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Effects of cellular adoptive immunotherapy in combination with immune checkpoint inhibition and TLR-agonists in soft tissue sarcoma mouse models

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Jonathan Bochtler
aus Donaueschingen

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Dekan: **Universitätsprofessor Dr. med. G. R. Fink**

1. Gutachter: **Universitätsprofessor Dr. med. Dr. nat. med. R. T. Ullrich,**
   Klinik I für Innere Medizin, Uniklinik Köln
2. Gutachterin: **Universitätsprofessorin Dr. rer. nat. R. Stripecke,**
   Klinik I für Innere Medizin, Uniklinik Köln

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CONTENT

ABBREVIATIONS

Ab	-	Antibody
ACT	-	Adoptive cellular therapy
BW	-	Body weight
CCR4	-	C-C chemokine receptor type 4
CD	-	cluster of differentiation
CIKs	-	Cytokine-Induced killer cells
CTLs	-	(tumor antigen-specific) cytotoxic T lymphocyte
CTLA-4	-	cytotoxic T-lymphocyte-associated protein 4
DMSO	-	dimethyl sulfoxide
FACS	-	fluorescence activated cell sorting
FAS-ligand	-	CD95 ligand
FC region	-	fragment crystallizable region
FCS	-	fetal calf serum
FELASA	-	Federation of European Laboratory Animal Science
G	-	gravitational constant
HGF	-	hepatocyte growth factor
i.m.	-	intramuscular/ly
i.p.	-	intraperitoneal/ly
IFN	-	interferone
IL2	-	interleukin 2
IL2R	-	interleukin 2 Receptor
IL6	-	interleukin 6
IL8	-	interleukin 8
KP	-	KRAS, tP53
LAG-3	-	lymphocyte activation gene 3
LAKs	-	lyphokine activated killer cells
LIF	-	leucemia inhibitory factor
M-CSF	-	macrophage colony-stimulating factor
mAb	-	murine antibodies
MAPK	-	mitogen-activated protein kinase
MCA	-	3-methylcholanthrene
MCP 3	-	Macrophage/Monocyte Chemotactic Protein 3

MFI	-	mean fluorescence intensity
MHC	-	Major Histocompatibility Complex
MOS	-	mean overall survival
NFκB	-	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NGS	-	next generation sequencing
NY-ESO-1	-	New York esophageal squamous cell carcinoma 1
PBS	-	phosphate buffered saline
PD-1	-	programmed cell death Protein 1
PD-L1	-	programmed death-ligand 1
PFA	-	paraformaldehyde
RT	-	room temperature
s.c.	-	subcutaneous/ly
STS	-	soft tissue sarcoma
TAM	-	tumor associated macrophages
TIM-3	-	T-cell immunoglobulin and mucin-domain containing-3
TNF-R	-	tumor necrosis factor receptor
VEGF	-	vascular endothelial growth factor

ZUSAMMENFASSUNG

Einleitung:

Weichteilsarkome sind eine heterogene Gruppe bösartiger Erkrankungen mesenchymalen Ursprungs. Sobald eine vollständige Resektion nicht mehr möglich ist, verschlechtert sich die Lebenserwartung drastisch auf eine mittlere Gesamtüberlebensrate von weniger als 12 Monaten unter der anthrazyklinbasierten Standard-Chemotherapie. Seit der Zulassung von Doxorubicin im Jahr 1974 hat sich das konservative Behandlungskonzept inoperabler Weichteilsarkome nicht wesentlich verbessert, sodass dringend nach besseren Alternativen gesucht werden muss (Italiano 2020). Gegenstand meiner Forschung ist die Bewertung der Wirksamkeit einer innovativen Immuntherapiekombination gegen undifferenzierte pleomorphe Sarkome in Zellkulturen sowie in heterotopen und orthotopen Mausmodellen.

Methoden:

Die adoptive Zelltherapie, die aus einer Mischung von Zytokin-induzierten Killerzellen, zytotoxischen T Lymphozyten, Lymphokin-aktivierten Killerzellen und γδ-T-Zellen besteht, wurde mit aPD-1-Antikörpern und TLR 3-, 7- und 9-Agonisten kombiniert (insgesamt: Triple-Therapie). Besonderes Augenmerk wurde bei den Analysen auf die induzierten Veränderungen im Tumormikromilieu gelegt, um die Mechanismen zu verstehen, die zur Antitumor -Wirksamkeit der Therapie beitragen könnten. Hierzu war neben diversen Assays und der Immunhistochemie die fluoreszenzaktivierte Durchflusszytometrie das wichtigste Analyseverfahren.

Ergebnisse:

Killing-Assays bestätigten die Annahme, dass die Antitumorwirkung gegen Sarkomzellen in vitro durch die Kombination aller vier ACT-Effektorzelltypen synergistische Effekte zeigte, verglichen mit ACT unter Verwendung jeweils nur eines Effektorzelltyps. Subkutane Mausmodelle für das undifferenzierte pleomorphe Sarkom zeigten deutlich verbesserte Gesamtüberlebensraten unter Kombinations-ACT-Behandlung im Vergleich zu unbehandelten Mäusen (20,6 d MOS [ACTmono] gegenüber 16,0 d MOS [Vehicle]). Diese positive Wirkung wurde durch eine Augmentierung des Behandlungsregimens durch aPD-1 und TLR-3, -7 und -9

Agonisten noch verstärkt (25,4 d MOS [Triple]). Das Triple-Therapieregimen zeigte bemerkenswerte Ansprechraten mit vollständigen Remissionen in 50 % der Tumoren an Tag 14 nach Beginn der Behandlung. In orthotopen undifferenzierten pleomorphen Sarkom-Mausmodellen konnten ähnlich günstige Antitumoreffekte in immuntherapeutisch behandelten Mäusen beobachtet werden. Bei der Analyse des Tumormikromilieus zeigte die Durchflusszytometrie eine verstärkte Infiltration und Aktivierung von T- und B-Zellen in Sarkomen, die auf die ACT mono- oder Triple-Therapie ansprechen, verglichen mit progressiven Tumoren. Darüber hinaus zeigt eine generell verstärkte Aktivierung antigenpräsentierender Zellen (APCs) die starke Stimulation des murinen Immunsystems.

Fazit und Ausblick:

Zusammenfassend lässt sich sagen, dass die mit der adoptiven Zelltherapie erzielten Antitumor -Effekte nicht nur auf direkten zytotoxischen Eigenschaften beruhen, sondern die Therapie vielmehr auch zu einer starken Interaktion zwischen dem angeborenen und dem adaptiven Immunsystem führt, die die undifferenzierten pleomorphen Sarkome in lebenden Modellen synergistisch effektiv bekämpfen. Da die meisten der induzierten positiven Veränderungen innerhalb des TME nach Tagen bis Wochen wieder verschwinden und die Tumoren wieder wachsen, ist es von entscheidender Bedeutung, die Tumor-Escape-Mechanismen zu verstehen. Daher muss das Tumormikromilieu progredienter undifferenzierter pleomorpher Sarkome, vor allem im Kontrast zu ansprechenden Tumoren, genauer analysiert werden. Hierdurch sollen Wege gefunden werden, die Sarkomzellen daran zu hindern, die durch die Immuntherapie-Kombinationen hervorgerufenen potenten Antitumor -Effekte zu überwinden.

1. SUMMARY

Soft tissue sarcomas are a heterogeneous group of malignancies deriving from mesenchymal tissues. If unresectable, life expectancy quickly deteriorates to a poor MOS rate of less than 12 months under anthracycline-based standard chemotherapy treatment.

Since the approval of doxorubicin in 1974, no significant improvement has been achieved in the treatment of inoperable soft tissue sarcomas, so there is an urgent need to find better alternatives (Italiano 2020).

Subject of my research is to evaluate the effectiveness of an innovative adoptive Immunotherapy combination against undifferentiated pleomorphic sarcomas in cell cultures as well as in heterotopic and orthotopic mouse models.

The ACT that consists of a mix of Cytokine-Induced Killer cells, Cytotoxic T Lymphocytes, Lymphokine Activated Killer cells and $\gamma\delta$-T cells was combined with aPD-1 antibodies and TLR-3, -7 and -9 agonists (Triple therapy).

Particular emphasis was placed on induced changes in the TME to understand the mechanisms that could contribute to the antitumor efficacy of the therapy.

Killing assays confirmed the preassumption that the killing efficacy against sarcoma cells in vitro was enhanced by the combination of all four ACT effector cell types compared to single cell type ACT.

