a recurrent variant fusion in clear cell sarcoma--association with gastrointestinal location and absence of melanocytic differentiation. *Clin. Cancer Res.* **12,** 5356–5362 (2006).
33. Aurias, A., Rimbaut, C., Buffe, D., Zucker, J. M. & Mazabraud, A. Translocation involving chromosome 22 in Ewing's sarcoma. A cytogenetic study of four fresh tumors. *Cancer Genet. Cytogenet.* **12,** 21–25 (1984).
34. Turc-Carel, C. *et al.* Chromosomes in Ewing's sarcoma. I. An evaluation of 85 cases of remarkable consistency of t(11;22)(q24;q12). *Cancer Genet. Cytogenet.* **32,** 229–238 (1988).
35. Douglass, E. C., Valentine, M., Green, A. A., Hayes, F. A. & Thompson, E. I. t(11;22) and other chromosomal rearrangements in Ewing's sarcoma. *J. Natl. Cancer Inst.* **77,** 1211–1215 (1986).
36. Delattre, O. *et al.* Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature* **359,** 162–165 (1992).
37. Zucman, J. *et al.* Cloning and characterization of the Ewing's sarcoma and peripheral neuroepithelioma t(11;22) translocation breakpoints. *Genes, Chromosomes and Cancer* **5,** 271–277 (1992).
38. Reid, R., de Silva, M. V. C., Paterson, L., Ryan, E. & Fisher, C. Low-grade fibromyxoid sarcoma and hyalinizing spindle cell tumor with giant rosettes share a common

t(7;16)(q34;p11) translocation. *Am. J. Surg. Pathol.* **27,** 1229–1236 (2003).
39. Storlazzi, C. T. *et al.* Fusion of the FUS and BBF2H7 genes in low grade fibromyxoid sarcoma. *Hum. Mol. Genet.* **12,** 2349–2358 (2003).
40. Crozat, A., Aman, P., Mandahl, N. & Ron, D. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature* **363,** 640–644 (1993).
41. Rabbitts, T. H., Forster, A., Larson, R. & Nathan, P. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. *Nature Genetics* **4,** 175–180 (1993).
42. Panagopoulos, I. *et al.* Fusion of the EWS and CHOP genes in myxoid liposarcoma. *Oncogene* **12,** 489–494 (1996).
43. Smith, S., Reeves, B. R., Wong, L. & Fisher, C. A consistent chromosome translocation in synovial sarcoma. *Cancer Genet. Cytogenet.* **26,** 179–180 (1987).
44. Reeves, B. R. *et al.* Characterization of the translocation between chromosomes X and 18 in human synovial sarcomas. *Oncogene* **4,** 373–378 (1989).
45. Knight, J. *et al.* Cytogenetic and molecular analysis of synovial sarcoma. *International Journal of Oncology* **1,** 747–752 (1992).
46. Ladanyi, M. Fusions of the SYT and SSX genes in synovial sarcoma. *Oncogene* **20,** 5755–5762 (2001).
47. Chmielecki, J. *et al.* Whole-exome sequencing identifies a recurrent NAB2-STAT6 fusion in solitary fibrous tumors. *Nature Genetics* **45,** 131–132 (2013).
48. Robinson, D. R. *et al.* Identification of recurrent NAB2-STAT6 gene fusions in solitary fibrous tumor by integrative sequencing. *Nature Genetics* **45,** 180–185 (2013).
49. Bridge, J. A., Neff, J. R. & Sandberg, A. A. Cytogenetic analysis of dermatofibrosarcoma protuberans. *Cancer Genet. Cytogenet.* **49,** 199–202 (1990).
50. Pedeutour, F. *et al.* Ring 22 chromosomes in dermatofibrosarcoma protuberans are low-level amplifiers of chromosome 17 and 22 sequences. *Clinical Cancer Research* **55,** 2400–2403 (1995).
51. Simon, M. P. *et al.* Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. *Nature Genetics* **15,** 95–98 (1997).
52. Dalprà, L. *et al.* First cytogenetic study of a recurrent familial chordoma of the clivus. *International Journal of Cancer* **81,** 24–30 (1999).
53. Scheil, S. *et al.* Genome-wide analysis of sixteen chordomas by comparative genomic hybridization and cytogenetics of the first human chordoma cell line, U-CH1. *Genes, Chromosomes and Cancer* **32,** 203–211 (2001).
54. Le, L. P. *et al.* Recurrent chromosomal copy number alterations in sporadic chordomas. *PLoS ONE* **6,** e18846 (2011).
55. Loh, W. E. *et al.* Human chromosome 11 contains two different growth suppressor genes for embryonal rhabdomyosarcoma. *Proceedings of the National Academy of Sciences* **89,** 1755–1759 (1992).
56. Besnard-Guérin, C., Newsham, I., Winqvist, R. & Cavenee, W. K. A common region of loss of heterozygosity in Wilms' tumor and embryonal rhabdomyosarcoma distal to the D11S988 locus on chromosome 11p15.5. *Hum. Genet.* **97,** 163–170 (1996).
57. Davicioni, E. *et al.* Molecular classification of rhabdomyosarcoma--genotypic and phenotypic determinants of diagnosis: a report from the Children's Oncology Group. *The*

American Journal of Pathology **174,** 550–564 (2009).
58. Visser, M. *et al.* Allelotype of pediatric rhabdomyosarcoma. *Oncogene* **15,** 1309–1314 (1997).
59. Gil-Benso, R. *et al.* Chromosomal and genetic changes produced in tumoral progression of embryonal rhabdomyosarcoma. *Histopathology* **62,** 816–819 (2013).
60. Sun, X. *et al.* Rhabdomyosarcoma: Advances in Molecular and Cellular Biology. *Sarcoma* **2015,** 232010–14 (2015).
61. Modena, P. *et al.* SMARCB1/INI1 tumor suppressor gene is frequently inactivated in epithelioid sarcomas. *Clinical Cancer Research* **65,** 4012–4019 (2005).
62. Flucke, U., Slootweg, P. J., Mentzel, T., Pauwels, P. & Hulsebos, T. J. M. Re: Infrequent SMARCB1/INI1 gene alteration in epithelioid sarcoma: a useful tool in distinguishing epithelioid sarcoma from malignant rhabdoid tumor: Direct evidence of mutational inactivation of SMARCB1/INI1 in epithelioid sarcoma. *Hum. Pathol.* **40,** 1361–2– author reply 1362–4 (2009).
63. Kohashi, K. *et al.* Infrequent SMARCB1/INI1 gene alteration in epithelioid sarcoma: a useful tool in distinguishing epithelioid sarcoma from malignant rhabdoid tumor. *Hum. Pathol.* **40,** 349–355 (2009).
64. Sullivan, L. M., Folpe, A. L., Pawel, B. R., Judkins, A. R. & Biegel, J. A. Epithelioid sarcoma is associated with a high percentage of SMARCB1 deletions. *Mod. Pathol.* **26,** 385–392 (2013).
65. Jamshidi, F. *et al.* The genomic landscape of epithelioid sarcoma cell lines and tumours. *The Journal of Pathology* **238,** 63–73 (2015).
66. Hirota, S. *et al.* Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* **279,** 577–580 (1998).
67. Lux, M. L. *et al.* KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. *The American Journal of Pathology* **156,** 791–795 (2000).
68. Rubin, B. P. *et al.* KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. *Clinical Cancer Research* **61,** 8118–8121 (2001).
69. Heinrich, M. C. *et al.* PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* **299,** 708–710 (2003).
70. Fletcher, C. D. *et al.* Correlation between clinicopathological features and karyotype in lipomatous tumors. A report of 178 cases from the Chromosomes and Morphology (CHAMP) Collaborative Study Group. *The American Journal of Pathology* **148,** 623–630 (1996).
71. Meis-Kindblom, J. M. *et al.* Cytogenetic and molecular genetic analyses of liposarcoma and its soft tissue simulators: recognition of new variants and differential diagnosis. *Virchows Arch.* **439,** 141–151 (2001).
72. Crago, A. M. & Singer, S. Clinical and molecular approaches to well differentiated and dedifferentiated liposarcoma. *Current Opinion in Oncology* **23,** 373–378 (2011).
73. Nilbert, M. Molecular and cytogenetics of soft tissue sarcomas. *Acta Orthop Scand Suppl* **273,** 60–67 (1997).
74. Nord, K. H. *et al.* Integrative genome and transcriptome analyses reveal two distinct types of ring chromosome in soft tissue sarcomas. *Hum. Mol. Genet.* **23,** 878–888 (2014).
75. Guillou, L. & Aurias, A. Soft tissue sarcomas with complex genomic profiles. *Virchows*

Arch. **456,** 201–217 (2010).
76. Dorfman, H. D. & Czerniak, B. Bone cancers. *Cancer* **75,** 203–210 (1995).
77. Brennan, M. F., Antonescu, C. R., Moraco, N. & Singer, S. Lessons learned from the study of 10,000 patients with soft tissue sarcoma. *Ann. Surg.* **260,** 416–21– discussion 421–2 (2014).
78. George, A. & Grimer, R. Early symptoms of bone and soft tissue sarcomas: could they be diagnosed earlier? *Ann R Coll Surg Engl* **94,** 261–266 (2012).
79. Ilaslan, H., Schils, J., Nageotte, W., Lietman, S. A. & Sundaram, M. Clinical presentation and imaging of bone and soft-tissue sarcomas. *Cleve Clin J Med* **77 Suppl 1,** S2–7 (2010).
80. Chotel, F. *et al.* Variability in the presentation of synovial sarcoma in children: a plea for greater awareness. *J Bone Joint Surg Br* **90,** 1090–1096 (2008).
81. Tanaka, K. & Ozaki, T. New TNM classification (AJCC eighth edition) of bone and soft tissue sarcomas: JCOG Bone and Soft Tissue Tumor Study Group. *Jpn. J. Clin. Oncol.* **49,** 103–107 (2019).
82. Trojani, M. *et al.* Soft-tissue sarcomas of adults; study of pathological prognostic variables and definition of a histopathological grading system. *International Journal of Cancer* **33,** 37–42 (1984).
83. Coindre, J. M. Grading of soft tissue sarcomas: review and update. *Arch. Pathol. Lab. Med.* **130,** 1448–1453 (2006).
84. Dancsok, A. R., Asleh-Aburaya, K. & Nielsen, T. O. Advances in sarcoma diagnostics and treatment. *Oncotarget* **8,** 7068–7093 (2017).
85. Hornick, J. L. Limited biopsies of soft tissue tumors: the contemporary role of immunohistochemistry and molecular diagnostics. *Mod. Pathol.* **11,** 24 (2019).
86. Italiano, A. *et al.* Clinical effect of molecular methods in sarcoma diagnosis (GENSARC): a prospective, multicentre, observational study. *Lancet Oncol.* **17,** 532–538 (2016).
87. ESMO / European Sarcoma Network Working Group. Soft tissue and visceral sarcomas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **23 Suppl 7,** vii92–9 (2012).
88. Gutowski, C. J., Basu-Mallick, A. & Abraham, J. A. Management of Bone Sarcoma. *Surg. Clin. North Am.* **96,** 1077–1106 (2016).
89. Gerrand, C. *et al.* UK guidelines for the management of bone sarcomas. *Clin Sarcoma Res* **6,** 7 (2016).
90. Trovik, C. S. *et al.* Surgical margins, local recurrence and metastasis in soft tissue sarcomas: 559 surgically-treated patients from the Scandinavian Sarcoma Group Register. *Eur. J. Cancer* **36,** 710–716 (2000).
91. Endo, M. & Lin, P. P. Surgical margins in the management of extremity soft tissue sarcoma. *Chin Clin Oncol* **7,** 37–37 (2018).
92. McDonald, D. J. Limb-salvage surgery for treatment of sarcomas of the extremities. *AJR Am J Roentgenol* **163,** 509–13– discussion 514–6 (1994).
93. Cahlon, O. *et al.* A postoperative nomogram for local recurrence risk in extremity soft tissue sarcomas after limb-sparing surgery without adjuvant radiation. *Ann. Surg.* **255,** 343–347 (2012).

94. Treasure, T., Fiorentino, F., Scarci, M., Møller, H. & Utley, M. Pulmonary metastasectomy for sarcoma: a systematic review of reported outcomes in the context of Thames Cancer Registry data. *BMJ Open* **2,** e001736 (2012).
95. Digesu, C. S., Wiesel, O., Vaporciyan, A. A. & Colson, Y. L. Management of Sarcoma Metastases to the Lung. *Surg. Oncol. Clin. N. Am.* **25,** 721–733 (2016).
96. Chao, C. & Goldberg, M. Surgical treatment of metastatic pulmonary soft-tissue sarcoma. *Oncology (Williston Park, N.Y.)* **14,** 835–41– discussion 842–4– 847 (2000).
97. Macherey, S. *et al.* [Surgery for Pulmonary Metastases in Patients with Advanced Soft Tissue and Osteosarcomas]. *Z Orthop Unfall* **155,** 567–574 (2017).
98. Billingsley, K. G. *et al.* Multifactorial analysis of the survival of patients with distant metastasis arising from primary extremity sarcoma. *Cancer* **85,** 389–395 (1999).
99. García Franco, C. E. *et al.* Long-term results after resection for bone sarcoma pulmonary metastases. *Eur J Cardiothorac Surg* **37,** 1205–1208 (2010).
100. Rosenberg, S. A. *et al.* The treatment of soft-tissue sarcomas of the extremities: prospective randomized evaluations of (1) limb-sparing surgery plus radiation therapy compared with amputation and (2) the role of adjuvant chemotherapy. *Ann. Surg.* **196,** 305–315 (1982).
101. Yang, J. C. *et al.* Randomized prospective study of the benefit of adjuvant radiation therapy in the treatment of soft tissue sarcomas of the extremity. *JCO* **16,** 197–203 (1998).
102. Pisters, P. W. *et al.* Long-term results of a prospective randomized trial of adjuvant brachytherapy in soft tissue sarcoma. *JCO* **14,** 859–868 (1996).
103. Harrison, L. B., Franzese, F., Gaynor, J. J. & Brennan, M. F. Long-term results of a prospective randomized trial of adjuvant brachytherapy in the management of completely resected soft tissue sarcomas of the extremity and superficial trunk. *Int. J. Radiat. Oncol. Biol. Phys.* **27,** 259–265 (1993).
104. O'Sullivan, B. *et al.* Preoperative versus postoperative radiotherapy in soft-tissue sarcoma of the limbs: a randomised trial. *Lancet* **359,** 2235–2241 (2002).
105. Zagars, G. K. *et al.* Prognostic factors for patients with localized soft-tissue sarcoma treated with conservation surgery and radiation therapy: an analysis of 1225 patients. *Cancer* **97,** 2530–2543 (2003).
106. Gundle, K. R. *et al.* Analysis of Margin Classification Systems for Assessing the Risk of Local Recurrence After Soft Tissue Sarcoma Resection. *J. Clin. Oncol.* **36,** 704–709 (2018).
107. Sheplan, L. J. & Juliano, J. J. Use of radiation therapy for patients with soft-tissue and bone sarcomas. *Cleve Clin J Med* **77 Suppl 1,** S27–9 (2010).
108. Akagunduz, O. O. *et al.* The role of radiotherapy in local control of nonextremity Ewing sarcomas. *Tumori* **102,** 162–167 (2016).
109. Dunst, J. & Schuck, A. Role of radiotherapy in Ewing tumors. *Pediatric Blood & Cancer* **42,** 465–470 (2004).
110. Weber, D. C. *et al.* Long term outcomes of patients with skull-base low-grade chondrosarcoma and chordoma patients treated with pencil beam scanning proton therapy. *Radiother Oncol* **120,** 169–174 (2016).
111. Schwarz, R., Bruland, O., Cassoni, A., Schomberg, P. & Bielack, S. The role of radiotherapy in oseosarcoma. *Cancer Treat. Res.* **152,** 147–164 (2009).