Subcutaneous undifferentiated pleomorphic sarcoma mouse models showed notably enhanced overall survival rates when treated with the combination ACT compared to untreated mice (20,6 d MOS [ACTmono] vs 16,0 d MOS [Vehicle]). This beneficial effect was even improved by additional treatment with aPD-1 and the TLR agonist mix (25,4 d MOS [Triple]). The Triple therapy regimen showed striking response rates with complete responses in 50 % of the specimens on day 14 of the treatment.

In orthotopic undifferentiated pleomorphic sarcoma mouse models, similarly beneficial antitumor effects in immunotherapeutically treated mice could be observed.

Analyzing the TME, FACS data revealed enhanced T cell and B cell infiltration and activation in sarcomas responsive to ACTmono or Triple therapy compared to progressive tumors.

Moreover, an enhanced generalized APC activation shows the potent stimulation of the murine immune system.

Summarizing, the implemented ACT antitumor effects do not rely only on direct cytotoxic properties but also lead to potent interactions between the innate and the adaptive immune system that synergistically combat the undifferentiated pleomorphic sarcomas in living models.

As most of the induced beneficial changes within the TME vanish after days to weeks and the tumors start to regrow, it is crucial to understand the tumor escape mechanisms. Therefore, more detailed analysis of the TME has to be undertaken to potentially find ways to prevent sarcoma cells from overcoming the great antitumor immune response that is induced by the immunotherapy combinations.

2 INTRODUCTION

2.1 The immune system

2.1.1 The innate immune system

The immune system is an extremely complex system of barriers, various organs, multiple types and subtypes of cells, molecular cascades comprising numerous molecules, and a prime example of interdisciplinary cooperation within those components.

The immune system can be functionally divided in two main compartments: The innate and the adaptive immune system.

The innate compartment of the immune system forms the phylogenetically oldest and nonspecific proportion**,** consisting among others of mechanical or chemical barriers, granulocytes, natural killer cells, antigen-presenting cells, cytokines and other proteins like complement factors (L.Krishnan 2019).

An important pillar of the innate immune system is represented by granulocytes and especially by neutrophils that are recruited and activated within damaged and infected tissue (Storey and Jordan 2008; Özcan and Boyman 2022). This cell type eliminates pathogens in three ways: degranulation of vesicles containing cytotoxic and proinflammatory proteins, phagocytosis and intracellular decomposition of foreign material and through the formation of “NETs” (Neutrophil extracellular traps) – enzyme-equipped DNA strands that can trap and destroy pathogens (Kolaczkowska and Kubes 2013; Aroca-Crevillén et al. 2024).

Cytokines are small proteins, capable of inducing and amplifying inflammation, facilitating vascular permeability, attracting leukocytes and inducing enhanced endothelial adhesion to promote diapedesis of these cells. Moreover, cytokines play an important role in inter- and intracellular communication and cellular maturation and differentiation.

The complement system, consisting of about 50 proteins and protein fragments, is activated by contact with pathogen associated surface molecules or by the antibody-Fc region. Extracellular activation cascades lead to opsonization of pathogens and chemotaxis of phagocytizing immune cells. Beyond that, the so-called membrane-attack complex can be formed by coordinated assembly of the complement factors 5b, 6, 7, 8 and 9 to directly eliminate unwanted intruders by osmotic lysis (Dranoff 2004; Storey and Jordan 2008). Natural killer cells are key important effector cells of the innate immune system, recognizing “non-self” structures, releasing proinflammatory or immunosuppressive agents such as IFNγ, TGFβ or IL10. Subsequently NK cells are effectively killing tumor cells and infected body cells by releasing perforins and granzymes. (Storey and Jordan 2008; S. Y. Wu et al. 2020; S. Chen, Zhu, and Jounaidi 2024).

Dendritic cells (DCs) and macrophages phagocytize foreign structures, intracellularly degrade them and present pathogen-fragments linked to MHC class I and II (Jhunjhunwala, Hammer,

and Delamarre 2021). Besides their stimulatory function via direct receptor-ligand interactions, DCs and macrophages release cytokines and complement factors mediating inflammation and attracting and activating other effector cells.

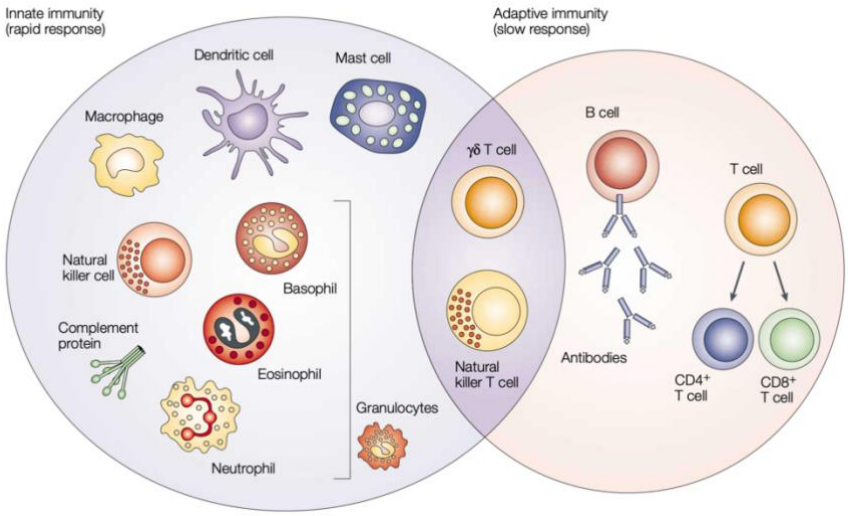


Figure 1.1

This figure shows typical immune cell types of the immune system.
DCs, Macrophages, NK cells as well soluble components and Granulocytes represent the innate immune system.
The adaptive immune system mainly consists of B and T lymphocytes.
γδ T cells and NKT cells can be classified as somewhere in between both of those systems as they show typical features of the innate as well as the adaptive immune cells

Adopted from Glenn Dranoffs Review "CYTOKINES IN CANCER PATHOGENESIS AND CANCER THERAPY" (Dranoff 2004)

2.1.2 The adaptive immune system

Despite a longer activation time, the adaptive immune system provides a highly effective defense against foreign antigens through its acquired, very specific immune response. As the sharp sword of a sophisticated immune reaction, it consists of lymphocytes and antibodies.
Naïve (non-activated) B and T lymphocytes are distributed within the bloodstream and many tissues but mostly in the lymphatic system (Alberts et al. 2002). T lymphocytes can be roughly subdivided into $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. Cytotoxic T cells are activated by APCs that present antigens bound to MHC class I and simultaneously transmit costimulatory signals. Because most body cells express intracellular antigens via MHC class I on their surface, $CD8^+$ T lymphocytes can detect infected or mutated cells and eliminate them by secreting cytotoxic granules or by FAS-ligand-induced apoptosis (Raskov et al. 2021). $CD4^+$ T helper cells recognize MHC class II presented antigens on APCs and are activated by them via costimulatory signals like CD28 (Zhu 2018). $CD4^+$ T lymphocyte activation results in clonal

expansion and differentiation into pro-inflammatory and anti-inflammatory subsets regulating the immune response (Zhu 2018). While $CD4^+$ regulatory T cells prevent the immune system from overshooting, $CD4^+$ T helper cells play essential roles in releasing cytokines and activating other T- and B-lymphocytes. B-cell activation requires an initial signal by direct specific antigen contact with their B-cell receptor (BCR). After BCR mediated endocytosis and lysosomal fragmentation of the antigen, B cells present antigen fragments via MHC class II on their surface. $CD4^+$ T cells then bind to MHC class II presented antigens with their T cell receptor and activate the B cells via additional costimulatory signaling like CD40-CD40L interactions and IL2,4, and 5 release (Storey and Jordan 2008; Rincon-Arevalo et al. 2024). Activated B cells clonally expand and by maturing into antibody-producing plasma cells they represent the cornerstone of the humoral adaptive immune response. These antibodies, which bind to specific epitopes on the antigen surface, attract and activate effector cells to their site and additionally induce the complement factor cascade (Goldberg and Ackerman 2020).
Both activated T and B lymphocytes are also developing into memory cells that induce rapid immune response in case of re-contact with the same antigens and thereby bypass the time-consuming activation cascade of the adaptive immune system.

2.2 Modification of the immune system

The competences of the immune system are therapeutically used for hundreds of years. Since the time when variolations and later vaccinations against the smallpox were applied, to save millions of lifes, the way was smoothed for the development of modern medicine (Boylston 2012). Even though the immune system is highly complex, modern research gains delicate insights into the roles and functions of thousands of cell types, receptors, small molecules or intracellular signaling pathways.
Vaccinations are one of the oldest ways to directly influence the immune system. By simply presenting specific antigens like weakened pathogens or synthesized particles to the immune system, an adaptive immune response is induced (Boylston 2012; Xing et al. 2025). Due to its capability of memorizing antigen contact, the primed immune system constitutes a very effective defense line against re-contact with that specific antigen (Zhang and Zhang 2020; Ayodele and Razak 2020).
Using deeper knowledge about how the immune system works, it was not only possible to specifically stimulate the immune system, but also to inhibit immune reactions to allow patients suffering from rheumatic diseases to live without pain or to enable allogenic organ transplantation without rejection (Burmester et al. 2017; I. Husain and Luo 2024). Besides immune inhibitory glucocorticoids and calcineurin inhibitors, other small molecules like cytostatic agents are used to suppress immune cell development. The modern use of

monoclonal antibodies against proinflammatory molecules or receptors, makes it possible to precisely inhibit certain mechanisms within the immune system and therefore bypassing certain serious side effects (Dufresne and Brahmi 2020; Galvez-Cancino et al. 2024).
In modern oncology, modulating the immune system in various ways has become a game changing cornerstone in antitumor treatment. In this context the following approaches are of particular importance heralding a paradigm shift in cancer therapy towards personalized treatment:

1. The deployment of artificially designed and produced monoclonal antibodies to precisely interfere with specific cellular signaling steps, or to label and eliminate cells with unique surface characteristics (Galvez-Cancino et al. 2024)
2. The use of small molecules to interact for example with immune checkpoints or tyrosine kinases to modify signaling pathways or to induce or attenuate epigenetic modifications (Cheng et al. 2025) Besides being way cheaper to produce, those drugs superceed monoclonal antibodies in many ways: They provide better tissue penetration and extra- and intracellular effects due to more than 100 times smaller sizes. Additionally they show better penetration of the blood-brain barrier and less immunogenicity. (Srivastava, Saxena, and Saxena 2024; X. Wu et al. 2024).
3. With cellular therapies it is possible to use living therapeutics that can adapt, replicate and combat cancer cells precisely.
   The most obvious approaches use extracted patient-own immune cells.
   Those cells are expanded, stimulated ex vivo and retransferred as so-called tumor infiltrating lymphocytes (TILs) to formerly lymphodepleted patients to enhance their efficacy (Rosenberg, Parkhurst, and Robbins 2023; König et al. 2024).
   By the use of genetical engineering techniques it is also possible to alter the efficacy of immune cells: In this way for example T cells can be modified to express chimeric antigen receptors (CAR) that don't require additional costimulatory signaling to be activated.Those so-called CAR T cells show striking antitumor effects and are commonly used, for example, against B cell malignancies. By the use of AI integration and synthetic biology, the field of designing cell based immunotherapy has expanded drastically. Supported by those kind of new technologies, increasingly robust and specific cell therapies are being synthesized and successfully used against broad varieties of cancer (Ali and DiPersio 2024; Strzelec and Helbig 2024; Boretti 2024).
4. Another very innovative approach in combating cancer is the use of vaccines that are specifically designed with the help of data from genome sequencing. Cancer vaccines aim to deliver specific cancer antigens to antigen-presenting cells, which in turn, as the most potent T cell activators, can trigger a strong immune response against tumour

tissue. Those vaccines that are often mRNA based and show beneficial responses against different malignancies such as Melanoma, NSCLC, Prostate cancer (Pail et al. 2025). The use against soft tissue sarcoma has shown disappointing results und thus has to be further investigated (Wood et al. 2024).

5. The concept of targeted drug delivery has been developed to highly precisely battle malignant cells and reduce collateral damage. Therefore cytotoxic agents, or radiotherapeutics or other effector drugs are hinged to monoclonal antibodies that provide cell damage only in certain tissue. Anti cancer drugs can also be encapsuled in lipide layers such as micelles or within nanoparticles and thus be pooled in certain tissues based on enhanced permeability or retention (Akram et al. 2025).

   Those kind of therapeutics show improved antitumor effects benext to decreased side effects compared to therapeutic standard therapies against multiple hematologic malignancies, different types of HER2 positive cancer, bladder cancer, oesophageal cancer and others (Etrych et al. 2022; Liu et al. 2024; Akram et al. 2025).

2.3 The tumor microenvironment

Cancers are not just conglomerates of malignant cells. Many different cell types such as fibroblasts, endothelial cells, or immune cells are components of malignant tumors. Interacting with the non-malignant cells via receptors and extracellular components such as cytokines or growth factors (Smolle et al. 2021), the cancer cells hijack them for nourishment, protection or other predominantly tumor-promoting reasons (Balkwill, Capasso, and Hagemann 2012). Those cooperating and self-promoting networks including all changes in biochemical conditions are known as the tumor microenvironment (TME) and depict an essential role in cancer development. Various oncogenic mutations such as RAS- or MYC- overexpression activate inflammatory cascades that induce a growth promoting environment which in turn shows mechanistic resemblances to wound healing (Balkwill, Capasso, and Hagemann 2012; M. Wang et al. 2024). Tumor associated fibroblasts for example are being stimulated by the cancer cells via cytokines (like IL1 or IL6), growth factors (such as TGFβ) or other stimuli. In return they promote cancer cell development by releasing mitogenic agents and remodel the extracellular matrix, favoring cancer cell invasion (Sahai et al. 2020; M. Wang et al. 2024).

The quantity and composition of TME-infiltrating immune cells strongly differ within different tumor entities as well as within individual tumors and metastases.

The increased presence of certain cell types like tumor associated macrophages (TAMs) are associated with tumor-promoting effects whereas for example dendritic cells or high amounts of B lymphocytes within the TME seem to come along with more favorable outcomes for the patients (Qian and Pollard 2010; Petitprez et al. 2020; Kirchner et al. 2024).

Also disturbed intercellular signaling and decreased cellular antitumor efficacy plays a significant role in how the TME affects tumor development.

The increased expression of immune checkpoint receptors like programmed cell death protein 1 (PD-1) on the surface of immune cells or the enhanced expression of its ligands (for example PD-L1 and PD-L2) on malignant cells, lead to mitigated immune response.

The PD-1 activation on T lymphocytes leads to phosphorylation of its two cytoplasmic tyrosine-based signaling motifs. Thus triggered, the recruitment of SHP 1 and SHP 2 proteins consequently attenuates the T cell activation (Sun, Mezzadra, and Schumacher 2018).

Similar effects are shown by the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) that is expressed on T lymphocytes interacting with its ligands CD80 and CD86 which are expressed by antigen-presenting cells (like DCs, B lymphocytes or macrophages). Due to its higher affinity to CD80 and CD86, this ligand-receptor contact inhibits T cell activation by CD80/CD86-CD28 interactions. Moreover, the formation of a CD80/CD86-CTLA-4 complex leads to antiproliferative effects on T lymphocytes and to inactivation of APCs (D. S. Chen and Mellman 2017; Franzese 2024).

These immune checkpoint mechanisms are physiologically important to prevent immune responses from overshooting. PD-L1 expression for example can be shown on hematopoietic cells and other blood cells, as well as on vascular endothelial cells, pancreatic island cells or on syncytiotrophoblast cells of the placenta (Sun, Mezzadra, and Schumacher 2018).

The exploitation of these important immunomodulatory signaling pathways by cancer cells plays a significant role for the tumor immune escape.

There is growing evidence for the prognostic value of many immune cells and soluble factors that can be found in the TME.

Besides immune cells like B cells or DCs that are associated with good prognosis (Petitprez et al. 2020; Sousa et al. 2021; Kirchner et al. 2024) or TAMs, whose presence indicates unfavorable outcomes for patients (Dancsok et al. 2020) there are numerous immunologically active molecules showing significant impact on tumor growth. Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) promote metastasis spread, tumor growth and angiogenesis (Masaru Katoh and Hitoshi Nakagamaim 2013). Seo et al. found high levels of IL6, and IL8 correlated with decreased survival rates (Seo et al. 2002).

The numerous aspects and the outstanding complexity of the TME is still subject of extensive research and plays a key role in cancer growth kinetics, metastasis formation, effective antitumor treatment or therapy resistance (Jin and Jin 2020; El-Tanani et al. 2024).