112. Mahajan, A. *et al.* Multimodality treatment of osteosarcoma: radiation in a high-risk cohort. *Pediatric Blood & Cancer* **50,** 976–982 (2008).
113. Errani, C. *et al.* Palliative therapy for osteosarcoma. *Expert Rev Anticancer Ther* **11,** 217–227 (2011).
114. Dahlin, D. C. & Coventry, M. B. Osteogenic sarcoma. A study of six hundred cases. *J Bone Joint Surg Am* **49,** 101–110 (1967).
115. Marcove, R. C., Miké, V., Hajek, J. V., Levin, A. G. & Hutter, R. V. Osteogenic sarcoma under the age of twenty-one. A review of one hundred and forty-five operative cases. *J Bone Joint Surg Am* **52,** 411–423 (1970).
116. Lindberg, R. D., Martin, R. G., Romsdahl, M. M. & Barkley, H. T. Conservative surgery and postoperative radiotherapy in 300 adults with soft-tissue sarcomas. *Cancer* **47,** 2391–2397 (1981).
117. Eilber, F. *et al.* Adjuvant chemotherapy for osteosarcoma: a randomized prospective trial. *JCO* **5,** 21–26 (1987).
118. Maki, R. G. Ifosfamide in the neoadjuvant treatment of osteogenic sarcoma. *J. Clin. Oncol.* **30,** 2033–2035 (2012).
119. Walczak, B. E. & Irwin, R. B. Sarcoma chemotherapy. *J Am Acad Orthop Surg* **21,** 480–491 (2013).
120. Bacci, G. *et al.* Long-term outcome for patients with nonmetastatic osteosarcoma of the extremity treated at the istituto ortopedico rizzoli according to the istituto ortopedico rizzoli/osteosarcoma-2 protocol: an updated report. *JCO* **18,** 4016–4027 (2000).
121. Bernthal, N. M. *et al.* Long-term results (>25 years) of a randomized, prospective clinical trial evaluating chemotherapy in patients with high-grade, operable osteosarcoma. *Cancer* **118,** 5888–5893 (2012).
122. Goorin, A. M. *et al.* Presurgical chemotherapy compared with immediate surgery and adjuvant chemotherapy for nonmetastatic osteosarcoma: Pediatric Oncology Group Study POG-8651. *JCO* **21,** 1574–1580 (2003).
123. Womer, R. B. *et al.* Randomized controlled trial of interval-compressed chemotherapy for the treatment of localized Ewing sarcoma: a report from the Children's Oncology Group. *J. Clin. Oncol.* **30,** 4148–4154 (2012).
124. Pervaiz, N. *et al.* A systematic meta-analysis of randomized controlled trials of adjuvant chemotherapy for localized resectable soft-tissue sarcoma. *Cancer* **113,** 573–581 (2008).
125. Woll, P. J. *et al.* Adjuvant chemotherapy with doxorubicin, ifosfamide, and lenograstim for resected soft-tissue sarcoma (EORTC 62931): a multicentre randomised controlled trial. *Lancet Oncol.* **13,** 1045–1054 (2012).
126. Jones, R. L., Fisher, C., Al-Muderis, O. & Judson, I. R. Differential sensitivity of liposarcoma subtypes to chemotherapy. *Eur. J. Cancer* **41,** 2853–2860 (2005).
127. Eilber, F. C. *et al.* The impact of chemotherapy on the survival of patients with high-grade primary extremity liposarcoma. *Ann. Surg.* **240,** 686–95– discussion 695–7 (2004).
128. Rosen, G. *et al.* Synovial sarcoma. Uniform response of metastases to high dose ifosfamide. *Cancer* **73,** 2506–2511 (1994).
129. Skubitz, K. M. & Haddad, P. A. Paclitaxel and pegylated-liposomal doxorubicin are both active in angiosarcoma. *Cancer* **104,** 361–366 (2005).
130. Penel, N. *et al.* Phase II trial of weekly paclitaxel for unresectable angiosarcoma: the

ANGIOTAX Study. *J. Clin. Oncol.* **26,** 5269–5274 (2008).
131. Maki, R. G. *et al.* Randomized phase II study of gemcitabine and docetaxel compared with gemcitabine alone in patients with metastatic soft tissue sarcomas: results of sarcoma alliance for research through collaboration study 002 [corrected]. *J. Clin. Oncol.* **25,** 2755–2763 (2007).
132. Bramwell, V. H., Anderson, D. & Charette, M. L. Doxorubicin-based chemotherapy for the palliative treatment of adult patients with locally advanced or metastatic soft-tissue sarcoma: a meta-analysis and clinical practice guideline. *Sarcoma* **4,** 103–112 (2000).
133. Arifi, S., Belbaraka, R., Rahhali, R. & Ismaili, N. Treatment of Adult Soft Tissue Sarcomas: An Overview. *Rare Cancers Ther* **3,** 69–87 (2015).
134. Italiano, A. *et al.* Trends in survival for patients with metastatic soft-tissue sarcoma. *Cancer* **117,** 1049–1054 (2011).
135. Borden, E. C. *et al.* Randomized comparison of three adriamycin regimens for metastatic soft tissue sarcomas. *JCO* **5,** 840–850 (1987).
136. Edmonson, J. H. *et al.* Randomized comparison of doxorubicin alone versus ifosfamide plus doxorubicin or mitomycin, doxorubicin, and cisplatin against advanced soft tissue sarcomas. *JCO* **11,** 1269–1275 (1993).
137. Judson, I. *et al.* Doxorubicin alone versus intensified doxorubicin plus ifosfamide for first-line treatment of advanced or metastatic soft-tissue sarcoma: a randomised controlled phase 3 trial. *Lancet Oncol.* **15,** 415–423 (2014).
138. Ratan, R. & Patel, S. R. Chemotherapy for soft tissue sarcoma. *Cancer* **122,** 2952–2960 (2016).
139. Schöffski, P. *et al.* Activity of eribulin mesylate in patients with soft-tissue sarcoma: a phase 2 study in four independent histological subtypes. *Lancet Oncol.* **12,** 1045–1052 (2011).
140. Demetri, G. D. *et al.* Efficacy and safety of trabectedin in patients with advanced or metastatic liposarcoma or leiomyosarcoma after failure of prior anthracyclines and ifosfamide: results of a randomized phase II study of two different schedules. *J. Clin. Oncol.* **27,** 4188–4196 (2009).
141. Schwartz, G. K. Trabectedin and the L-Sarcomas: A Decade-Long Odyssey. *J. Clin. Oncol.* **34,** 769–771 (2016).
142. van Oosterom, A. T. *et al.* Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. *Lancet* **358,** 1421–1423 (2001).
143. Demetri, G. D. *et al.* Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N. Engl. J. Med.* **347,** 472–480 (2002).
144. Casper, E. S. Gastrointestinal stromal tumors. *Curr Treat Options Oncol* **1,** 267–273 (2000).
145. Blanke, C. D. *et al.* Phase III randomized, intergroup trial assessing imatinib mesylate at two dose levels in patients with unresectable or metastatic gastrointestinal stromal tumors expressing the kit receptor tyrosine kinase: S0033. *J. Clin. Oncol.* **26,** 626–632 (2008).
146. Dematteo, R. P. *et al.* Adjuvant imatinib mesylate after resection of localised, primary gastrointestinal stromal tumour: a randomised, double-blind, placebo-controlled trial. *Lancet* **373,** 1097–1104 (2009).
147. Joensuu, H. *et al.* Effect of the tyrosine kinase inhibitor STI571 in a patient with a

metastatic gastrointestinal stromal tumor. *N. Engl. J. Med.* **344,** 1052–1056 (2001).
148. Raut, C. P. *et al.* Efficacy and Tolerability of 5-Year Adjuvant Imatinib Treatment for Patients With Resected Intermediate- or High-Risk Primary Gastrointestinal Stromal Tumor: The PERSIST-5 Clinical Trial. *JAMA Oncol* **4,** e184060–e184060 (2018).
149. Demetri, G. D. *et al.* Efficacy and safety of sunitinib in patients with advanced gastrointestinal stromal tumour after failure of imatinib: a randomised controlled trial. *Lancet* **368,** 1329–1338 (2006).
150. Demetri, G. D. *et al.* Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* **381,** 295–302 (2013).
151. Sleijfer, S. *et al.* Pazopanib, a multikinase angiogenesis inhibitor, in patients with relapsed or refractory advanced soft tissue sarcoma: a phase II study from the European organisation for research and treatment of cancer-soft tissue and bone sarcoma group (EORTC study 62043). *J. Clin. Oncol.* **27,** 3126–3132 (2009).
152. van der Graaf, W. T. A. *et al.* Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* **379,** 1879–1886 (2012).
153. Endo, M. & Nielsen, T. O. Pazopanib for metastatic soft-tissue sarcoma. *Lancet* **380,** 801–author reply 801 (2012).
154. Samuels, B. L. *et al.* Results of a prospective phase 2 study of pazopanib in patients with advanced intermediate-grade or high-grade liposarcoma. *Cancer* **123,** 4640–4647 (2017).
155. Mehren, von, M. *et al.* Phase 2 Southwest Oncology Group-directed intergroup trial (S0505) of sorafenib in advanced soft tissue sarcomas. *Cancer* **118,** 770–776 (2012).
156. Santoro, A. *et al.* Phase II prospective study with sorafenib in advanced soft tissue sarcomas after anthracycline-based therapy. *Ann. Oncol.* **24,** 1093–1098 (2013).
157. Mahmood, S. T. *et al.* Phase II study of sunitinib malate, a multitargeted tyrosine kinase inhibitor in patients with relapsed or refractory soft tissue sarcomas. Focus on three prevalent histologies: leiomyosarcoma, liposarcoma and malignant fibrous histiocytoma. *International Journal of Cancer* **129,** 1963–1969 (2011).
158. Mir, O. *et al.* Safety and efficacy of regorafenib in patients with advanced soft tissue sarcoma (REGOSARC): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Oncol.* **17,** 1732–1742 (2016).
159. Wang, J., Coltrera, M. D. & Gown, A. M. Cell proliferation in human soft tissue tumors correlates with platelet-derived growth factor B chain expression: an immunohistochemical and in situ hybridization study. *Clinical Cancer Research* **54,** 560–564 (1994).
160. Penniman, L., Parmar, S. & Patel, K. Olaratumab (Lartruvo): An Innovative Treatment for Soft Tissue Sarcoma. *P T* **43,** 267–270 (2018).
161. Olaratumab for STS Disappoints in Phase III. *Cancer Discov* (2019).
162. Demetri, G. D. *et al.* Results of an international randomized phase III trial of the mammalian target of rapamycin inhibitor ridaforolimus versus placebo to control metastatic sarcomas in patients after benefit from prior chemotherapy. *J. Clin. Oncol.* **31,** 2485–2492 (2013).
163. Louis-Brennetot, C. *et al.* The CDKN2A/CDKN2B/CDK4/CCND1 pathway is pivotal in

well-differentiated and dedifferentiated liposarcoma oncogenesis: an analysis of 104 tumors. *Genes, Chromosomes and Cancer* **50,** 896–907 (2011).
164. Ingham, M. & Schwartz, G. K. Cell-Cycle Therapeutics Come of Age. *J. Clin. Oncol.* **35,** 2949–2959 (2017).
165. Dickson, M. A. *et al.* Phase II trial of the CDK4 inhibitor PD0332991 in patients with advanced CDK4-amplified well-differentiated or dedifferentiated liposarcoma. *J. Clin. Oncol.* **31,** 2024–2028 (2013).
166. Dickson, M. A. *et al.* Progression-Free Survival Among Patients With Well-Differentiated or Dedifferentiated Liposarcoma Treated With CDK4 Inhibitor Palbociclib: A Phase 2 Clinical Trial. *JAMA Oncol* **2,** 937–940 (2016).
167. Bauer, T. M. *et al.* A phase 1 study of MDM2 inhibitor DS-3032b in patients with well/de-differentiated liposarcoma (WD/DD LPS), solid tumors (ST) and lymphomas (L). *JCO* **36,** 11514–11514 (2018).
168. Laroche-Clary, A. *et al.* Combined targeting of MDM2 and CDK4 is synergistic in dedifferentiated liposarcomas. *J Hematol Oncol* **10,** 123 (2017).
169. Laporte, A. N., Ji, J. X., Ma, L., Nielsen, T. O. & Brodin, B. A. Identification of cytotoxic agents disrupting synovial sarcoma oncoprotein interactions by proximity ligation assay. *Oncotarget* **7,** 34384–34394 (2016).
170. Laporte, A. N. *et al.* Death by HDAC Inhibition in Synovial Sarcoma Cells. *Mol Cancer Ther* **16,** 2656–2667 (2017).
171. Bernhart, E. *et al.* Histone deacetylase inhibitors vorinostat and panobinostat induce G1 cell cycle arrest and apoptosis in multidrug resistant sarcoma cell lines. *Oncotarget* **8,** 77254–77267 (2017).
172. Tavallai, S., Hamed, H. A., Grant, S., Poklepovic, A. & Dent, P. Pazopanib and HDAC inhibitors interact to kill sarcoma cells. *Cancer Biol. Ther.* **15,** 578–585 (2014).
173. Laporte, A. N. *et al.* HDAC and Proteasome Inhibitors Synergize to Activate Pro-Apoptotic Factors in Synovial Sarcoma. *PLoS ONE* **12,** e0169407 (2017).
174. Cassier, P. A. *et al.* A phase II trial of panobinostat in patients with advanced pretreated soft tissue sarcoma. A study from the French Sarcoma Group. *Br. J. Cancer* **109,** 909–914 (2013).
175. Chu, Q. S.-C. *et al.* A phase II study of SB939, a novel pan-histone deacetylase inhibitor, in patients with translocation-associated recurrent/metastatic sarcomas-NCIC-CTG IND 200†. *Ann. Oncol.* **26,** 973–981 (2015).
176. Schmitt, T. *et al.* Vorinostat in refractory soft tissue sarcomas - Results of a multi-centre phase II trial of the German Soft Tissue Sarcoma and Bone Tumour Working Group (AIO). *Eur. J. Cancer* **64,** 74–82 (2016).
177. Tang, F., Choy, E., Tu, C., Hornicek, F. & Duan, Z. Therapeutic applications of histone deacetylase inhibitors in sarcoma. *Cancer Treat. Rev.* **59,** 33–45 (2017).
178. Gounder, M. M. *et al.* Phase 2 multicenter study of the EZH2 inhibitor tazemetostat in adults with INI1 negative epithelioid sarcoma (NCT02601950). *JCO* **35,** 11058–11058 (2017).
179. Shankaran, V. *et al.* IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410,** 1107–1111 (2001).
180. Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nature Reviews Immunology* **6,** 715–727