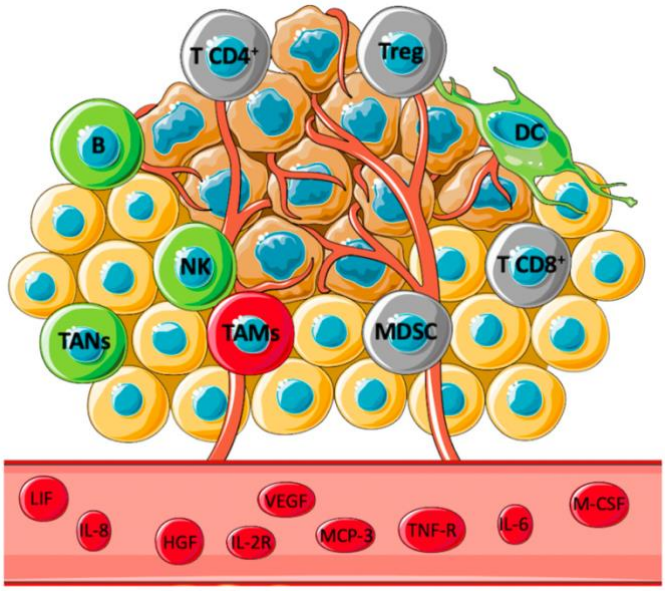


Figure 1.2

This figure displays TME infiltrating immune cells.
The prognostic value of those cells penetrating the tumor environment is labeled by color code:
Green: Immune cells associated with favorable prognosis (B cells [B], dendritic cells [DC], Natural killer cells [NK], Tumor Associated Neutrophiles [TANs],
Red: Immune cells / soluble factors associated with unfavorable influence on prognosis (Tumor Associated Macrophages [TAMs] / Leukemia Inhibitory Factor [LIF], IL8, Hepatocyte Growth Factor [HGF], IL2R, Vascular Endothelial Growth Factor [VEGF], Macrophage/Monocyte Chemotactic Protein 3 [MCP 3], Tumor Necrosis Factor R [TNF R], IL6, Macrophage Colony Stimulating Factor [M CSF])
Grey: Immune cells whose impact on prognosis is currently unclear.

Adopted from Sousa et al. "Tumor and Peripheral Immune Status in Soft Tissue Sarcoma: Implications for Immunotherapy" (Sousa et al. 2021)

2.4 Modifying the tumor microenvironment

One of the biggest challenges in antitumor therapy is to overcome immunosuppressive mechanisms within the TME.

As already mentioned above (1.2), several approaches have been discovered to modulate the immune system and many of them are used successfully in cancer treatment (Dufresne and Brahmi 2020; Hou, Chen, and Chen 2021; Srivastava, Saxena, and Saxena 2024; Wood et al. 2024).

In modifying the TME, the following approaches are of particular importance:

First, there are immunotherapeutics interacting with immune checkpoints.

In 2018 James Patrick Allison and Tasuku Honjo were awarded the Nobel Prize for their groundbreaking discoveries of immune checkpoints in 1995 and following (Ishida et al. 1992; Freeman et al. 2000; Iwai, Terawaki, and Honjo 2005).

Since then, this knowledge was harnessed to develop immune checkpoint inhibitors: Monoclonal Antibodies or small molecules that function by blocking CTLA-4 (e.g. Ipilimumab, Tremelimumab [not yet approved]), PD-1 (for example Nivolumab and Pembrolizumab) or PD-L1 (e.g. Atezulizumab, Durvalumab) and therefore inhibit the attenuation of the antitumor immune response. Even though these drugs show good efficacy against some cancer entities like melanomas or renal cell carcinoma, many other malignancies can hardly be tackled by them due to primary or acquired resistances (Keung et al. 2018; Cheng et al. 2025).

For several years, there are also therapeutics against other immune checkpoints (like TIM-3 or LAG-3) under investigation (Gaynor, Crown, and Collins 2022).

Apart from that it is possible to stimulate antigen-presenting cells such as dendritic cells using TLR-agonists, small molecules that interact with Toll-like receptors (TLRs) in an agonistic manner. TLRs are extracellular (TLR-1,-2,-4,-5,-6,-10) - or intracellular (TLR-3,-7,-8,-9) receptors and function as pattern recognition receptors (PRRs). Those receptors can register so-called PAMPs (pathogen-associated molecular patterns) – which are molecular structures (e.g. lipids, lipoproteins, nucleic acids), foreign to the immune system (Kawai and Akira 2007; Kaczanowska, Joseph, and Davila 2013; Jeon et al. 2024).

The stimulation of both extra- or intracellularly located TLRs activates intracellular signaling including the NFκB pathway or MAPK derived cascades that result in the altered expression of proinflammatory proteins (like cytokines or INFα) (Kawai and Akira 2007). Stimulating TLRs is an elegant way to activate APCs and thereby enhancing the immune response against malignancies.

Another approach to induce effective antitumor immune response in the TME is the adoptive cellular therapy (ACT). After isolating patient's own immune cells they are expanded and stimulated or modified ex vivo. As "living drugs" those cells are reinfused into the patient to directly tackle the malignant tissue.

Although these methods show remarkable success against some cancer entities, there are problems to impede the comprehensive use in modern cancer treatment.

When using ACTs, especially TILs, against solid tumors, the effector cells often show low survival rates after transfusion and poor homing and reproduction tendencies resulting in limited persistence (Mata and Gottschalk 2015; Bear et al. 2021; König et al. 2024).

To overcome therapy specific weak spots, combining immunotherapies appears to be a promising approach to provide more compatible and effective therapeutic alternatives to conventional, highly toxic approaches and probably vanquishing more and more malignancies in the future.

2.5 Soft tissue sarcoma

Sarcomas are a group of rare mesenchymal malignancies, representing about 1 % of all malignant neoplasms in adult patients (Bourcier et al. 2019; The Cancer Genome Atlas Research Network 2017; Tseng, Somaiah, and Engleman 2014). Sarcomas can be divided into two main groups: Bone sarcomas and soft tissue sarcomas (STS) of which the latter accounts for about 86 % (Stiller et al. 2013). Showing incidence rates of 1,1-5,6 per 100.000 in 2013 in Germany (Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft, Deutsche Krebshilfe 2021) soft tissue sarcomas are a very heterogeneous group of cancer in terms of localization, histological features and prognosis. About 70 different subtypes are defined histologically and can emerge virtually everywhere within the body including fatty tissue, muscle tissue, fibrous tissue, from blood vessels or nervous tissue (Bourcier et al. 2019).

Even though the mutational load and the number of potentially targetable genetic alterations in soft tissue sarcomas are generally various, there might be other variables predicting favorable outcome and response to immunotherapeutic interventions. Petitprez et al. invented an immune classification for soft tissue sarcomas based on the histological composition and vasculature of the TME.

They found that a generally high expression of immune cell related gene signatures is being favorable for the patient outcome. More precisely, especially the high expression of B lineage signature, the altered presence of DCs and the presence of tertiary lymphoid structures (TLS) within the tumor tissue, were associated with beneficial response to immunotherapeutics and better overall survival (Petitprez et al. 2020)

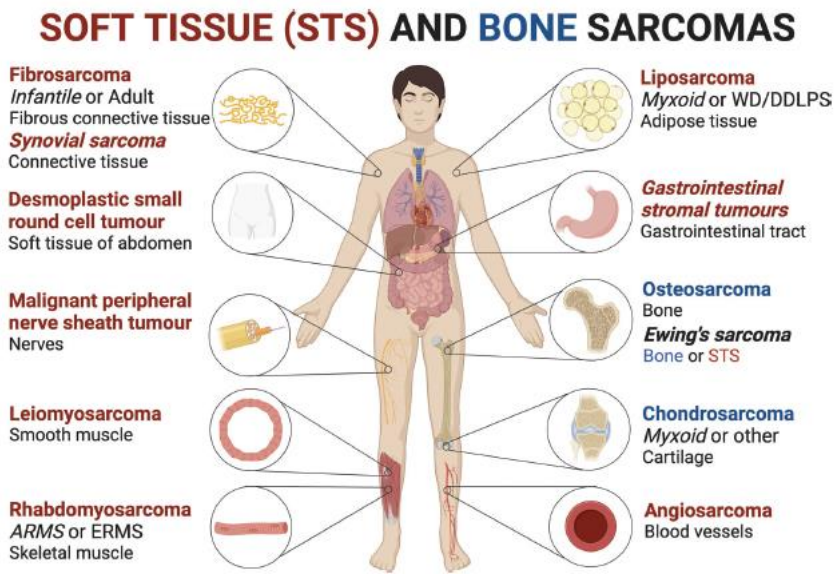


Figure 1.3

Picture showing the most common locations and exemplary entities for soft tissue sarcomas (red) and bone sarcomas (blue) respectively.
ARMS - Alveolar rhabdomyosarcoma; ERMS - Embryonal rhabdomyosarcoma; WD- /DDLPS - Well differentiated-/ Dedifferentiated liposarcoma and pleomorphic sarcoma

Adopted from Demerell, Pepper and Prince "Molecular mechanisms underpinning sarcomas and implications for current and future therapy" (Damerell, Pepper, and Prince 2021)

2.6 Combating soft tissue sarcoma

2.6.1 Chemotherapy

It is important to note that the distinct heterogeneity of soft tissue sarcoma makes it very difficult to define accurate incidence or mortality rates and currently almost unthinkable to find a therapy regimen to tackle every entity with acceptable efficacy.