(2006).
181. Hanahan, D. & Weinberg, R. A. The Hallmarks of Cancer. *Cell* **100,** 57–70 (2000).
182. Dunn, G. P., Old, L. J. & Schreiber, R. D. The three Es of cancer immunoediting. *Annu. Rev. Immunol.* **22,** 329–360 (2004).
183. Smyth, M. J., Dunn, G. P. & Schreiber, R. D. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv. Immunol.* **90,** 1–50 (2006).
184. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* **331,** 1565–1570 (2011).
185. Dunn, G. P., Old, L. J. & Schreiber, R. D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21,** 137–148 (2004).
186. BURNET, M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. *Br Med J* **1,** 841–847 (1957).
187. Burnet, F. M. The concept of immunological surveillance. *Prog Exp Tumor Res* **13,** 1–27 (1970).
188. Stutman, O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science* **183,** 534–536 (1974).
189. Stutman, O. Spontaneous tumors in nude mice: effect of the viable yellow gene. *Exp. Cell Biol.* **47,** 129–135 (1979).
190. Stutman, O. Chemical carcinogenesis in nude mice: comparison between nude mice from homozygous matings and heterozygous matings and effect of age and carcinogen dose. *J. Natl. Cancer Inst.* **62,** 353–358 (1979).
191. Hunig, T. T-cell function and specificity in athymic mice. *Immunol. Today* **4,** 84–87 (1983).
192. Maleckar, J. R. & Sherman, L. A. The composition of the T cell receptor repertoire in nude mice. *J. Immunol.* **138,** 3873–3876 (1987).
193. Dighe, A. S., Richards, E., Old, L. J. & Schreiber, R. D. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* **1,** 447–456 (1994).
194. Kaplan, D. H. *et al.* Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proceedings of the National Academy of Sciences* **95,** 7556–7561 (1998).
195. van den Broek, M. E. *et al.* Decreased tumor surveillance in perforin-deficient mice. *Journal of Experimental Medicine* **184,** 1781–1790 (1996).
196. Smyth, M. J. *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *Journal of Experimental Medicine* **191,** 661–668 (2000).
197. Smyth, M. J. *et al.* Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *Journal of Experimental Medicine* **192,** 755–760 (2000).
198. Shinkai, Y. *et al.* RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68,** 855–867 (1992).
199. Penn, I. & Starzl, T. E. Malignant tumors arising de novo in immunosuppressed organ transplant recipients. *Transplantation* **14,** 407–417 (1972).
200. Gatti, R. A. & Good, R. A. Occurrence of malignancy in immunodeficiency diseases. A literature review. *Cancer* **28,** 89–98 (1971).
201. Boshoff, C. & Weiss, R. AIDS-related malignancies. *Nat. Rev. Cancer* **2,** 373–382

(2002).
202. Novellino, L., Castelli, C. & Parmiani, G. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol. Immunother.* **54,** 187–207 (2005).
203. Bodey, B., Siegel, S. E. & Kaiser, H. E. Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. *Anticancer Res.* **20,** 2665–2676 (2000).
204. Escors, D. Tumour immunogenicity, antigen presentation and immunological barriers in cancer immunotherapy. *New J Sci* **2014,** 1–25 (2014).
205. Spurrell, E. L. & Lockley, M. Adaptive immunity in cancer immunology and therapeutics. *Ecancermedicalscience* **8,** 441 (2014).
206. Staveley-O'Carroll, K. *et al.* Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proceedings of the National Academy of Sciences* **95,** 1178–1183 (1998).
207. Kägi, D. *et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369,** 31–37 (1994).
208. Martínez-Lostao, L., Anel, A. & Pardo, J. How Do Cytotoxic Lymphocytes Kill Cancer Cells? *Clin. Cancer Res.* **21,** 5047–5056 (2015).
209. Pardo, J., Balkow, S., Anel, A. & Simon, M. M. Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control. *European Journal of Immunology* **32,** 2881–2887 (2002).
210. Duluc, D. *et al.* Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *International Journal of Cancer* **125,** 367–373 (2009).
211. Doyle, C. & Strominger, J. L. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330,** 256–259 (1987).
212. Swain, S. L., McKinstry, K. K. & Strutt, T. M. Expanding roles for CD4⁺ T cells in immunity to viruses. *Nature Reviews Immunology* **12,** 136–148 (2012).
213. Mosmann, T. R. & Coffman, R. L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7,** 145–173 (1989).
214. Coffman, R. L. & Mosmann, T. R. CD4+ T-cell subsets: regulation of differentiation and function. *Res. Immunol.* **142,** 7–9 (1991).
215. Romagnani, S. Understanding the role of Th1/Th2 cells in infection. *Trends Microbiol.* **4,** 470–473 (1996).
216. Romagnani, S. Th1/Th2 cells. *Inflamm. Bowel Dis.* **5,** 285–294 (1999).
217. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **155,** 1151–1164 (1995).
218. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nature Reviews Immunology* **6,** 295–307 (2006).
219. Curiel, T. J. Tregs and rethinking cancer immunotherapy. *J. Clin. Invest.* **117,** 1167–1174 (2007).
220. Kim, C. H. FOXP3 and its role in the immune system. *Adv. Exp. Med. Biol.* **665,** 17–29 (2009).
221. Piccirillo, C. A. & Shevach, E. M. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J. Immunol.* **167,** 1137–1140 (2001).

222. Levings, M. K., Sangregorio, R. & Roncarolo, M. G. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *Journal of Experimental Medicine* **193,** 1295–1302 (2001).
223. Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19,** 683–765 (2001).
224. Su, L.-C., Liu, X.-Y., Huang, A.-F. & Xu, W.-D. Emerging role of IL-35 in inflammatory autoimmune diseases. *Autoimmun Rev* **17,** 665–673 (2018).
225. Collison, L. W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450,** 566–569 (2007).
226. Marie, J. C., Letterio, J. J., Gavin, M. & Rudensky, A. Y. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *Journal of Experimental Medicine* **201,** 1061–1067 (2005).
227. Schmidt, A., Oberle, N. & Krammer, P. H. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol* **3,** 51 (2012).
228. Cassard, L. *et al.* Fc gamma receptors and cancer. *Springer Semin. Immunopathol.* **28,** 321–328 (2006).
229. DiLillo, D. J., Yanaba, K. & Tedder, T. F. B cells are required for optimal CD4+ and CD8+ T cell tumor immunity: therapeutic B cell depletion enhances B16 melanoma growth in mice. *J. Immunol.* **184,** 4006–4016 (2010).
230. Reuschenbach, M., Knebel Doeberitz, von, M. & Wentzensen, N. A systematic review of humoral immune responses against tumor antigens. *Cancer Immunol. Immunother.* **58,** 1535–1544 (2009).
231. Li, Q., Teitz-Tennenbaum, S., Donald, E. J., Li, M. & Chang, A. E. In vivo sensitized and in vitro activated B cells mediate tumor regression in cancer adoptive immunotherapy. *J. Immunol.* **183,** 3195–3203 (2009).
232. Kemp, T. J., Moore, J. M. & Griffith, T. S. Human B cells express functional TRAIL/Apo-2 ligand after CpG-containing oligodeoxynucleotide stimulation. *J. Immunol.* **173,** 892–899 (2004).
233. Jahrsdörfer, B. *et al.* B-chronic lymphocytic leukemia cells and other B cells can produce granzyme B and gain cytotoxic potential after interleukin-21-based activation. *Blood* **108,** 2712–2719 (2006).
234. Rosser, E. C. & Mauri, C. Regulatory B cells: origin, phenotype, and function. *Immunity* **42,** 607–612 (2015).
235. Woo, S.-R., Corrales, L. & Gajewski, T. F. Innate immune recognition of cancer. *Annu. Rev. Immunol.* **33,** 445–474 (2015).
236. Paul, S. & Lal, G. The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Front Immunol* **8,** 1124 (2017).
237. Raulet, D. H. Roles of the NKG2D immunoreceptor and its ligands. *Nature Reviews Immunology* **3,** 781–790 (2003).
238. Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285,** 727–729 (1999).
239. Cerwenka, A. *et al.* Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* **12,** 721–727 (2000).
240. Diefenbach, A., Jamieson, A. M., Liu, S. D., Shastri, N. & Raulet, D. H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and

macrophages. *Nat. Immunol.* **1,** 119–126 (2000).
241. Kruse, P. H., Matta, J., Ugolini, S. & Vivier, E. Natural cytotoxicity receptors and their ligands. *Immunol. Cell Biol.* **92,** 221–229 (2014).
242. Veillette, A. Immune regulation by SLAM family receptors and SAP-related adaptors. *Nature Reviews Immunology* **6,** 56–66 (2006).
243. Chen, C.-W. *et al.* Cutting Edge: 2B4-Mediated Coinhibition of CD4+ T Cells Underlies Mortality in Experimental Sepsis. *J. Immunol.* **199,** 1961–1966 (2017).
244. Tangye, S. G., Cherwinski, H., Lanier, L. L. & Phillips, J. H. 2B4-mediated activation of human natural killer cells. *Mol. Immunol.* **37,** 493–501 (2000).
245. Rah, S.-Y., Kwak, J.-Y., Chung, Y.-J. & Kim, U.-H. ADP-ribose/TRPM2-mediated Ca2+ signaling is essential for cytolytic degranulation and antitumor activity of natural killer cells. *Sci Rep* **5,** 9482 (2015).
246. Welte, S., Kuttruff, S., Waldhauer, I. & Steinle, A. Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction. *Nat. Immunol.* **7,** 1334–1342 (2006).
247. Davis, J. E., Smyth, M. J. & Trapani, J. A. Granzyme A and B-deficient killer lymphocytes are defective in eliciting DNA fragmentation but retain potent in vivo anti-tumor capacity. *European Journal of Immunology* **31,** 39–47 (2001).
248. Hayakawa, Y. *et al.* Cutting edge: tumor rejection mediated by NKG2D receptor-ligand interaction is dependent upon perforin. *J. Immunol.* **169,** 5377–5381 (2002).
249. Osińska, I., Popko, K. & Demkow, U. Perforin: an important player in immune response. *Cent Eur J Immunol* **39,** 109–115 (2014).
250. Sonar, S. & Lal, G. Role of Tumor Necrosis Factor Superfamily in Neuroinflammation and Autoimmunity. *Front Immunol* **6,** 364 (2015).
251. Johnsen, A. C. *et al.* Regulation of APO-2 ligand/trail expression in NK cells-involvement in NK cell-mediated cytotoxicity. *Cytokine* **11,** 664–672 (1999).
252. Lee, R. K., Spielman, J., Zhao, D. Y., Olsen, K. J. & Podack, E. R. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells. *J. Immunol.* **157,** 1919–1925 (1996).
253. Zamai, L. *et al.* Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *Journal of Experimental Medicine* **188,** 2375–2380 (1998).
254. Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331,** 44–49 (2011).
255. Castro, F., Cardoso, A. P., Gonçalves, R. M., Serre, K. & Oliveira, M. J. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Front Immunol* **9,** 847 (2018).
256. O'Sullivan, T. *et al.* Cancer immunoediting by the innate immune system in the absence of adaptive immunity. *J. Exp. Med.* **209,** 1869–1882 (2012).
257. Peter, M. E. & Krammer, P. H. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ.* **10,** 26–35 (2003).
258. Braumüller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494,** 361–365 (2013).
259. Ribeiro, V. S. G. *et al.* Cutting edge: Thymic NK cells develop independently from T cell precursors. *J. Immunol.* **185,** 4993–4997 (2010).

260. Beetz, S. *et al.* Innate immune functions of human gammadelta T cells. *Immunobiology* **213,** 173–182 (2008).
261. Hayday, A. C. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol.* **18,** 975–1026 (2000).
262. Kabelitz, D., Glatzel, A. & Wesch, D. Antigen recognition by human gammadelta T lymphocytes. *Int. Arch. Allergy Immunol.* **122,** 1–7 (2000).
263. Wrobel, P. *et al.* Lysis of a broad range of epithelial tumour cells by human gamma delta T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. *Scand. J. Immunol.* **66,** 320–328 (2007).
264. Oliaro, J. *et al.* Vgamma9Vdelta2 T cells use a combination of mechanisms to limit the spread of the pathogenic bacteria Brucella. *J. Leukoc. Biol.* **77,** 652–660 (2005).
265. Koizumi, H. *et al.* Expression of perforin and serine esterases by human gamma/delta T cells. *Journal of Experimental Medicine* **173,** 499–502 (1991).
266. Paul, S. & Lal, G. Regulatory and effector functions of gamma-delta (γδ) T cells and their therapeutic potential in adoptive cellular therapy for cancer. *International Journal of Cancer* **139,** 976–985 (2016).
267. Ye, J. *et al.* Specific recruitment of γδ regulatory T cells in human breast cancer. *Clinical Cancer Research* **73,** 6137–6148 (2013).
268. Lafont, V. *et al.* Plasticity of γδ T Cells: Impact on the Anti-Tumor Response. *Front Immunol* **5,** 622 (2014).
269. Presti, Lo, E., Dieli, F. & Meraviglia, S. Tumor-Infiltrating γδ T Lymphocytes: Pathogenic Role, Clinical Significance, and Differential Programing in the Tumor Microenvironment. *Front Immunol* **5,** 607 (2014).
270. Zhao, Y., Niu, C. & Cui, J. Gamma-delta (γδ) T cells: friend or foe in cancer development? *J Transl Med* **16,** 3 (2018).
271. Van Overmeire, E., Laoui, D., Keirsse, J., Van Ginderachter, J. A. & Sarukhan, A. Mechanisms driving macrophage diversity and specialization in distinct tumor microenvironments and parallelisms with other tissues. *Front Immunol* **5,** 127 (2014).
272. Bingle, L., Brown, N. J. & Lewis, C. E. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of Pathology* **196,** 254–265 (2002).
273. Zhang, Q.-W. *et al.* Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. *PLoS ONE* **7,** e50946 (2012).
274. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S. & Ruco, L. The origin and function of tumor-associated macrophages. *Immunol. Today* **13,** 265–270 (1992).
275. Atri, C., Guerfali, F. Z. & Laouini, D. Role of Human Macrophage Polarization in Inflammation during Infectious Diseases. *Int J Mol Sci* **19,** 1801 (2018).
276. MACKANESS, G. B. THE IMMUNOLOGICAL BASIS OF ACQUIRED CELLULAR RESISTANCE. *Journal of Experimental Medicine* **120,** 105–120 (1964).
277. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25,** 677–686 (2004).
278. Hong, I.-S. Stimulatory versus suppressive effects of GM-CSF on tumor progression in multiple cancer types. *Exp. Mol. Med.* **48,** e242–e242 (2016).
279. Beyer, M. *et al.* High-resolution transcriptome of human macrophages. *PLoS ONE* **7,** e45466 (2012).

280. Italiani, P. *et al.* Transcriptomic profiling of the development of the inflammatory response in human monocytes in vitro. *PLoS ONE* **9,** e87680 (2014).
281. Martinez, F. O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol.* **177,** 7303–7311 (2006).
282. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* **11,** 889–896 (2010).
283. Gordon, S. Alternative activation of macrophages. *Nature Reviews Immunology* **3,** 23–35 (2003).
284. Vogel, D. Y. S. *et al.* Human macrophage polarization in vitro: maturation and activation methods compared. *Immunobiology* **219,** 695–703 (2014).
285. Ferracini, M., Rios, F. J. O., Pecenin, M. & Jancar, S. Clearance of apoptotic cells by macrophages induces regulatory phenotype and involves stimulation of CD36 and platelet-activating factor receptor. *Mediators Inflamm.* **2013,** 950273–8 (2013).
286. Xu, W. *et al.* IL-10-producing macrophages preferentially clear early apoptotic cells. *Blood* **107,** 4930–4937 (2006).
287. Murray, P. J. *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* **41,** 14–20 (2014).
288. Ferrante, C. J. *et al.* The adenosine-dependent angiogenic switch of macrophages to an M2-like phenotype is independent of interleukin-4 receptor alpha (IL-4Rα) signaling. *Inflammation* **36,** 921–931 (2013).
289. Wang, Q. *et al.* Fra-1 protooncogene regulates IL-6 expression in macrophages and promotes the generation of M2d macrophages. *Cell Res.* **20,** 701–712 (2010).
290. Duluc, D. *et al.* Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood* **110,** 4319–4330 (2007).
291. Tarique, A. A. *et al.* Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am. J. Respir. Cell Mol. Biol.* **53,** 676–688 (2015).
292. Verreck, F. A. W. *et al.* Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proceedings of the National Academy of Sciences* **101,** 4560–4565 (2004).
293. Verreck, F. A. W., de Boer, T., Langenberg, D. M. L., van der Zanden, L. & Ottenhoff, T. H. M. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. *J. Leukoc. Biol.* **79,** 285–293 (2006).
294. Guiducci, C., Vicari, A. P., Sangaletti, S., Trinchieri, G. & Colombo, M. P. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Clinical Cancer Research* **65,** 3437–3446 (2005).
295. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* **3,** 991–998 (2002).
296. Uyttenhove, C. *et al.* Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat. Med.* **9,** 1269–1274 (2003).

297. Urban, J. L., Holland, J. M., Kripke, M. L. & Schreiber, H. Immunoselection of tumor cell variants by mice suppressed with ultraviolet radiation. *Journal of Experimental Medicine* **156,** 1025–1041 (1982).
298. Beatty, G. L. & Gladney, W. L. Immune escape mechanisms as a guide for cancer immunotherapy. *Clin. Cancer Res.* **21,** 687–692 (2015).
299. Garrido, F. *et al.* Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol. Today* **18,** 89–95 (1997).
300. Hicklin, D. J., Marincola, F. M. & Ferrone, S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol Med Today* **5,** 178–186 (1999).
301. Restifo, N. P. *et al.* Identification of human cancers deficient in antigen processing. *Journal of Experimental Medicine* **177,** 265–272 (1993).
302. Rotem-Yehudar, R., Groettrup, M., Soza, A., Kloetzel, P. M. & Ehrlich, R. LMP-associated proteolytic activities and TAP-dependent peptide transport for class 1 MHC molecules are suppressed in cell lines transformed by the highly oncogenic adenovirus 12. *Journal of Experimental Medicine* **183,** 499–514 (1996).
303. Johnsen, A. K., Templeton, D. J., Sy, M. & Harding, C. V. Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis. *J. Immunol.* **163,** 4224–4231 (1999).
304. Maeurer, M. J. *et al.* Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. *J. Clin. Invest.* **98,** 1633–1641 (1996).
305. Sharma, P., Hu-Lieskovan, S., Wargo, J. A. & Ribas, A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* **168,** 707–723 (2017).
306. Turcotte, S. *et al.* Tumor MHC class I expression improves the prognostic value of T-cell density in resected colorectal liver metastases. *Cancer Immunol Res* **2,** 530–537 (2014).
307. Zhang, J. *et al.* Loss of expression of MHC class I-related chain A (MICA) is a frequent event and predicts poor survival in patients with hepatocellular carcinoma. *Int J Clin Exp Pathol* **7,** 3123–3131 (2014).
308. Zhao, Y. *et al.* Prognostic value of MICA/B in cancers: a systematic review and meta-analysis. *Oncotarget* **8,** 96384–96395 (2017).
309. Baumeister, S. H., Freeman, G. J., Dranoff, G. & Sharpe, A. H. Coinhibitory Pathways in Immunotherapy for Cancer. *Annu. Rev. Immunol.* **34,** 539–573 (2016).
310. Hurwitz, A. A., Kwon, E. D. & van Elsas, A. Costimulatory wars: the tumor menace. *Current Opinion in Immunology* **12,** 589–596 (2000).
311. Zippelius, A. *et al.* Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. *Clinical Cancer Research* **64,** 2865–2873 (2004).
312. Baruah, P. *et al.* Decreased levels of alternative co-stimulatory receptors OX40 and 4-1BB characterise T cells from head and neck cancer patients. *Immunobiology* **217,** 669–675 (2012).
313. Chong, H., Hutchinson, G., Hart, I. R. & Vile, R. G. Expression of co-stimulatory molecules by tumor cells decreases tumorigenicity but may also reduce systemic

antitumor immunity. *Hum. Gene Ther.* **7,** 1771–1779 (1996).
314. Airoldi, I. *et al.* Expression of costimulatory molecules in human neuroblastoma. Evidence that CD40+ neuroblastoma cells undergo apoptosis following interaction with CD40L. *Br. J. Cancer* **88,** 1527–1536 (2003).
315. Thompson, C. B. & Allison, J. P. The emerging role of CTLA-4 as an immune attenuator. *Immunity* **7,** 445–450 (1997).
316. Huang, P.-Y. *et al.* Tumor CTLA-4 overexpression predicts poor survival in patients with nasopharyngeal carcinoma. *Oncotarget* **7,** 13060–13068 (2016).
317. Santoni, G. *et al.* High CTLA-4 expression correlates with poor prognosis in thymoma patients. *Oncotarget* **9,** 16665–16677 (2018).
318. Jacobs, J. F. M., Nierkens, S., Figdor, C. G., de Vries, I. J. M. & Adema, G. J. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol.* **13,** e32–42 (2012).
319. Lind, M. H. *et al.* Tumor necrosis factor receptor 1-mediated signaling is required for skin cancer development induced by NF-kappaB inhibition. *Proceedings of the National Academy of Sciences* **101,** 4972–4977 (2004).
320. Lin, E. Y., Gouon-Evans, V., Nguyen, A. V. & Pollard, J. W. The macrophage growth factor CSF-1 in mammary gland development and tumor progression. *J Mammary Gland Biol Neoplasia* **7,** 147–162 (2002).
321. Maeda, H. & Shiraishi, A. TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J. Immunol.* **156,** 73–78 (1996).
322. Klein, S. C., Jücker, M., Abts, H. & Tesch, H. IL6 and IL6 receptor expression in Burkitt's lymphoma and lymphoblastoid cell lines: promotion of IL6 receptor expression by EBV. *Hematol Oncol* **13,** 121–130 (1995).
323. Matsuda, M. *et al.* Interleukin 10 pretreatment protects target cells from tumor- and allo-specific cytotoxic T cells and downregulates HLA class I expression. *Journal of Experimental Medicine* **180,** 2371–2376 (1994).
324. Curiel, T. J. *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* **10,** 942–949 (2004).
325. Lee, I. *et al.* Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. *Journal of Experimental Medicine* **201,** 1037–1044 (2005).
326. Yokokawa, J. *et al.* Enhanced functionality of CD4+CD25(high)FoxP3+ regulatory T cells in the peripheral blood of patients with prostate cancer. *Clin. Cancer Res.* **14,** 1032–1040 (2008).
327. Gasparoto, T. H. *et al.* Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment. *Cancer Immunol. Immunother.* **59,** 819–828 (2010).
328. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* **23,** 549–555 (2002).
329. Wang, H. *et al.* Interactions between colon cancer cells and tumor-infiltrated macrophages depending on cancer cell-derived colony stimulating factor 1. *Oncoimmunology* **5,** e1122157 (2016).

330. Li, Y. *et al.* Chemokines CCL2, 3, 14 stimulate macrophage bone marrow homing, proliferation, and polarization in multiple myeloma. *Oncotarget* **6,** 24218–24229 (2015).
331. Ambade, A. *et al.* Hepatocellular carcinoma is accelerated by NASH involving M2 macrophage polarization mediated by hif-1αinduced IL-10. *Oncoimmunology* **5,** e1221557 (2016).
332. Zhao, P. *et al.* Response gene to complement 32 (RGC-32) expression on M2-polarized and tumor-associated macrophages is M-CSF-dependent and enhanced by tumor-derived IL-4. *Cell. Mol. Immunol.* **12,** 692–699 (2015).
333. Couzin-Frankel, J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* **342,** 1432–1433 (2013).
334. Goldstein, D. & Laszlo, J. The role of interferon in cancer therapy: a current perspective. *CA Cancer J Clin* **38,** 258–277 (1988).
335. Ratain, M. J. *et al.* Treatment of hairy cell leukemia with recombinant alpha 2 interferon. *Blood* **65,** 644–648 (1985).
336. Amato, R. Modest effect of interferon alfa on metastatic renal-cell carcinoma. *Lancet* **353,** 6–7 (1999).
337. Krown, S. E. AIDS-associated Kaposi's sarcoma: is there still a role for interferon alfa? *Cytokine Growth Factor Rev.* **18,** 395–402 (2007).
338. Sckisel, G. D. *et al.* Out-of-Sequence Signal 3 Paralyzes Primary CD4(+) T-Cell-Dependent Immunity. *Immunity* **43,** 240–250 (2015).
339. Levin, A. M. *et al.* Exploiting a natural conformational switch to engineer an interleukin-2 'superkine'. *Nature* **484,** 529–533 (2012).
340. Boyman, O., Kovar, M., Rubinstein, M. P., Surh, C. D. & Sprent, J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* **311,** 1924–1927 (2006).
341. Senzer, N. N. *et al.* Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J. Clin. Oncol.* **27,** 5763–5771 (2009).
342. Kochenderfer, J. N. *et al.* Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. *J. Clin. Oncol.* **33,** 540–549 (2015).
343. Jiang, S.-S. *et al.* A phase I clinical trial utilizing autologous tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma. *Oncotarget* **6,** 41339–41349 (2015).
344. Currie, G. A. Eighty years of immunotherapy: a review of immunological methods used for the treatment of human cancer. *Br. J. Cancer* **26,** 141–153 (1972).
345. Banchereau, J. & Palucka, K. Immunotherapy: Cancer vaccines on the move. *Nat Rev Clin Oncol* **15,** 9–10 (2018).
346. Guo, C. *et al.* Therapeutic cancer vaccines: past, present, and future. *Adv. Cancer Res.* **119,** 421–475 (2013).
347. Carreno, B. M. *et al.* Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science* **348,** 803–808 (2015).
348. Mohammed, S., Bakshi, N., Chaudri, N., Akhter, J. & Akhtar, M. Cancer Vaccines: Past, Present, and Future. *Adv Anat Pathol* **23,** 180–191 (2016).

349. Ott, P. A. *et al.* An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **547,** 217–221 (2017).
350. Sahin, U. *et al.* Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **547,** 222–226 (2017).
351. MITCHISON, N. A. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *Journal of Experimental Medicine* **102,** 157–177 (1955).
352. Greenberg, P. D., Reusser, P., Goodrich, J. M. & Riddell, S. R. Development of a treatment regimen for human cytomegalovirus (CMV) infection in bone marrow transplantation recipients by adoptive transfer of donor-derived CMV-specific T cell clones expanded in vitro. *Ann. N. Y. Acad. Sci.* **636,** 184–195 (1991).
353. Brown, C. E. & Mackall, C. L. CAR T cell therapy: inroads to response and resistance. *Nature Reviews Immunology* **19,** 73–74 (2019).
354. Sotillo, E. *et al.* Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy. *Cancer Discovery* **5,** 1282–1295 (2015).
355. Bagashev, A. *et al.* CD19 Alterations Emerging after CD19-Directed Immunotherapy Cause Retention of the Misfolded Protein in the Endoplasmic Reticulum. *Mol. Cell. Biol.* **38,** 4134 (2018).
356. Orlando, E. J. *et al.* Genetic mechanisms of target antigen loss in CAR19 therapy of acute lymphoblastic leukemia. *Nat. Med.* **24,** 1504–1506 (2018).
357. Neelapu, S. S. *et al.* Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N. Engl. J. Med.* **377,** 2531–2544 (2017).
358. Lamprecht, M. & Dansereau, C. CAR T-Cell Therapy: Update on the State of the Science. *Clin J Oncol Nurs* **23,** 6–12 (2019).
359. Fry, T. J. *et al.* CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat. Med.* **24,** 20–28 (2018).
360. Qin, H. *et al.* Preclinical Development of Bivalent Chimeric Antigen Receptors Targeting Both CD19 and CD22. *Mol Ther Oncolytics* **11,** 127–137 (2018).
361. Hodi, F. S. *et al.* Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proceedings of the National Academy of Sciences* **100,** 4712–4717 (2003).
362. Phan, G. Q. *et al.* Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proceedings of the National Academy of Sciences* **100,** 8372–8377 (2003).
363. Hodi, F. S. *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* **363,** 711–723 (2010).
364. Burugu, S., Dancsok, A. R. & Nielsen, T. O. Emerging targets in cancer immunotherapy. *Semin. Cancer Biol.* (2017). doi:10.1016/j.semcancer.2017.10.001
365. Nishimura, H., Nose, M., Hiai, H., Minato, N. & Honjo, T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* **11,** 141–151 (1999).
366. Greenwald, R. J., Freeman, G. J. & Sharpe, A. H. The B7 family revisited. *Annu. Rev. Immunol.* **23,** 515–548 (2005).
367. Thibult, M.-L. *et al.* PD-1 is a novel regulator of human B-cell activation. *Int. Immunol.*