The main pillar in soft tissue sarcoma treatment nowadays is the surgical intervention aiming for the residue-free removal of the tumor. If a complete resection isn't achievable, the overall survical decreases drastically to a median survival rate of about 12 months under anthracycline-based chemotherapy (Italiano 2020).

Additionally radiation therapy or special treatments like isolated limb perfusion are playing a role in combating soft tissue sarcoma (Bourcier et al. 2019).

Doxorubicin-based sarcoma treatment was first approved in 1974, and since then, only sligh improvement could be achieved by new therapeutic approaches for most unresectable soft tissue sarcoma.

Doxorubicin is a cytostatic drug that intercalates into DNA and therefore inhibits the topoisomerase II-DNA complex inducing double-stranded breaks which in turn impede RNA translation and transcription (Agudelo et al. 2016). Due to the nonspecific cytotoxicity, severe

side effects occur and high and very toxic doses have to be administered to induce an adequate antitumor response.

The current standard chemotherapeutic regimen, which includes doxorubicin with or without ifosfamide or dacarbazine prolongs the time to tumor relapse, but there is no unambiguous evidence for significant improvement in overall survival (Pervaiz et al. 2008).

2.6.2 Immunomodulatory therapy

Genetic analysis as a milestone in molecular diagnostics nowadays is routinely performed in addition to histological examinations. Even though targetable oncogenic gene aberrations in soft tissue sarcoma are rare, fundamental research unraveled different tumor-promoting mutations and based on those insights there are many attempts to deploy immunotherapeutic regimens against different soft tissue sarcoma entities:

The overexpression of EZH2 has been detected in some soft tissue sarcoma subtypes like clear cell sarcomas of the kidney, rhabdomyosarcoma, rynovial sarcoma or epithelioid sarcoma (Hoy 2020; Italiano 2020; Ma et al. 2020; Mart et al. 2020).

Based on these findings, EZH2 inhibitors have been tested to combat those tumors, demonstrating that epithelioid sarcomas showed a beneficial response to the EZH2 inhibitor tazemetostat (Hoy 2020; US Food and Drug Admisitration (FDA) 2020).

Those results show that modulating the epigenetics has the potential to play a role in future sarcoma treatment against certain entities of soft tissue sarcomas.

Immune checkpoint inhibition is a revolutionary technique with great successes against some cancer entities.

Also, in soft tissue sarcoma treatment, immune checkpoint blockade came into focus of research. Unfortunately, there is not much data due to the rareness and heterogeneity of soft tissue sarcoma, but several phase 2 studies are in progress, about to be published within the next view years. The data that could be acquired so far, is contradictory regarding the effectiveness of immune checkpoint inhibition against different types of soft tissue sarcoma (D'Angelo et al. 2018; Keung et al. 2018).

In metastatic undifferentiated pleomorphic sarcoma, dedifferentiated liposarcoma and GIST the MOS was improved when treated with nivolumab in combination with ipilimumab. However, the improvement in MOS remained within reasonable limits as when treated with nivolumab only 6,6 months and 15,2 months when treated with nivolumab combined with ipilimumab. (Seligson et al. 2024).

Tawbi et al. showed successful treatment with pembrolizumab of undifferentiated pleomorphic sarcoma, dedifferentiated liposarcoma and synovial sarcoma, achieving at least partial

response in 18 % of the cases even though altered PD-L1 expression could be observed in only 5 % of the cancers within the study (Tawbi et al. 2017).
In comparison to those promising data synovial sarcoma seems not to respond to CTLA-4 inhibition with Ipilimumab: Only 4 of 6 patients included in a small pilot study could even receive the intended 3 doses of Ipilimumab and all of those patients featured progressive disease during and after treatment (Maki et al. 2013; Petitprez et al. 2020). There are some ongoing clinical trials that explore the combination of pembrolizumab and cabozantinib (a tyrosinekinase inhibitor) in patients with advanced sarcomas (NCT05182164).

2.6.3 Cellular immunotherapy

High levels of tumor-infiltrating lymphocytes (TILs) are known to have a potentially beneficial effect on patient survival outcomes in many cancer entities like urological malignancies, Ewing sarcoma or retroperitoneal sarcoma (Berghuis et al. 2011; Miyake et al. 2020). Due to insufficient activation or tumor penetration, these cells are often limited in their ability to exert their full antitumor potential.
TILs can either be extracted from tumor tissue, expanded, and stimulated invitro or cultured from PBMCs that are activated by certain antigen presentation invitro.
After lymphodepletion, reinfusion of these cells may overcome the inactivation of these effector cells, making it a potential way to treat malignancies.
As the efficacy of such TIL transfer has been proven for malignancies like metastatic melanoma (Rosenberg et al. 2011; Miyake et al. 2020), it may also show beneficial yet limited effect on soft tissue sarcoma (Wood et al. 2024).
To avoid the dependence of HLA restriction, NK cell-based Immunotherapy is another promising approach.
Ratnavelu et al. described impressive results in his case report about a man with therapy refractory, locally advanced epithelioid sarcoma. (S. Chen, Zhu, and Jounaidi 2024) By reinfusing NK cells, produced from patient's own peripheral blood, following extraction, stimulation and propagation, his team could not only obtain improvement in quality of life but also force the sarcoma into stable disease and thus enhance the patient's prognosis (Ratnavelu et al. 2013).
CD3 / CD56 double-positive, natural killer group 2 member D receptor (NKG2D) mediated cytokine-induced killer cells (CIKs) can be used to combat cancer. CIKs have been used against several cancer entities with varying but partly high therapeutic success rates in murine models as well as in human trials (Sangiolo et al. 2014; Cappuzzello et al. 2023; Jiang et al. 2024).

Ishihara et al. treated patients with NY-ESO-1 expressing sarcoma, breast cancer, ovarian cancer and malignant salivary tumor with NY-ESO-1-specific TCR bearing, engineered T cells. They discovered significant tumor response especially in synovial sarcoma that expressed high levels of NY-ESO-1 (Ishihara et al. 2022).
In 2024 Afamicel, a MAGE-A4-Protein targeting, engineered T cell immunotherapy, was approved by the FDA for metastatic synovial sarcoma. It showed a response rate of 38.6 % with a median duration time of 11.2 months (Chawla et al. 2025).
Ongoing trials are investigating the effects of EGFR-specific and CAR T cell therapy against different types of sarcoma (NCT03618381 and NCT03635632). In the NCT01803152, NCT00923351, NCT00405327 and NCT02496520 clinical trials, autologous dendritic cell vaccination is used against different types of sarcoma (Chawla et al. 2025).