25, 129–137 (2013).
368. Lim, T. S. *et al.* PD-1 expression on dendritic cells suppresses CD8+ T cell function and antitumor immunity. *Oncoimmunology* **5,** e1085146 (2016).
369. Rodrigues, C. P. *et al.* Tolerogenic IDO(+) Dendritic Cells Are Induced by PD-1-Expressing Mast Cells. *Front Immunol* **7,** 9 (2016).
370. Dong, H., Zhu, G., Tamada, K. & Chen, L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat. Med.* **5,** 1365–1369 (1999).
371. Freeman, G. J. *et al.* Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *Journal of Experimental Medicine* **192,** 1027–1034 (2000).
372. Latchman, Y. *et al.* PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* **2,** 261–268 (2001).
373. Tseng, S. Y. *et al.* B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *Journal of Experimental Medicine* **193,** 839–846 (2001).
374. Brown, J. A. *et al.* Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J. Immunol.* **170,** 1257–1266 (2003).
375. Chemnitz, J. M., Parry, R. V., Nichols, K. E., June, C. H. & Riley, J. L. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J. Immunol.* **173,** 945–954 (2004).
376. Parry, R. V. *et al.* CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol. Cell. Biol.* **25,** 9543–9553 (2005).
377. Patsoukis, N. *et al.* Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation. *Sci Signal* **5,** ra46–ra46 (2012).
378. Sheppard, K.-A. *et al.* PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett.* **574,** 37–41 (2004).
379. Nurieva, R. *et al.* T-cell tolerance or function is determined by combinatorial costimulatory signals. *The EMBO Journal* **25,** 2623–2633 (2006).
380. Inman, B. A. *et al.* PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata: associations with localized stage progression. *Cancer* **109,** 1499–1505 (2007).
381. Ahmadzadeh, M. *et al.* Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* **114,** 1537–1544 (2009).
382. Hino, R. *et al.* Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* **116,** 1757–1766 (2010).
383. Hamanishi, J. *et al.* Safety and Antitumor Activity of Anti-PD-1 Antibody, Nivolumab, in Patients With Platinum-Resistant Ovarian Cancer. *J. Clin. Oncol.* **33,** 4015–4022 (2015).
384. Kim, J. W. *et al.* Prognostic implications of immunosuppressive protein expression in tumors as well as immune cell infiltration within the tumor microenvironment in gastric cancer. *Gastric Cancer* **19,** 42–52 (2016).

385. Honda, Y. *et al.* Infiltration of PD-1-positive cells in combination with tumor site PD-L1 expression is a positive prognostic factor in cutaneous angiosarcoma. *Oncoimmunology* **6,** e1253657 (2017).
386. Zhang, Y. *et al.* Prognostic significance of programmed cell death 1 (PD-1) or PD-1 ligand 1 (PD-L1) Expression in epithelial-originated cancer: a meta-analysis. *Medicine (Baltimore)* **94,** e515 (2015).
387. Nishimura, H. *et al.* Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* **291,** 319–322 (2001).
388. Wang, J. *et al.* Establishment of NOD-Pdcd1-/- mice as an efficient animal model of type I diabetes. *Proceedings of the National Academy of Sciences* **102,** 11823–11828 (2005).
389. Lucas, J. A. *et al.* Programmed death ligand 1 regulates a critical checkpoint for autoimmune myocarditis and pneumonitis in MRL mice. *J. Immunol.* **181,** 2513–2521 (2008).
390. Iwai, Y. *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proceedings of the National Academy of Sciences* **99,** 12293–12297 (2002).
391. Iwai, Y., Terawaki, S. & Honjo, T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int. Immunol.* **17,** 133–144 (2005).
392. Curiel, T. J. *et al.* Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat. Med.* **9,** 562–567 (2003).
393. Hirano, F. *et al.* Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Clinical Cancer Research* **65,** 1089–1096 (2005).
394. Strome, S. E. *et al.* B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Clinical Cancer Research* **63,** 6501–6505 (2003).
395. Brahmer, J. R. *et al.* Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J. Clin. Oncol.* **28,** 3167–3175 (2010).
396. Topalian, S. L., Drake, C. G. & Pardoll, D. M. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* **27,** 450–461 (2015).
397. Topalian, S. L. *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* **366,** 2443–2454 (2012).
398. Larkin, J. *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N. Engl. J. Med.* **373,** 23–34 (2015).
399. Tang, J. *et al.* Trial watch: The clinical trial landscape for PD1/PDL1 immune checkpoint inhibitors. *Nat Rev Drug Discov* **17,** 854–855 (2018).
400. Wolchok, J. D. *et al.* Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol.* **11,** 155–164 (2010).
401. Anderson, A. C., Joller, N. & Kuchroo, V. K. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity* **44,** 989–1004 (2016).
402. Scurr, M. *et al.* Highly prevalent colorectal cancer-infiltrating LAP^{+} Foxp3^{-} T cells exhibit more potent immunosuppressive activity than Foxp3^{+} regulatory T cells.

Mucosal Immunol **7,** 428–439 (2014).
403. Zhang, Q. & Vignali, D. A. A. Co-stimulatory and Co-inhibitory Pathways in Autoimmunity. *Immunity* **44,** 1034–1051 (2016).
404. Williams, J. B. *et al.* The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8+ T cells in the tumor microenvironment. *J. Exp. Med.* **214,** 381–400 (2017).
405. Casati, C. *et al.* Soluble human LAG-3 molecule amplifies the in vitro generation of type 1 tumor-specific immunity. *Clinical Cancer Research* **66,** 4450–4460 (2006).
406. Shapiro, M. *et al.* Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia. *Haematologica* **102,** 874–882 (2017).
407. Tassi, E. *et al.* Early Effector T Lymphocytes Coexpress Multiple Inhibitory Receptors in Primary Non-Small Cell Lung Cancer. *Clinical Cancer Research* **77,** 851–861 (2017).
408. Bottai, G. *et al.* An immune stratification reveals a subset of PD-1/LAG-3 double-positive triple-negative breast cancers. *Breast Cancer Res.* **18,** 121 (2016).
409. Burugu, S., Gao, D., Leung, S., Chia, S. K. & Nielsen, T. O. LAG-3+ tumor infiltrating lymphocytes in breast cancer: clinical correlates and association with PD-1/PD-L1+ tumors. *Ann. Oncol.* **28,** 2977–2984 (2017).
410. Woo, S.-R. *et al.* Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Clinical Cancer Research* **72,** 917–927 (2012).
411. Huang, R.-Y. *et al.* LAG3 and PD1 co-inhibitory molecules collaborate to limit CD8+ T cell signaling and dampen antitumor immunity in a murine ovarian cancer model. *Oncotarget* **6,** 27359–27377 (2015).
412. Huang, R.-Y., Francois, A., McGray, A. R., Miliotto, A. & Odunsi, K. Compensatory upregulation of PD-1, LAG-3, and CTLA-4 limits the efficacy of single-agent checkpoint blockade in metastatic ovarian cancer. *Oncoimmunology* **6,** e1249561 (2017).
413. Brignone, C., Grygar, C., Marcu, M., Schäkel, K. & Triebel, F. A soluble form of lymphocyte activation gene-3 (IMP321) induces activation of a large range of human effector cytotoxic cells. *J. Immunol.* **179,** 4202–4211 (2007).
414. Brignone, C., Escudier, B., Grygar, C., Marcu, M. & Triebel, F. A phase I pharmacokinetic and biological correlative study of IMP321, a novel MHC class II agonist, in patients with advanced renal cell carcinoma. *Clin. Cancer Res.* **15,** 6225–6231 (2009).
415. Duhoux, F. P. *et al.* Combination of paclitaxel and a LAG-3 fusion protein (eftilagimod alpha), as a first-line chemoimmunotherapy in patients with metastatic breast carcinoma (MBC): Final results from the run-in phase of a placebo-controlled randomized phase II. *JCO* **36,** 1050–1050 (2018).
416. Ascierto, P. *et al. LBA18Efficacy of BMS-986016, a monoclonal antibody that targets lymphocyte activation gene-3 (LAG-3), in combination with nivolumab in pts with melanoma who progressed during prior anti–PD-1/PD-L1 therapy (mel prior IO) in all-comer and biomarker-enriched populations*. **28,**
417. Hong, D. S. *et al.* Phase I/II study of LAG525 ± spartalizumab (PDR001) in patients (pts) with advanced malignancies. *JCO* **36,** 3012–3012 (2018).
418. Monney, L. *et al.* Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* **415,** 536–541 (2002).

419. Gao, X. *et al.* TIM-3 Expression Characterizes Regulatory T Cells in Tumor Tissues and Is Associated with Lung Cancer Progression. *PLoS ONE* **7,** e30676 (2012).
420. Yan, J. *et al.* Tim-3 expression defines regulatory T cells in human tumors. *PLoS ONE* **8,** e58006 (2013).
421. Gleason, M. K. *et al.* Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. *Blood* **119,** 3064–3072 (2012).
422. Ndhlovu, L. C. *et al.* Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. *Blood* **119,** 3734–3743 (2012).
423. Anderson, A. C. *et al.* Promotion of Tissue Inflammation by the Immune Receptor Tim-3 Expressed on Innate Immune Cells. *Science* **318,** 1141–1143 (2007).
424. Wada, J. & Kanwar, Y. S. Identification and characterization of galectin-9, a novel beta-galactoside-binding mammalian lectin. *J. Biol. Chem.* **272,** 6078–6086 (1997).
425. Zhu, C. *et al.* The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* **6,** 1245–1252 (2005).
426. Sabatos, C. A. *et al.* Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat. Immunol.* **4,** 1102–1110 (2003).
427. Sánchez-Fueyo, A. *et al.* Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat. Immunol.* **4,** 1093–1101 (2003).
428. Jones, R. B. *et al.* Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J. Exp. Med.* **205,** 2763–2779 (2008).
429. Hafler, D. A. & Kuchroo, V. TIMs: central regulators of immune responses. *J. Exp. Med.* **205,** 2699–2701 (2008).
430. Golden-Mason, L. *et al.* Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J. Virol.* **83,** 9122–9130 (2009).
431. Takamura, S. *et al.* Premature terminal exhaustion of Friend virus-specific effector CD8+ T cells by rapid induction of multiple inhibitory receptors. *J. Immunol.* **184,** 4696–4707 (2010).
432. Jin, H.-T. *et al.* Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 14733–14738 (2010).
433. Fourcade, J. *et al.* Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J. Exp. Med.* **207,** 2175–2186 (2010).
434. Baitsch, L. *et al.* Exhaustion of tumor-specific $CD8^+$ T cells in metastases from melanoma patients. *J. Clin. Invest.* **121,** 2350–2360 (2011).
435. Yang, Z.-Z. *et al.* IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J. Clin. Invest.* **122,** 1271–1282 (2012).
436. Zong, Y. & He, S. Identification of Co-inhibitory Receptors PD-1 and TIM-3 on T Cells from Gastric Cancer Patients. *Immunotherapy: Open Access* **01,** (2016).
437. Lu, X. *et al.* Tumor antigen-specific CD8(+) T cells are negatively regulated by PD-1 and Tim-3 in human gastric cancer. *Cell. Immunol.* **313,** 43–51 (2017).
438. Burugu, S., Gao, D., Leung, S., Chia, S. K. & Nielsen, T. O. TIM-3 expression in breast

cancer. *Oncoimmunology* **7,** e1502128 (2018).
439. Linedale, R. *et al.* Elevated frequencies of CD8 T cells expressing PD-1, CTLA-4 and Tim-3 within tumour from perineural squamous cell carcinoma patients. *PLoS ONE* **12,** e0175755 (2017).
440. Li, Z., Liu, X., Guo, R. & Wang, P. TIM-3 plays a more important role than PD-1 in the functional impairments of cytotoxic T cells of malignant Schwannomas. *Tumour Biol.* **39,** 1010428317698352 (2017).
441. Shayan, G. *et al.* Adaptive resistance to anti-PD1 therapy by Tim-3 upregulation is mediated by the PI3K-Akt pathway in head and neck cancer. *Oncoimmunology* **6,** e1261779 (2017).
442. Ceresoli, G. L. & Mantovani, A. Immune checkpoint inhibitors in malignant pleural mesothelioma. *Lancet Oncol.* **18,** 559–561 (2017).
443. Sakuishi, K. *et al.* Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J. Exp. Med.* **208,** 1331–1331 (2011).
444. Zhou, Q. *et al.* Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* **117,** 4501–4510 (2011).
445. Gautron, A.-S., Dominguez-Villar, M., de Marcken, M. & Hafler, D. A. Enhanced suppressor function of TIM-3+ FoxP3+ regulatory T cells. *European Journal of Immunology* **44,** 2703–2711 (2014).
446. Gupta, S. *et al.* Allograft rejection is restrained by short-lived TIM-3+PD-1+Foxp3+ Tregs. *J. Clin. Invest.* **122,** 2395–2404 (2012).
447. da Silva, I. P. *et al.* Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. *Cancer Immunol Res* **2,** 410–422 (2014).
448. Kang, C.-W. *et al.* Apoptosis of tumor infiltrating effector TIM-3+CD8+ T cells in colon cancer. *Sci Rep* **5,** 15659 (2015).
449. Ngiow, S. F. *et al.* Anti-TIM3 Antibody Promotes T Cell IFN-γ–Mediated Antitumor Immunity and Suppresses Established Tumors. *Clinical Cancer Research* **71,** 3540–3551 (2011).
450. Koyama, S. *et al.* Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. *Nat Commun* **7,** 10501 (2016).
451. Brown, E. J. & Frazier, W. A. Integrin-associated protein (CD47) and its ligands. *Trends Cell Biol.* **11,** 130–135 (2001).
452. Brown, E., Hooper, L., Ho, T. & Gresham, H. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J. Cell Biol.* **111,** 2785–2794 (1990).
453. Lindberg, F. P. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alpha v beta 3-dependent ligand binding. *J. Cell Biol.* **123,** 485–496 (1993).
454. Wang, X. Q. & Frazier, W. A. The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. *Molecular Biology of the Cell* **9,** 865–874 (1998).
455. Reinhold, M. I. *et al.* In vivo expression of alternatively spliced forms of integrin-associated protein (CD47). *J. Cell. Sci.* **108 (Pt 11),** 3419–3425 (1995).
456. Oldenborg, P. A. *et al.* Role of CD47 as a marker of self on red blood cells. *Science* **288,**

2051–2054 (2000).
457. Fossati-Jimack, L. *et al.* Selective increase of autoimmune epitope expression on aged erythrocytes in mice: implications in anti-erythrocyte autoimmune responses. *J. Autoimmun.* **18,** 17–25 (2002).
458. Khandelwal, S., van Rooijen, N. & Saxena, R. K. Reduced expression of CD47 during murine red blood cell (RBC) senescence and its role in RBC clearance from the circulation. *Transfusion* **47,** 1725–1732 (2007).
459. Olsson, M., Nilsson, A. & Oldenborg, P.-A. Dose-dependent inhibitory effect of CD47 in macrophage uptake of IgG-opsonized murine erythrocytes. *Biochem. Biophys. Res. Commun.* **352,** 193–197 (2007).
460. Ishikawa-Sekigami, T. *et al.* SHPS-1 promotes the survival of circulating erythrocytes through inhibition of phagocytosis by splenic macrophages. *Blood* **107,** 341–348 (2006).
461. Yamao, T. *et al.* Negative regulation of platelet clearance and of the macrophage phagocytic response by the transmembrane glycoprotein SHPS-1. *J. Biol. Chem.* **277,** 39833–39839 (2002).
462. Fujioka, Y. *et al.* A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol. Cell. Biol.* **16,** 6887–6899 (1996).
463. Tsai, R. K. & Discher, D. E. Inhibition of 'self' engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. *J. Cell Biol.* **180,** 989–1003 (2008).
464. Van, V. Q. *et al.* Expression of the self-marker CD47 on dendritic cells governs their trafficking to secondary lymphoid organs. *The EMBO Journal* **25,** 5560–5568 (2006).
465. Grimbert, P. *et al.* Thrombospondin/CD47 interaction: a pathway to generate regulatory T cells from human CD4+ CD25- T cells in response to inflammation. *J. Immunol.* **177,** 3534–3541 (2006).
466. Baumgartner, J. M., Palmer, B. E. & Banerjee, A. Role of melanoma secreted thrombospondin-1 on induction of immunosuppressive regulatory T cells through CD47. *J Cancer* (2008).
467. Avice, M. N., Rubio, M., Sergerie, M., Delespesse, G. & Sarfati, M. CD47 ligation selectively inhibits the development of human naive T cells into Th1 effectors. *J. Immunol.* **165,** 4624–4631 (2000).
468. Manna, P. P. & Frazier, W. A. CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. *Clinical Cancer Research* **64,** 1026–1036 (2004).
469. Rendtlew Danielsen, J. M., Knudsen, L. M., Dahl, I. M., Lodahl, M. & Rasmussen, T. Dysregulation of CD47 and the ligands thrombospondin 1 and 2 in multiple myeloma. *Br. J. Haematol.* **138,** 756–760 (2007).
470. Kim, M. J. *et al.* Association of CD47 with natural killer cell-mediated cytotoxicity of head-and-neck squamous cell carcinoma lines. *Tumour Biol.* **29,** 28–34 (2008).
471. Jaiswal, S. *et al.* CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell* **138,** 271–285 (2009).
472. Chan, K. S. *et al.* Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 14016–14021 (2009).

473. Chao, M. P. *et al.* Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* **142,** 699–713 (2010).
474. Edris, B. *et al.* Antibody therapy targeting the CD47 protein is effective in a model of aggressive metastatic leiomyosarcoma. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 6656–6661 (2012).
475. Willingham, S. B. *et al.* The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 6662–6667 (2012).
476. Majeti, R. *et al.* CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* **138,** 286–299 (2009).
477. Tothill, R. W. *et al.* Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin. Cancer Res.* **14,** 5198–5208 (2008).
478. Chung, C. H. *et al.* Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression. *Cancer Cell* **5,** 489–500 (2004).
479. Gravendeel, L. A. M. *et al.* Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology. *Clinical Cancer Research* **69,** 9065–9072 (2009).
480. Nutt, C. L. *et al.* Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. *Clinical Cancer Research* **63,** 1602–1607 (2003).
481. Freije, W. A. *et al.* Gene expression profiling of gliomas strongly predicts survival. *Clinical Cancer Research* **64,** 6503–6510 (2004).
482. Lee, Y. *et al.* Gene expression analysis of glioblastomas identifies the major molecular basis for the prognostic benefit of younger age. *BMC Med Genomics* **1,** 52 (2008).
483. Smith, J. J. *et al.* Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. *Gastroenterology* **138,** 958–968 (2010).
484. Xiao, Z. *et al.* Antibody mediated therapy targeting CD47 inhibits tumor progression of hepatocellular carcinoma. *Cancer Letters* **360,** 302–309 (2015).
485. Liu, J. *et al.* Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential. *PLoS ONE* **10,** e0137345 (2015).
486. Takenaka, K. *et al.* Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. *Nat. Immunol.* **8,** 1313–1323 (2007).
487. Yamauchi, T. *et al.* Polymorphic Sirpa is the genetic determinant for NOD-based mouse lines to achieve efficient human cell engraftment. *Blood* **121,** 1316–1325 (2013).
488. Liu, X. *et al.* CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. *Nat. Med.* **21,** 1209–1215 (2015).
489. Advani, R. *et al.* CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma. *N. Engl. J. Med.* **379,** 1711–1721 (2018).
490. Sikic, B. I. *et al.* A first-in-human, first-in-class phase I trial of the anti-CD47 antibody Hu5F9-G4 in patients with advanced cancers. *JCO* **34,** 3019–3019 (2016).
491. Ansell, S. *et al.* A Phase 1 Study of TTI-621, a Novel Immune Checkpoint Inhibitor Targeting CD47, in Patients with Relapsed or Refractory Hematologic Malignancies. *Blood* **128,** 1812 (2016).
492. Gibney, G. T., Weiner, L. M. & Atkins, M. B. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. *Lancet Oncol.* **17,** e542–e551 (2016).

493. Mahoney, K. M. & Atkins, M. B. Prognostic and predictive markers for the new immunotherapies. *Oncology (Williston Park, N.Y.)* **28 Suppl 3,** 39–48 (2014).
494. Taube, J. M. *et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin. Cancer Res.* **20,** 5064–5074 (2014).
495. Daud, A. I. *et al.* Abstract CT104: Antitumor activity of the anti-PD-1 monoclonal antibody MK-3475 in melanoma(MEL): Correlation of tumor PD-L1 expression with outcome. *Cancer Res* **74,** CT104 (2014).
496. Patel, R., Bock, M., Polotti, C. F. & Elsamra, S. Pharmacokinetic drug evaluation of atezolizumab for the treatment of locally advanced or metastatic urothelial carcinoma. *Expert Opin Drug Metab Toxicol* **13,** 225–232 (2017).
497. Gajewski, T. F., Schreiber, H. & Fu, Y.-X. Innate and adaptive immune cells in the tumor microenvironment. *Nat. Immunol.* **14,** 1014–1022 (2013).
498. Leighl, N. B. *et al.* Pembrolizumab in patients with advanced non-small-cell lung cancer (KEYNOTE-001): 3-year results from an open-label, phase 1 study. *Lancet Respir Med* **7,** 347–357 (2019).
499. Tumeh, P. C. *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515,** 568–571 (2014).
500. Chen, P.-L. *et al.* Analysis of Immune Signatures in Longitudinal Tumor Samples Yields Insight into Biomarkers of Response and Mechanisms of Resistance to Immune Checkpoint Blockade. *Cancer Discovery* **6,** 827–837 (2016).
501. Galon, J. & Bruni, D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov* **18,** 197–218 (2019).
502. Castle, J. C. *et al.* Exploiting the mutanome for tumor vaccination. *Clinical Cancer Research* **72,** 1081–1091 (2012).
503. Rooney, M. S., Shukla, S. A., Wu, C. J., Getz, G. & Hacohen, N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* **160,** 48–61 (2015).
504. Schumacher, T. N. & Schreiber, R. D. Neoantigens in cancer immunotherapy. *Science* **348,** 69–74 (2015).
505. Le, D. T. *et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N. Engl. J. Med.* **372,** 2509–2520 (2015).
506. Le, D. T. *et al.* Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **357,** 409–413 (2017).
507. Snyder, A. *et al.* Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N. Engl. J. Med.* **371,** 2189–2199 (2014).
508. Van Allen, E. M. *et al.* Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science* **350,** 207–211 (2015).
509. Rizvi, N. A. *et al.* Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* **348,** 124–128 (2015).
510. Johnson, D. B. *et al.* Targeted Next Generation Sequencing Identifies Markers of Response to PD-1 Blockade. *Cancer Immunol Res* **4,** 959–967 (2016).
511. Riaz, N. *et al.* Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. *Cell* **171,** 934–949.e16 (2017).
512. Arnaud-Haond, S., Duarte, C. M., Alberto, F. & Serrão, E. A. Standardizing methods to

address clonality in population studies. *Mol. Ecol.* **16,** 5115–5139 (2007).
513. Roh, W. *et al.* Integrated molecular analysis of tumor biopsies on sequential CTLA-4 and PD-1 blockade reveals markers of response and resistance. *Sci Transl Med* **9,** eaah3560 (2017).
514. Forde, P. M., Chaft, J. E. & Pardoll, D. M. Neoadjuvant PD-1 Blockade in Resectable Lung Cancer. *N. Engl. J. Med.* **379,** e14 (2018).
515. Inoue, H. *et al.* Intratumoral expression levels of PD-L1, GZMA, and HLA-A along with oligoclonal T cell expansion associate with response to nivolumab in metastatic melanoma. *Oncoimmunology* **5,** e1204507 (2016).
516. Reuben, A. *et al.* Genomic and immune heterogeneity are associated with differential responses to therapy in melanoma. *NPJ Genom Med* **2,** 2507 (2017).
517. Postow, M. A. *et al.* Peripheral T cell receptor diversity is associated with clinical outcomes following ipilimumab treatment in metastatic melanoma. *J Immunother Cancer* **3,** 23 (2015).
518. Snyder, A. *et al.* Contribution of systemic and somatic factors to clinical response and resistance to PD-L1 blockade in urothelial cancer: An exploratory multi-omic analysis. *PLoS Med.* **14,** e1002309 (2017).
519. Hopkins, A. C. *et al.* T cell receptor repertoire features associated with survival in immunotherapy-treated pancreatic ductal adenocarcinoma. *JCI Insight* **3,** (2018).
520. Havel, J. J., Chowell, D. & Chan, T. A. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat. Rev. Cancer* **19,** 133–150 (2019).
521. Coley, W. B. II. Contribution to the Knowledge of Sarcoma. *Ann. Surg.* **14,** 199–220 (1891).
522. McCarthy, E. F. *The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. The Iowa orthopaedic journal* **26,** 154–158 (University of Iowa, 2006).
523. Pollack, S. M., Ingham, M., Spraker, M. B. & Schwartz, G. K. Emerging Targeted and Immune-Based Therapies in Sarcoma. *J. Clin. Oncol.* **36,** 125–135 (2018).
524. Balch, C. M. *et al.* Patterns of human tumor-infiltrating lymphocytes in 120 human cancers. *Arch Surg* **125,** 200–205 (1990).
525. D'Angelo, S. P. *et al.* Prevalence of tumor-infiltrating lymphocytes and PD-L1 expression in the soft tissue sarcoma microenvironment. *Hum. Pathol.* **46,** 357–365 (2015).
526. van Erp, A. E. M. *et al.* Expression and clinical association of programmed cell death-1, programmed death-ligand-1 and CD8+ lymphocytes in primary sarcomas is subtype dependent. *Oncotarget* **8,** 71371–71384 (2017).
527. Fujii, H. *et al.* $CD8^+$ tumor-infiltrating lymphocytes at primary sites as a possible prognostic factor of cutaneous angiosarcoma. *International Journal of Cancer* **134,** 2393–2402 (2014).
528. Simard, F. A. *et al.* Description of the immune microenvironment of chondrosarcoma and contribution to progression. *Oncoimmunology* **6,** e1265716 (2017).
529. Zou, M.-X. *et al.* Clinical Impact of the Immune Microenvironment in Spinal Chordoma: Immunoscore as an Independent Favorable Prognostic Factor. *Neurosurgery* **26,** 2168 (2018).
530. Feng, Y. *et al.* Expression of programmed cell death ligand 1 (PD-L1) and prevalence of tumor-infiltrating lymphocytes (TILs) in chordoma. *Oncotarget* **6,** 11139–11149 (2015).

531. Machado, I., López-Guerrero, J. A., Scotlandi, K., Picci, P. & Llombart-Bosch, A. Immunohistochemical analysis and prognostic significance of PD-L1, PD-1, and CD8+ tumor-infiltrating lymphocytes in Ewing's sarcoma family of tumors (ESFT). *Virchows Arch.* **472,** 815–824 (2018).
532. Blakely, A. M. *et al.* Role of immune microenvironment in gastrointestinal stromal tumours. *Histopathology* **72,** 405–413 (2018).
533. Cohen, J. E., Eleyan, F., Zick, A., Peretz, T. & Katz, D. Intratumoral immune-biomarkers and mismatch repair status in leiyomyosarcoma -potential predictive markers for adjuvant treatment: a pilot study. *Oncotarget* **9,** 30847–30854 (2018).
534. Shurell, E. *et al.* Characterizing the immune microenvironment of malignant peripheral nerve sheath tumor by PD-L1 expression and presence of CD8+ tumor infiltrating lymphocytes. *Oncotarget* **7,** 64300–64308 (2016).
535. Ogura, K. *et al.* Integrated genetic and epigenetic analysis of myxofibrosarcoma. *Nat Commun* **9,** 2765 (2018).
536. Palmerini, E. *et al.* Tumoral immune-infiltrate (IF), PD-L1 expression and role of CD8/TIA-1 lymphocytes in localized osteosarcoma patients treated within protocol ISG-OS1. *Oncotarget* **8,** 111836–111846 (2017).
537. Fritzsching, B. *et al.* CD8(+)/FOXP3(+)-ratio in osteosarcoma microenvironment separates survivors from non-survivors: a multicenter validated retrospective study. *Oncoimmunology* **4,** e990800 (2015).
538. Nowicki, T. S. *et al.* Infiltration of CD8 T Cells and Expression of PD-1 and PD-L1 in Synovial Sarcoma. *Cancer Immunol Res* **5,** 118–126 (2017).
539. Keung, E. Z. *et al.* Analysis of the immune infiltrate in undifferentiated pleomorphic sarcoma of the extremity and trunk in response to radiotherapy: Rationale for combination neoadjuvant immune checkpoint inhibition and radiotherapy. *Oncoimmunology* **7,** e1385689 (2018).
540. Tseng, W. W. *et al.* Analysis of the intratumoral adaptive immune response in well differentiated and dedifferentiated retroperitoneal liposarcoma. *Sarcoma* **2015,** 547460–9 (2015).
541. Berghuis, D. *et al.* Pro-inflammatory chemokine-chemokine receptor interactions within the Ewing sarcoma microenvironment determine CD8(+) T-lymphocyte infiltration and affect tumour progression. *The Journal of Pathology* **223,** 347–357 (2011).
542. Oike, N. *et al.* Prognostic impact of the tumor immune microenvironment in synovial sarcoma. *Cancer Sci.* **109,** 3043–3054 (2018).
543. Rusakiewicz, S. *et al.* Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. *Clinical Cancer Research* **73,** 3499–3510 (2013).
544. Sorbye, S. W. *et al.* Prognostic impact of peritumoral lymphocyte infiltration in soft tissue sarcomas. *BMC Clin Pathol* **12,** 5 (2012).
545. D'Angelo, S. P. *et al.* Combined KIT and CTLA-4 Blockade in Patients with Refractory GIST and Other Advanced Sarcomas: A Phase Ib Study of Dasatinib plus Ipilimumab. *Clin. Cancer Res.* **23,** 2972–2980 (2017).
546. Kim, C. *et al.* Prognostic implications of PD-L1 expression in patients with soft tissue sarcoma. *BMC Cancer 2015 15:1* **16,** 434 (2016).
547. Lee, C.-H. *et al.* Prognostic significance of macrophage infiltration in leiomyosarcomas. *Clin. Cancer Res.* **14,** 1423–1430 (2008).