2.6.4 Immunotherapy combinations

As many types of soft tissue sarcoma are considered immunogenically “cold” tumors, the use of only one therapeutic approach often proved to be insufficient.
The combination of different therapeutical modalities opens an almost endless variety of promising therapeutic approaches that could potentially develop synergistic effects and successfully overcome tumor immune escape mechanisms.
There are several trials ongoing, that investigate the combination of different therapeutic approaches with immunotherapy (M. Husain et al. 2023).
For example a randomized phase I/II trial is testing the combination of doxorubicin and pembrolizumab in advanced anthracycline-naive sarcoma. It showed a median progression-free survival of 8.1 months (95% CI, 7.6–10.8) and median overall survival of 27.6 months (95% CI, 18.7–not reached) at the time of analysis. 2/3 of Patient with undifferentiated pleomorphic sarcoma and 1/2 of Patient with dedifferentiated liposarcoma showed durable partial response (Pollack et al. 2020).
In the NCT03967223 trial TCR-T cell therapies targeting NY-ESO-1 (a human cancer / testis antigen) in combination with nivolumab or ipilimumab are tested against synovial sarcoma or myxoid/round cell liposarcoma aiming to improve antigen-specific T cell activity and to overcome tumor-induced immunosuppression.
An oncolytic virus (T-VEC) is combined with pembrolizumab against advanced sarcoma in the NCT03069378 phase II clinical trial.
According to Chu et al. the administration of IL15 combined with NK based adoptive cellular immunotherapy and dinutuximab (a monoclonal antibody against GD2 - a disialoganglioside expressed on neuroectodermal tumors) leads to significant inhibition of osteosarcoma cell

growth and improves the survival in osteosarcoma xenografted mouse models (Chu et al. 2021).

2.7 Preliminary work

Borchmann et al established the so-called TRI score to classify the immune infiltration of solid tumor TMEs by the presence of T cells, NK cells and γδ-T cells. One point was given for the presence of one of the described immune cell types.
It could be shown that especially the TME infiltration of all three immune cell types lead to significantly increased survival rates, especially in sarcoma patients (Fig. 1.4).

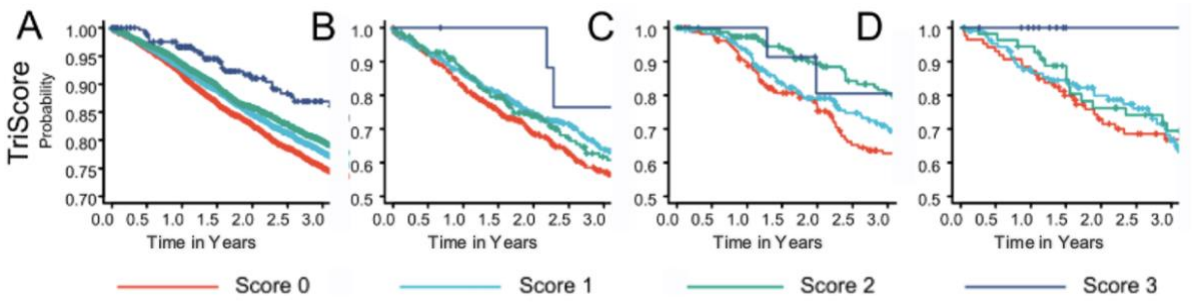


Figure 1.4

This figure shows survival rates depicted as Kaplan Meyer plots depending on the TME infiltration by T cells, NK cells and γδ-T cells in different human solid cancers (A), lung cancer (B), melanoma (C) and sarcoma (D) patients.
The Tri Score (Score 0- 3) describes the TME immune infiltration with T cells, NK cells and γδ-T cells. One point was given for the presence of each immune cell type respectively.

Adopted from Journal for ImmunoTherapy of Cancer "Tripartite antigen- agnostic combination immunotherapy cures established poorly immunogenic tumors" (Borchmann et al. 2022)

To mimic that infiltration pattern and achieve an optimal antitumor effect, a novel tripartide immunotherapy was developed consisting of four different ACT effector celltypes (LAKs, CIKs, CTLs and γδ-T cells), that were administered after a pre ACT lymphodepletion and combined with a mix of TLR-3, -7 and -9 agonists and anti PD-1 antibodies.
LAKs are IL2 stimulated lymphocytes (NK cells and T cells) that derive from peripheral blood. Eventhough there have been some postivive effects in clinical trials in human for example against lung cancer and sarcomas, the side effects have often shown to be severe due to the high dosages of IL2 that were administered in the therapeutic regimens.
Moreover, the antitumor effects were disappointing in most studies in vivo. Therefore LAKs

are not commonly used in modern oncologic treatments (Zhou et al. 2013; Ganina et al. 2024).

CIKs are $CD3^+$ T cells, $CD3^+CD56^+$ NKT like cells, and NK like cells that are activated from peripheral blood lymphocytes, by the use of anti-CD3, IL-2, IFNγ. As they exert broad MHC-unrestricted cytotoxicity, many studies have found good antitumor effects for example against lung cancer, HCC and renal cancer (Jiang et al. 2024; Zhong, Tang, and Wang 2024; Cappuzzello et al. 2023).

TILs are lymphocytes that are activated by cancer tissue and can either be harvested from tumors or bred ex vivo from Immune cells and tumor tissue.

Often tested in comparison to or in combination with aPD-1 inhibitors against melanoma, good improvements of survival rates could be shown in many trials (Rosenberg, Parkhurst, and Robbins 2023; Rohaan et al. 2022). In an ongoing Phase II clinical trial (NCT03449108), TILs are tested against different refractory or relapsed solid cancer entities such as Breast- and ovarian cancer as well as bone- and soft tissue sarcomas.

$\gamma\delta$-T cells are a lymphocytes subset that has often proven to be highly effective in killing infected or neoplastic cells by direct elimination. Their activation occurs rapidly, without prior antigen contact and is induced via a variety of tumor ligants.

As the $\gamma\delta$-T cell activation is not restricted to HLA recognition, it can overcome tumor immune evasion mechanisms. Furthermore, the stimulation of IFNγ and TNFα and the interconnection of $\gamma\delta$-T cells with other immune cells create a tumor suppressive microenvironment. Therefore theoretically they are an extremely interesting candidate for future cell based antitumor therapies (Chan et al. 2022; C. Q. Wang, Lim, and Tan 2023).

$\gamma\delta$-T cells have thus been tested agains many malignancies but despite the favorable safety profile, those cells alone could not show promising antitumor effects. There are some ongoing clinical trials, testing the effects of $\gamma\delta$-T cells against B cell malignancies and different types of solid malignancies. Also those special T cell subsets are tested against different tumors in in some combination therapy regimens. (Subhi-Issa et al. 2025)

Those 4 different effector cell types have shown synergistic effects against different solid malignancies. This could be proven by in vitro experiments and later shown to be applicable to in vivo mouse models by Borchman et al (Borchmann et al. 2022).

The Application of TLR3 agonist could be proven to induce an inflammative antitumor immune response that leads to an autovaccination, increased immune cell proliferation, $\gamma\delta$-T cell toxicity and excretion of IFNγ and TNFα (Salazar et al. 2014). TLR7 agonists lead to increased IL2 and IFNγ production and enhances the immune cell invasion in the TME (Frega et al. 2020) TLR9 improves the expression of IL2 and the synthesis of the IL2R.

Moreover, it stimulates $CD4^+$ T cell function and inhibits the influence of regulatory T cells (Gallotta et al. 2018; Kaczanowska, Joseph, and Davila 2013).
Borchmann et al. demonstrated that the comprehensive application of all four effector cell types in the adoptive cell therapy (ACT) resulted in significantly improved survival and response rates across various solid tumor mouse models, compared to regimens incorporating only one, two, or three of these cell types. The additional administration of aPD-1 inhibitors and the TLR agonist mix could be shown to further significantly improve the antitumor effects.
The combination immunotherapy led to massive tumor shrinkage in melanoma- and lung cancer mouse models as well as in breast cancer and soft tissue sarcoma mouse models. (Borchmann et al. 2022).
The scientific work behind this doctoral thesis was developed from the idea to further explore the effects of this new therpeutic approach in murine soft tissue sarcoma in vitro and in vivo. The existing limitations, potential strategies to overcome those, and the opportunities for further investigation in the context of human soft tissue sarcoma should be analyzed.

2.8 Research questions and Hypotheses

As there is increasing evidence for effective immunotherapeutic interventions to combat soft tissue sarcoma, this dissertation aims to gain additional knowledge about the complex cellular interactions within the sarcoma, more precisely, the undifferentiated pleomorphic sarcoma (UPS) TME.
Additionally, the beneficial or detrimental effects of modifying the TME via immunotherapeutic combination therapies on undifferentiated pleomorphic sarcoma mouse models should be explored.
These objectives were converted into the following research questions:

- Does classical doxorubicin treatment affect undifferentiated pleomorphic sarcoma cell line proliferation?

  Hypothesis 1: *Doxorubicin significantly affects undifferentiated pleomorphic sarcoma cell line proliferation in vitro*

- Can ACT and its cellular components mediate an antitumor response in undifferentiated pleomorphic sarcoma cells in vitro?