548. Espinosa, I. *et al.* Coordinate expression of colony-stimulating factor-1 and colony-stimulating factor-1-related proteins is associated with poor prognosis in gynecological and nongynecological leiomyosarcoma. *The American Journal of Pathology* **174,** 2347–2356 (2009).
549. Ganjoo, K. N. *et al.* The prognostic value of tumor-associated macrophages in leiomyosarcoma: a single institution study. *Am. J. Clin. Oncol.* **34,** 82–86 (2011).
550. Nabeshima, A. *et al.* Tumour-associated macrophages correlate with poor prognosis in myxoid liposarcoma and promote cell motility and invasion via the HB-EGF-EGFR-PI3K/Akt pathways. *Br. J. Cancer* **112,** 547–555 (2015).
551. Fujiwara, T. *et al.* Macrophage infiltration predicts a poor prognosis for human ewing sarcoma. *The American Journal of Pathology* **179,** 1157–1170 (2011).
552. van Dongen, M. *et al.* Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. *International Journal of Cancer* **127,** 899–909 (2010).
553. Pollack, S. M. *et al.* T-cell infiltration and clonality correlate with programmed cell death protein 1 and programmed death-ligand 1 expression in patients with soft tissue sarcomas. *Cancer* **123,** 3291–3304 (2017).
554. Bindea, G. *et al.* Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* **39,** 782–795 (2013).
555. Kostine, M. *et al.* Analysis of PD-L1, T-cell infiltrate and HLA expression in chondrosarcoma indicates potential for response to immunotherapy specifically in the dedifferentiated subtype. *Mod. Pathol.* **29,** 1028–1037 (2016).
556. Bertucci, F. *et al.* PDL1 expression is a poor-prognosis factor in soft-tissue sarcomas. *Oncoimmunology* **6,** e1278100 (2017).
557. Zhu, M. M. T., Dancsok, A. R. & Nielsen, T. O. Indoleamine Dioxygenase Inhibitors: Clinical Rationale and Current Development. *Curr Oncol Rep* **21,** 2 (2019).
558. Toulmonde, M. *et al.* Integrative assessment of expression and prognostic value of PDL1, IDO, and kynurenine in 371 primary soft tissue sarcomas with genomic complexity. *JCO* **34,** 11008–11008 (2016).
559. Chapman, P. B. *et al.* Vaccination with a bivalent G(M2) and G(D2) ganglioside conjugate vaccine: a trial comparing doses of G(D2)-keyhole limpet hemocyanin. *Clin. Cancer Res.* **6,** 4658–4662 (2000).
560. Dillman, R. *et al.* Phase I/II trial of autologous tumor cell line-derived vaccines for recurrent or metastatic sarcomas. *Cancer Biother. Radiopharm.* **19,** 581–588 (2004).
561. Dillman, R. O. *et al.* Phase II trial of dendritic cells loaded with antigens from self-renewing, proliferating autologous tumor cells as patient-specific antitumor vaccines in patients with metastatic melanoma: final report. *Cancer Biother. Radiopharm.* **24,** 311–319 (2009).
562. Mackall, C. L. *et al.* A pilot study of consolidative immunotherapy in patients with high-risk pediatric sarcomas. *Clin. Cancer Res.* **14,** 4850–4858 (2008).
563. Suminoe, A., Matsuzaki, A., Hattori, H., Koga, Y. & Hara, T. Immunotherapy with autologous dendritic cells and tumor antigens for children with refractory malignant solid tumors. *Pediatr Transplant* **13,** 746–753 (2009).
564. Geiger, J. D. *et al.* Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. *Clinical*

Cancer Research **61,** 8513–8519 (2001).
565. Kawaguchi, S. *et al.* SYT-SSX breakpoint peptide vaccines in patients with synovial sarcoma: a study from the Japanese Musculoskeletal Oncology Group. *Cancer Sci.* **103,** 1625–1630 (2012).
566. Endo, M. *et al.* NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. *Mod. Pathol.* **28,** 587–595 (2015).
567. Jungbluth, A. A. *et al.* Monophasic and biphasic synovial sarcomas abundantly express cancer/testis antigen NY-ESO-1 but not MAGE-A1 or CT7. *International Journal of Cancer* **94,** 252–256 (2001).
568. Pollack, S. M. *et al.* NY-ESO-1 is a ubiquitous immunotherapeutic target antigen for patients with myxoid/round cell liposarcoma. *Cancer* **118,** 4564–4570 (2012).
569. Odegard, J. M. *et al.* A novel HSV-2 subunit vaccine induces GLA-dependent CD4 and CD8 T cell responses and protective immunity in mice and guinea pigs. *Vaccine* **34,** 101–109 (2016).
570. Albershardt, T. C. *et al.* LV305, a dendritic cell-targeting integration-deficient ZVex(TM)-based lentiviral vector encoding NY-ESO-1, induces potent anti-tumor immune response. *Mol Ther Oncolytics* **3,** 16010 (2016).
571. Somaiah, N. *et al.* Single-agent LV305 to induce anti-tumor immune and clinical responses in patients with advanced or metastatic sarcoma and other cancers expressing NY-ESO-1. *JCO* **34,** 3093–3093 (2016).
572. Somaiah, N. *et al.* Immune response, safety, and survival impact from CMB305 in NY-ESO-1+ recurrent soft tissue sarcomas (STS). *JCO* **35,** 11006–11006 (2017).
573. Pollack, S. M. The potential of the CMB305 vaccine regimen to target NY-ESO-1 and improve outcomes for synovial sarcoma and myxoid/round cell liposarcoma patients. *Expert Rev Vaccines* **17,** 107–114 (2018).
574. Pollack, S. M. *et al.* First-in-Human Treatment With a Dendritic Cell-targeting Lentiviral Vector-expressing NY-ESO-1, LV305, Induces Deep, Durable Response in Refractory Metastatic Synovial Sarcoma Patient. *J. Immunother.* **40,** 302–306 (2017).
575. Somaiah, N. *et al.* Immune response, safety, and survival impact from CMB305 in NY-ESO-1+ recurrent soft tissue sarcomas (STS). *JCO* **35,** 11006–11006 (2017).
576. Chawla, S. *et al.* 1480PDA phase 2 study of CMB305 and atezolizumab in NY-ESO-1+ soft tissue sarcoma: Interim analysis of immunogenicity, tumor control and survival. *Annals of Oncology* **28,** (2017).
577. Pollack, S. M. *et al.* Tetramer guided, cell sorter assisted production of clinical grade autologous NY-ESO-1 specific CD8(+) T cells. *J Immunother Cancer* **2,** 36 (2014).
578. Robbins, P. F. *et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J. Clin. Oncol.* **29,** 917–924 (2011).
579. Robbins, P. F. *et al.* A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin. Cancer Res.* **21,** 1019–1027 (2015).
580. Ahmed, N. *et al.* Human Epidermal Growth Factor Receptor 2 (HER2) -Specific Chimeric Antigen Receptor-Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma. *J. Clin. Oncol.* **33,** 1688–1696 (2015).
581. Tawbi, H. A. *et al.* Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma

(SARC028): a multicentre, two-cohort, single-arm, open-label, phase 2 trial. *Lancet Oncol.* **18,** 1493–1501 (2017).
582. D'Angelo, S. P. *et al.* Nivolumab with or without ipilimumab treatment for metastatic sarcoma (Alliance A091401): two open-label, non-comparative, randomised, phase 2 trials. *Lancet Oncol.* **19,** 416–426 (2018).
583. Toulmonde, M. *et al.* Use of PD-1 Targeting, Macrophage Infiltration, and IDO Pathway Activation in Sarcomas: A Phase 2 Clinical Trial. *JAMA Oncol* **4,** 93–97 (2018).
584. Young, R. J. *et al.* First-line anthracycline-based chemotherapy for angiosarcoma and other soft tissue sarcoma subtypes: Pooled analysis of eleven European Organisation for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group trials. *European Journal of Cancer* **50,** 3178–3186 (2014).
585. Isakoff, M. S., Bielack, S. S., Meltzer, P. & Gorlick, R. Osteosarcoma: Current Treatment and a Collaborative Pathway to Success. *JCO* **33,** 3029–3035 (2015).
586. In, G. K., Hu, J. S. & Tseng, W. W. Treatment of advanced, metastatic soft tissue sarcoma: latest evidence and clinical considerations. *Ther Adv Med Oncol* **9,** 533–550 (2017).
587. Le Cesne, A. *et al.* Phase II study of ET-743 in advanced soft tissue sarcomas: a European Organisation for the Research and Treatment of Cancer (EORTC) soft tissue and bone sarcoma group trial. *JCO* **23,** 576–584 (2005).
588. Nielsen, O. S. *et al.* Effect of high-dose ifosfamide in advanced soft tissue sarcomas. A multicentre phase II study of the EORTC Soft Tissue and Bone Sarcoma Group. *European Journal of Cancer* **36,** 61–67 (2000).
589. Leahy, M. *et al.* Chemotherapy treatment patterns and clinical outcomes in patients with metastatic soft tissue sarcoma. The SArcoma treatment and Burden of Illness in North America and Europe (SABINE) study. *Ann. Oncol.* **23,** 2763–2770 (2012).
590. Ryan, C. W. *et al.* PICASSO III: A Phase III, Placebo-Controlled Study of Doxorubicin With or Without Palifosfamide in Patients With Metastatic Soft Tissue Sarcoma. *J. Clin. Oncol.* **34,** 3898–3905 (2016).
591. Savina, M. *et al.* Patterns of care and outcomes of patients with METAstatic soft tissue SARComa in a real-life setting: the METASARC observational study. *BMC Med* **15,** 78 (2017).
592. Dudley, M. E. *et al.* Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* **298,** 850–854 (2002).
593. Dudley, M. E. *et al.* Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J. Clin. Oncol.* **26,** 5233–5239 (2008).
594. Porter, D. L., Levine, B. L., Kalos, M., Bagg, A. & June, C. H. Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia. *N. Engl. J. Med.* **365,** 725–733 (2011).
595. Robert, C. *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N. Engl. J. Med.* **364,** 2517–2526 (2011).
596. Ott, P. A. *et al.* Pembrolizumab in Patients With Extensive-Stage Small-Cell Lung Cancer: Results From the Phase Ib KEYNOTE-028 Study. *J. Clin. Oncol.* **35,** 3823–3829 (2017).
597. Herbst, R. S. *et al.* Pembrolizumab versus docetaxel for previously treated, PD-L1-

positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* **387,** 1540–1550 (2016).
598. Plimack, E. R. *et al.* Safety and activity of pembrolizumab in patients with locally advanced or metastatic urothelial cancer (KEYNOTE-012): a non-randomised, open-label, phase 1b study. *Lancet Oncol.* **18,** 212–220 (2017).
599. Ben-Ami, E. *et al.* Immunotherapy with single agent nivolumab for advanced leiomyosarcoma of the uterus: Results of a phase 2 study. *Cancer* **123,** 3285–3290 (2017).
600. Maki, R. G. *et al.* A Pilot Study of Anti-CTLA4 Antibody Ipilimumab in Patients with Synovial Sarcoma. *Sarcoma* **2013,** 168145–8 (2013).
601. Paoluzzi, L. *et al.* Response to anti-PD1 therapy with nivolumab in metastatic sarcomas. *Clin Sarcoma Res* **6,** 24 (2016).
602. Jenkins, R. W., Barbie, D. A. & Flaherty, K. T. Mechanisms of resistance to immune checkpoint inhibitors. *Br. J. Cancer* **118,** 9–16 (2018).
603. Datar, I. *et al.* Measurement of PD-1, TIM-3 and LAG-3 protein in non-small cell lung carcinomas (NSCLCs) with acquired resistance to PD-1 axis blockers. *JCO* **35,** e14611–e14611 (2017).
604. Nielsen, T. O. *et al.* Tissue Microarray Validation of Epidermal Growth Factor Receptor and SALL2 in Synovial Sarcoma with Comparison to Tumors of Similar Histology. *The American Journal of Pathology* **163,** 1449–1456 (2003).
605. Ng, T. L., Gown, A. M., Barry, T. S. & Cheang, M. Nuclear beta-catenin in mesenchymal tumors. *Modern …* (2005).
606. Steigen, S. E. *et al.* Clinicopathologic factors and nuclear morphometry as independent prognosticators in KIT-positive gastrointestinal stromal tumors. *J. Histochem. Cytochem.* **56,** 139–145 (2008).
607. Cheng, H. *et al.* Validation of immature adipogenic status and identification of prognostic biomarkers in myxoid liposarcoma using tissue microarrays. *Hum. Pathol.* **40,** 1244–1251 (2009).
608. Pacheco, M. & Nielsen, T. O. Histone deacetylase 1 and 2 in mesenchymal tumors. *Modern Pathology* **25,** 222–230 (2011).
609. Endo, M., Su, L. & Nielsen, T. O. Activating transcription factor 2 in mesenchymal tumors. *Hum. Pathol.* **45,** 276–284 (2014).
610. Banito, A. *et al.* The SS18-SSX Oncoprotein Hijacks KDM2B-PRC1.1 to Drive Synovial Sarcoma. *Cancer Cell* **33,** 527–541.e8 (2018).
611. Terry, J. *et al.* TLE1 as a diagnostic immunohistochemical marker for synovial sarcoma emerging from gene expression profiling studies. *Am. J. Surg. Pathol.* **31,** 240–246 (2007).
612. Demicco, E. G. *et al.* Extensive survey of STAT6 expression in a large series of mesenchymal tumors. *Am J Clin Pathol* **143,** 672–682 (2015).
613. Garon, E. B. *et al.* Pembrolizumab for the treatment of non-small-cell lung cancer. *N. Engl. J. Med.* **372,** 2018–2028 (2015).
614. Pirici, D. *et al.* Antibody elution method for multiple immunohistochemistry on primary antibodies raised in the same species and of the same subtype. *J. Histochem. Cytochem.* **57,** 567–575 (2009).
615. Constantinidou, A., Miah, A., Pollack, S. & Jones, R. L. New drugs and clinical trial