  Hypothesis 2: *ACT components show antitumor response against undifferentiated pleomorphic sarcoma cells in vitro and there is an additional effect if the effector cells types are combined*

- Is the ACT treatment, as established in our study, associated with improved survival and tumor growth kinetics compared to standard chemotherapy regimens in KRAS trp 53 mutated undifferentiated pleomorphic sarcoma bearing mice?

  Hypothesis 3: *The ACT treatment can improve survival in our soft tissue sarcoma mouse models in comparison to standard chemotherapy regimens*

- What are the advantages of combining immunotherapeutic approaches for KRAS trp 53 mutated undifferentiated pleomorphic sarcoma bearing mice in terms of tumor growth kinetics and overall survival?

  Hypothesis 4: *The ACT combination therapy with antiPD-1 antibodies and TLR agonsists shows additional effects on improving survival and growth kinetics in comparison to ACT monotherapy*

- What changes are induced by ACT combination therapy in the TME in progressing and responding KRAS trp 53 mutated undifferentiated pleomorphic sarcoma in vivo?

  Hypothesis 5: *Mice, responding to our combination therapy, show an initial increase in general immune infiltration and -activation. Tumor escape mechanisms counteract those effects over time.*

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Cell lines

The tumorous cells that are subject of the following experiments are six murine soft tissue sarcoma cell lines.
The cell lines 403460, 403492, and 420085 originate from genetically engineered 129/SVJaeJ mice bearing floxed homozygous alleles of trp53 and heterozygous KRAS G12D knock in mutation. By injection of 25 µl of a solution containing adenovirus expressing Cre recombinase in the thigh muscle of the animal, a trp53 knock out, and KRAS knock in is achieved.
The cell lines 403765, 403692, and 403111 originate from 129/SVJaeJ mice also bearing Cre-inducible homozygous trp53 knock out alleles, but no further genetic modification. To induce tumor growth in those mice, sequential intramuscular injection of 25 µl of the same solution as mentioned above containing cre recombinase expressing adenovirus and 300 µg of MCA (3-methylcholanthrene) in the thigh muscle was necessary. (Lee et al. 2019; Wisdom et al. 2020)
These two approaches lead to the formation of cancer cells that can be classified as undifferenciated pleomorphic sarcoma cells by key features like pleomorphic spindle cells, high mitotic activity, necrotic areas and the absence of differentiation markers. The mutational burden in the KRAS induced sarcoma cell lines can be considered high, the mutational burden of the MCA induced sarcoma cell lines can be considered moderate.
For the experiments the cells were used at lowest possible passage.

3.1.2 Mice

Wildtype mice (mus musculus) with 129/SVJaeJ background were used to perform the experiments. The husbandry, breeding and therapeutic interventions were in accordance with FELASA recommendations and with approval of the local ethics committee of animal experiments (2018.A368).
Mouse breeding was conducted by our own researching group and the Cologne animal facility. Light conditions, day-night rhythm, temperature, food and water supply were kept as consistently as possible. Potentially distressing procedures are performed under local anesthesia which is performed by intraperitoneal injection of xylazine and ketamine or by insufflation of 2,5% Isoflurane. If an animal showed signs of suffering, pain or worsening of the clinical condition (criteria for this were defined in advance and monitored daily) the animal was euthanized by cervical dislocation.

A regular administration of painkiller was not necessary due to a expected low level of pain for the animals in the conducted experiments.
The 3Rs principle (Replace, Reduce, Refine) was applied during the planning of the experiment to minimize any form of avoidable suffering for living beings.

3.1.3 Cell culture materials

To culture the cells in fluid, adhesive cultures, the sterile nutrient medium based on DMEM (1X) + GlutaMAX™ -I (+4,5 g/L D-Glucose) from Gibco / Life Technologies contained 10 % FCS and 1 % of a Penicillin- Streptomycin mix [5.000 U/ml Penicillin + 5.000 µg/ml Streptomycin].
For culturing murine immune cell cultures the nutrient medium was based on a 1:1 composition of RPMI Medium 1640 (1X)+ GlutaMAX™ and DMEM/F12 (1:1) (1X) (+L-Glutamine + 15 mM HEPES). Additionally 10 % of FCS, 1 % of the Penicillin- Streptomycin mix [5.000 U/ml Penicillin and 5.000 µg/ml Streptomycin] and b2-Mercaptoethanol [0,05 mol/l] were added.

Material	**Producer**
anti-mouse CD28 Monoclonal antibody	BioLegend, San Diego, USA
anti-mouse CD3 Monoclonal antibody	BioLegend, San Diego, USA
ACK Lysing Buffer	Lonza Group AG, Basel, Swiss
anti-mouse gd TCR Monoclonal Antibody	BioLegend, San Diego, USA
Axiovert 40 C Microscope	Carl Zeiss AG, Oberkochen, Germany
Cell culture flasks, filter screw cap (T25/ T75/ T175)	Greiner Bio One GMBH, Leipzig, Germany
Cell Scraper with 2-Position Blade	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cellstar™ 96 well microplate, U-bottom (white/ clear)	Greiner Bio One GMBH, Leipzig, Germany
Cell Strainer 40 µm	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cell-Titer Glo Luminescent cell viability	Promega Co., Fitchburg, USA
Centrifuge Tube (15 ml/ 50 ml)	Corning Science Mexico, Reynosa, Mexico
CO2 Incubator	Binder GmbH, Tuttlingen, Germany
CRYO.S™ Cryovials	Greiner Bio One GMBH, Leipzig, Germany
DPBS	Thermo Fisher Scientific Inc., Waltham, USA
Eppendorf Centrifuge 5417 R	Eppendorf SE, Hamburg, Germany
Eppendorf Centrifuge 5702	Eppendorf SE, Hamburg, Germany
FCS	Thermo Fisher Scientific Inc., Waltham, USA
Dimethyl Sulfoxide - Freezing agent	Applichem GMBH, Darmstadt, Germany

Hidex Sense Multiplate Reader Hidex	Hidex Deutschland Vertrieb GmbH, Mainz, Germany
IFNγ	PeproTech Germany, Hamburg, Germany
IL2	Thermo Fisher Scientific Inc., Waltham, USA
IL7	Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany
Injekt-H 1ml Syringe, Luer Duo, 26 G	B.Braun SE, Melsungen, Germany
Integra Pipetboy 2	INTEGRA Holding AG, Wallisellen, Swiss
Luciferase assay system	Promega Co., Fitchburg, USA
Mars Biological safety cabinets	LaboGene, Lillerød, Dänemark
Cellstar™ 6 well plate	Greiner Bio One GMBH, Leipzig, Germany
Mitomycin	Medac GmbH, Wedel, Germany
Nanozoomer S360 Digital slide scanner	Hamamatsu Photonics, Hamamatsu, Japan
Petri Dishes (3,5 cm/ 6 cm/ 10 cm)	Sarstedt AG & Co. KG, Nümbrecht, Germany
PFA	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Pipetting Reservoir 50 ml, RNAse free	Antylia Sciantific, Vernon Hills, USA
Round bottom Falcon tubes 5 ml	Thermo Fisher Scientific Inc., Waltham, USA
Safe-Lock Tubes (1,5 ml/ 2 ml/ 5 ml)	Eppendorf SE, Hamburg, Germany
Serological Pipettes (5 ml, 10 ml, 25 ml)	Greiner Bio One GMBH, Leipzig, Germany
Stuart Digital water bath SWB6D	Antylia Sciantific, Vernon Hills, USA
Tazemetostat (EPZ-6438) EZH2-Inhibitor	Absource Diagnostics GmbH, Munich, Germany
Triton™ X100 Permeabilization detergent	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Trypan Blue Stain (0,4 %)	Thermo Fisher Scientific Inc., Waltham, USA
Trypsin-EDTA, Dissociating reagent 0,05 %	Thermo Fisher Scientific Inc., Waltham, USA
Virkon S Granulat	LANXESS AG, Cologne, Germany
Vortex Mixer VTX 3000 L	LMS Consult GmbH & Co. KG, Brigachtal, Germany
Vortex V1	Biosan, Riga, Latvia
VWR Pipet tips with filter (10 µl/ 100 µl/ 200 µl/ 1000 µl)	VWR International, Radnor, USA

3.1.4 Drugs

Drug	**Producer**
Dexpanthenol-ointment (Bepanthen®)	Bayer AG, Leverkusen, Germany
Carprofen	Pfizer GmbH, Berlin, Germany
Isoflurane Piramal [100 %]	Piramal Critical Care Deutschland GmbH, Hallbermoos, Germany
Doxorubicin [2 mg/ml]	Stadapharm GmbH, Bad Vilbel, Germany

anti-mouse PD-1	Bio X Cell, Lebanon, USA
Gardiquimod	Cayman Chemical, Ann Arbor, Michigan, USA
Poly I:C	Merck Millipore, Burlington, Massachusetts, USA
ODN-2395	IDT, Coralville, Iowa, USA
Cyclophosphamide	HEXAL, Holzkirchen, Germany
Fludarabine	Sanofi Genzyme, Paris, France
Rompun, Xylazin Injection solution [2 %]	Bayer AG, Leverkusen, Germany
Ketaset, Ketamin injections solution [100 mg/ml]	Zoetis Inc., Parsippany-Troy Hills, USA

3.1.5 Flow cytometry

The FACS buffer was based on PBS and contained EDTA [5 mM] and FCS [2 %]. The Fixation buffer was composed by adding PFA [1 %] to the FACS buffer. Permeabilization buffer was based on FACS buffer with Triton [0,1 %] added.