design in advanced sarcoma: have we made any progress? *Future Oncology* **9,** 1409–1411 (2013).
616. Han, Q., Shi, H. & Liu, F. CD163(+) M2-type tumor-associated macrophage support the suppression of tumor-infiltrating T cells in osteosarcoma. *Int. Immunopharmacol.* **34,** 101–106 (2016).
617. Laginestra, M. A. *et al.* Distinctive Histogenesis and Immunological Microenvironment Based on Transcriptional Profiles of Follicular Dendritic Cell Sarcomas. *Mol. Cancer Res.* **15,** 541–552 (2017).
618. Paydas, S., Bagir, E. K., Deveci, M. A. & Gonlusen, G. Clinical and prognostic significance of PD-1 and PD-L1 expression in sarcomas. *Med. Oncol.* **33,** 93 (2016).
619. Torabi, A., Amaya, C. N., Wians, F. H., Jr. & Bryan, B. A. PD-1 and PD-L1 expression in bone and soft tissue sarcomas. *Modern Pathology* **49,** 506–513 (2017).
620. Boxberg, M. *et al.* PD-L1 and PD-1 and characterization of tumor-infiltrating lymphocytes in high grade sarcomas of soft tissue - prognostic implications and rationale for immunotherapy. *Oncoimmunology* **7,** e1389366 (2018).
621. Spurny, C. *et al.* Programmed cell death ligand 1 (PD-L1) expression is not a predominant feature in Ewing sarcomas. *Pediatric Blood & Cancer* **65,** e26719 (2018).
622. Thorsson, V. *et al.* The Immune Landscape of Cancer. *Immunity* **48,** 812–830.e14 (2018).
623. Merchant, M. S. *et al.* Phase I Clinical Trial of Ipilimumab in Pediatric Patients with Advanced Solid Tumors. *Clin. Cancer Res.* **22,** 1364–1370 (2016).
624. Ghisoli, M. *et al.* Three-year Follow up of GMCSF/bi-shRNA furin DNA-transfected Autologous Tumor Immunotherapy (Vigil) in Metastatic Advanced Ewing's Sarcoma. *Molecular Therapy* **24,** 1478–1483 (2016).
625. Miwa, S. *et al.* Phase 1/2 study of immunotherapy with dendritic cells pulsed with autologous tumor lysate in patients with refractory bone and soft tissue sarcoma. *Cancer* **123,** 1576–1584 (2017).
626. Research, A. A. F. C. High TMB Predicts Immunotherapy Benefit. *Cancer Discovery* **8,** 668–668 (2018).
627. Hellmann, M. D. *et al.* Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. *N. Engl. J. Med.* **378,** 2093–2104 (2018).
628. Hellmann, M. D. *et al.* Tumor Mutational Burden and Efficacy of Nivolumab Monotherapy and in Combination with Ipilimumab in Small-Cell Lung Cancer. *Cancer Cell* **33,** 853–861.e4 (2018).
629. Liao, Y. *et al.* Targeting programmed cell death ligand 1 by CRISPR/Cas9 in osteosarcoma cells. *Oncotarget* **8,** 30276–30287 (2017).
630. Kim, J. R. *et al.* Tumor infiltrating PD1-positive lymphocytes and the expression of PD-L1 predict poor prognosis of soft tissue sarcomas. *PLoS ONE* **8,** e82870 (2013).
631. Costa Arantes, D. A. *et al.* Evaluation of HLA-G, HLA-E, and PD-L1 proteins in oral osteosarcomas. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* **123,** e188–e196 (2017).
632. Chowdhury, F. *et al.* PD-L1 and CD8 +PD1 +lymphocytes exist as targets in the pediatric tumor microenvironment for immunomodulatory therapy. *Oncoimmunology* **4,** e1029701 (2015).
633. Que, Y. *et al.* PD-L1 Expression Is Associated with FOXP3+ Regulatory T-Cell

Infiltration of Soft Tissue Sarcoma and Poor Patient Prognosis. *J Cancer* **8,** 2018–2025 (2017).
634. Budczies, J. *et al.* PD-L1 (CD274) copy number gain, expression, and immune cell infiltration as candidate predictors for response to immune checkpoint inhibitors in soft-tissue sarcoma. *Oncoimmunology* **6,** e1279777 (2017).
635. Sundara, Y. T. *et al.* Increased PD-L1 and T-cell infiltration in the presence of HLA class I expression in metastatic high-grade osteosarcoma: a rationale for T-cell-based immunotherapy. *Cancer Immunol. Immunother.* **66,** 119–128 (2017).
636. Shen, J. K. *et al.* Programmed cell death ligand 1 expression in osteosarcoma. *Cancer Immunol Res* **2,** 690–698 (2014).
637. Koirala, P. *et al.* Immune infiltration and PD-L1 expression in the tumor microenvironment are prognostic in osteosarcoma. *Sci Rep* **6,** 30093 (2016).
638. Calles, A. *et al.* Expression of PD-1 and Its Ligands, PD-L1 and PD-L2, in Smokers and Never Smokers with KRAS-Mutant Lung Cancer. *J Thorac Oncol* **10,** 1726–1735 (2015).
639. Krawczyk, P. *et al.* Immunohistochemical assays incorporating SP142 and 22C3 monoclonal antibodies for detection of PD-L1 expression in NSCLC patients with known status of EGFR and ALK genes. *Oncotarget* **8,** 64283–64293 (2017).
640. Xu, H. *et al.* Assessment of Concordance between 22C3 and SP142 Immunohistochemistry Assays regarding PD-L1 Expression in Non-Small Cell Lung Cancer. *Sci Rep* **7,** 16956 (2017).
641. Gameiro, S. R. *et al.* Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing. *Oncotarget* **5,** 403–416 (2014).
642. Golden, E. B. & Apetoh, L. Radiotherapy and immunogenic cell death. *Semin Radiat Oncol* **25,** 11–17 (2015).
643. Ng, J. & Dai, T. Radiation therapy and the abscopal effect: a concept comes of age. *Ann Transl Med* **4,** 118–118 (2016).
644. Gide, T. N., Wilmott, J. S., Scolyer, R. A. & Long, G. V. Primary and Acquired Resistance to Immune Checkpoint Inhibitors in Metastatic Melanoma. *Clin. Cancer Res.* **24,** 1260–1270 (2018).
645. Leek, R. D. *et al.* Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. *Br. J. Cancer* **77,** 2246–2251 (1998).
646. Lee, A. H., Happerfield, L. C., Bobrow, L. G. & Millis, R. R. Angiogenesis and inflammation in invasive carcinoma of the breast. *J. Clin. Pathol.* **50,** 669–673 (1997).
647. Lissbrant, I. F. *et al.* Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *International Journal of Oncology* **17,** 445–451 (2000).
648. Fujimoto, J., Sakaguchi, H., Aoki, I. & Tamaya, T. Clinical implications of expression of interleukin 8 related to angiogenesis in uterine cervical cancers. *Clinical Cancer Research* **60,** 2632–2635 (2000).
649. Koukourakis, M. I. *et al.* Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. *Br. J. Cancer* **77,** 1696–1703 (1998).

650. Miao, D. *et al.* Response and oligoclonal resistance to pembrolizumab in uterine leiomyosarcoma: Genomic, neoantigen, and immunohistochemical evaluation. *JCO* **34,** 11043–11043 (2016).
651. Xu, J.-F. *et al.* CD47 blockade inhibits tumor progression human osteosarcoma in xenograft models. *Oncotarget* **6,** 23662–23670 (2015).
652. doi:10.1016/j.immuni.2018.03.023/attachment/22b1df93-e78b-40b9-bceb-2e69aa3f55d2/mmc2.xlsx
653. Maleki Vareki, S. High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint inhibitors. *J Immunother Cancer* **6,** 157 (2018).
654. Thomas, A. *et al.* Tumor mutational burden is a determinant of immune-mediated survival in breast cancer. *Oncoimmunology* **7,** e1490854 (2018).
655. Greillier, L., Tomasini, P. & Barlesi, F. The clinical utility of tumor mutational burden in non-small cell lung cancer. *Transl Lung Cancer Res* **7,** 639–646 (2018).
656. Gamrekelashvili, J., Greten, T. F. & Korangy, F. Immunogenicity of necrotic cell death. *Cell. Mol. Life Sci.* **72,** 273–283 (2015).
657. Sachet, M., Liang, Y. Y. & Oehler, R. The immune response to secondary necrotic cells. *Apoptosis* **22,** 1189–1204 (2017).
658. Dvorak, H. F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* **315,** 1650–1659 (1986).
659. Broz, M. L. *et al.* Dissecting the Tumor Myeloid Compartment Reveals Rare Activating Antigen-Presenting Cells Critical for T Cell Immunity. *Cancer Cell* **26,** 938 (2014).
660. Engelhardt, J. J. *et al.* Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. *Cancer Cell* **21,** 402–417 (2012).
661. Peranzoni, E. *et al.* Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. *Proc. Natl. Acad. Sci. U.S.A.* **115,** E4041–E4050 (2018).
662. Herwig, M. C., Bergstrom, C., Wells, J. R., Höller, T. & Grossniklaus, H. E. M2/M1 ratio of tumor associated macrophages and PPAR-gamma expression in uveal melanomas with class 1 and class 2 molecular profiles. *Exp. Eye Res.* **107,** 52–58 (2013).
663. Jackute, J. *et al.* Distribution of M1 and M2 macrophages in tumor islets and stroma in relation to prognosis of non-small cell lung cancer. *BMC Immunol.* **19,** 3 (2018).
664. Edin, S. *et al.* The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer. *PLoS ONE* **7,** e47045 (2012).
665. Yahaya, M. A. F., Lila, M. A. M., Ismail, S., Zainol, M. & Afizan, N. A. R. N. M. Tumour-Associated Macrophages (TAMs) in Colon Cancer and How to Reeducate Them. *J Immunol Res* **2019,** 2368249–9 (2019).
666. Zheng, X. *et al.* Redirecting tumor-associated macrophages to become tumoricidal effectors as a novel strategy for cancer therapy. *Oncotarget* **8,** 48436–48452 (2017).
667. Land, W. G. The Role of Damage-Associated Molecular Patterns in Human Diseases: Part I - Promoting inflammation and immunity. *Sultan Qaboos Univ Med J* **15,** e9–e21 (2015).
668. Hernandez, C., Huebener, P. & Schwabe, R. F. Damage-associated molecular patterns in

cancer: a double-edged sword. *Oncogene* **35,** 5931–5941 (2016).
669. He, Y. *et al.* Tissue damage-associated 'danger signals' influence T-cell responses that promote the progression of preneoplasia to cancer. *Clinical Cancer Research* **73,** 629–639 (2013).
670. Shi, L., Bian, Z. & Liu, Y. Dual role of SIRPα in macrophage activation: inhibiting M1 while promoting M2 polarization via selectively activating SHP-1 and SHP-2 signal. *J. Immunol.* **198,** 67.12 (2017).
671. Voduc, D., Kenney, C. & Nielsen, T. O. Tissue microarrays in clinical oncology. *Semin Radiat Oncol* **18,** 89–97 (2008).
672. Barros, M. H. M., Hauck, F., Dreyer, J. H., Kempkes, B. & Niedobitek, G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS ONE* **8,** e80908 (2013).
673. Rath, M., Müller, I., Kropf, P., Closs, E. I. & Munder, M. Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. *Front Immunol* **5,** 532 (2014).
674. Lisi, L. *et al.* Expression of iNOS, CD163 and ARG-1 taken as M1 and M2 markers of microglial polarization in human glioblastoma and the surrounding normal parenchyma. *Neurosci. Lett.* **645,** 106–112 (2017).
675. Yoshida, K. *et al.* CD47 is an adverse prognostic factor and a therapeutic target in gastric cancer. *Cancer Med* **4,** 1322–1333 (2015).
676. Ye, X. *et al.* CD47 as a potential prognostic marker for oral leukoplakia and oral squamous cell carcinoma. *Oncol Lett* **15,** 9075–9080 (2018).
677. Suzuki, S. *et al.* CD47 expression regulated by the miR-133a tumor suppressor is a novel prognostic marker in esophageal squamous cell carcinoma. *Oncol. Rep.* **28,** 465–472 (2012).
678. Zhao, H. *et al.* CD47 Promotes Tumor Invasion and Metastasis in Non-small Cell Lung Cancer. *Sci Rep* **6,** 29719 (2016).
679. Fu, W., Li, J., Zhang, W. & Li, P. High expression of CD47 predicts adverse prognosis in Chinese patients and suppresses immune response in melanoma. *Biomed. Pharmacother.* **93,** 1190–1196 (2017).
680. Mulder, D. T. *et al.* CapTCR-seq: hybrid capture for T-cell receptor repertoire profiling. *Blood Adv* **2,** 3506–3514 (2018).
681. Thavaneswaran, S. *et al.* Cancer Molecular Screening and Therapeutics (MoST): a framework for multiple, parallel signal-seeking studies of targeted therapies for rare and neglected cancers. *Med. J. Aust.* **209,** 354–355 (2018).
682. Bolotin, D. A. *et al.* MiXCR: software for comprehensive adaptive immunity profiling. *Nat. Methods* **12,** 380–381 (2015).
683. Nazarov, V. I. *et al.* tcR: an R package for T cell receptor repertoire advanced data analysis. *BMC Bioinformatics* **16,** 175 (2015).
684. Shannon, C. E. *The mathematical theory of communication. 1963. M.D. computing : computers in medical practice* **14,** 306–317 (1997).
685. Kansara, M. *et al.* Activity of tremelimumab and durvalumab in advanced sarcomas: preliminary results of a signal-seeking phase 2 trial. *Connective Tissue Oncology Society CTOS Annual Meeting* (2018).
686. Penel, N. *et al.* Presentation and outcome of frequent and rare sarcoma histologic

subtypes: A study of 10,262 patients with localized visceral/soft tissue sarcoma managed in reference centers. *Cancer* **124,** 1179–1187 (2018).