Cytometer	Producer
Cytoflex™ Flow Cytometer	Beckman Coulter, Brea, USA
Gallios™ Flow Cytometer	Beckman Coulter, Brea, USA

3.1.5.1 Cell culture analysis

Cytochrome	Target	Isotype	Dilution	Producer
Aqua	Zombie		1:200	BioLegend, San Diego, USA
FITC	anti-mouse CD274 (PD-L2)	rat	1:200	BioLegend, San Diego, USA
PerCP-Cy5.5	anti-mouse I-A/I-E (MHC II)	rat	1:100	BioLegend, San Diego, USA
PerCP-Cy5.5	anti-mouse H2Kb/H2Db (MHC I)	Mouse	1:100	BioLegend, San Diego, USA
PE-Cy7	anti-mouse CD273 (PD-L1)	rat	1:100	BioLegend, San Diego, USA

3.1.5.2 Murine tissue analysis

Cytochrome	Target	Isotype	Dilution	Producer
Alexa Fluor 700	anti-mouse F4/80	Rat	1:200	BioLegend, San Diego, USA
Alexa Fluor 700	anti-mouse IFNγ	Rat	1:200	BioLegend, San Diego, USA
Alexa Fluor 700	anti-mouse/human CD45R/B220	Rat	1:200	BioLegend, San Diego, USA

APC	anti-mouse CD366 (TIM3)	Rat	1:200	BioLegend, San Diego, USA
APC	anti-mouse CD86	Rat	1:200	BioLegend, San Diego, USA
APC/Fire 750	anti-mouse TCR$\gamma\partial$	armenian hamster	1:200	BioLegend, San Diego, USA
APC/Fire 750	anti-mouse Ly-6G/Ly-6C (Gr-1)	rat	1:200	BioLegend, San Diego, USA
APC/Fire 750	anti-mouse CD24	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 421	anti-mouse CD8a	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 421	anti-mouse CD192 (CCR2)	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 421	anti-mouse CD326 (Ep-CAM)	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 510	anti-mouse CD279 (aPD-1)	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 510	anti-mouse I-A/I-E	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 605	anti-mouse/human CD11b	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 785	anti-mouse CD4	rat	1:200	BioLegend, San Diego, USA
Brilliant Violet 785	anti-mouse CX3CR1	mouse	1:200	BioLegend, San Diego, USA
Brilliant Violet 785	anti-mouse CD19	rat	1:200	BioLegend, San Diego, USA
Brillliant Violet 605	anti-mouse Ki-67	rat	1:200	BioLegend, San Diego, USA
BUV 737	anti-mouse CD69	armenian hamster	1:200	BD Biosciences, San Jose/ Milpitas, Kalifornien
FITC	anti-mouse CD80	armenian hamster	1:200	BioLegend, San Diego, USA
PE	anti-mouse CD163	rat	1:200	BioLegend, San Diego, USA
PE	anti-mouse/rat/human FOXP3	mouse	1:200	BioLegend, San Diego, USA
PE	anti-mouse Galectin-9	rat	1:200	BioLegend, San Diego, USA
PE/Cy7	anti-mouse CD3epsilon	armenian hamster	1:200	BioLegend, San Diego, USA
PE/Cy7	anti-mouse 206	rat	1:200	BioLegend, San Diego, USA
PE/Cy7	anti-mouse CD274 (PD-L1)	rat	1:200	BioLegend, San Diego, USA
PE/Dazzle 594	anti-mouse CD49b	rat	1:200	BioLegend, San Diego, USA
PE/Dazzle 594	anti-mouse 11c	armenian hamster	1:200	BioLegend, San Diego, USA
PE/Dazzle 594	anti-mouse CD40	rat	1:200	BioLegend, San Diego, USA
PerCP/Cyanine 5.5	anti-mouse CD45	rat	1:200	BioLegend, San Diego, USA
UV	Zombie		1:1000	BioLegend, San Diego, USA

3.1.6 Statistics and Evaluation

Software	Producer
Kaluza Flow Cytometry Analyzing software	Beckman Coulter, Brea, USA
Prism 8	Graphpad Software, Inc., San Diego, USA
Excel	Microsoft Co., Redmond, USA
NDP view 2.0	Hamamatsu Photonics, Hamamatsu, Japan

3.2 Methods

Any work with the cell cultures were performed under as sterile and consistent conditions as possible. All work were performed under the compliance with S1 guidelines and under a biosafety cabinet. The operating ranges were cleaned and disinfected daily as well as before and after use and following every contamination.

3.2.1 In Vitro

3.2.1.1 Cell culture

All soft tissue sarcoma cell lines were cultured under as constant circumstances as achievable in adhesive fluid cultures. The T75 cell culture flasks were stored in the incubator at 37 °C and a CO_2 concentration of 5 % with high humidity. The cell lines were passaged every 2-3 days when reaching a confluency of about 80 %. Before starting to passage the cells, the 0,05 % Trypsin-EDTA dissociating reagent, the nutrient medium and the PBS was heated to 37 °C. The nutrient medium was aspirated and discarded. The cells were washed by rinsing 10 ml DPBS over the cells and panning gently. After taking off and discarding the DPBS, 2 ml of the 0,05 % Trypsin-EDTA dissociating reagent was distributed into the flasks, which were then incubated for 5 min until the cells were fully detached. By adding 8 ml of the nutrient medium, the Trypsin-EDTA was inactivated, and the cells could be flushed off. The cell dilution was resuspended and transferred to a 15 ml falcon tube, then centrifuged for 3 min at 800 rpm. The supernatant was discarded, and the cell pellet was resuspended in 5 ml nutrition medium. 10 % of the cell suspension was then transferred into a new T75 cell culture flask and 12 ml of nutrient medium was added.

3.2.1.2 Doxorubicin Cell viability assay with soft tissue sarcoma cell lines

Doxorubicin is the standard therapy for unresectable soft tissue sarcoma. To evaluate its killing effect in vitro on the 6 soft tissue sarcoma cell lines, a CellTiter-Glo toxicity assay was performed.

Therefore, the cells were harvested when attaining a confluency of about 80 %.

After discarding the medium and rinsing the cells with PBS, the cells were dissociated using 2 ml of Trypsin-EDTA, as shown above.

When fully detached, the cells were resuspended in 8 additional ml of nutrient medium and transferred into a falcon tube. The cells were then centrifuged at 800 rpm for 3 min.

After discarding the supernatant and resuspending the cells in 10 ml of nutrient medium, the cells were counted by using the Thoma-chamber.

To seed the cells, a white, non-transparent U-bottom 96 well plate was used.

In three consecutive rows (B,C,D or E,F,G) each well of the columns 2-11 was filled with 90 µl of a cell suspension containing 1500 cells. The outer wells were filled with 90 µl of nutritional medium containing no cells.

After incubating the seeded cells at 37 °C for 24 h, 10µl of doxorubicin dilutions in different concentrations were added (100 µM, 30 µM, 10 µM, 3 µM, 1 µM, 0,3 µM 0,1 µM, 0,03 µM, 0,01 µM and 0 µM (PBS only)) to columnwise well triplets respectively (B-D or E-G ; column 2-11) (see Illustration 1).

After Incubating the cells in the 96 well plates for another 48 h, 100 µl of the CellTiter-Glo-solution was pipetted into each well respectively.

The plate was left to equilibrate for 30 min in the dark at RT. The luminescence was then measured using the luminometer.

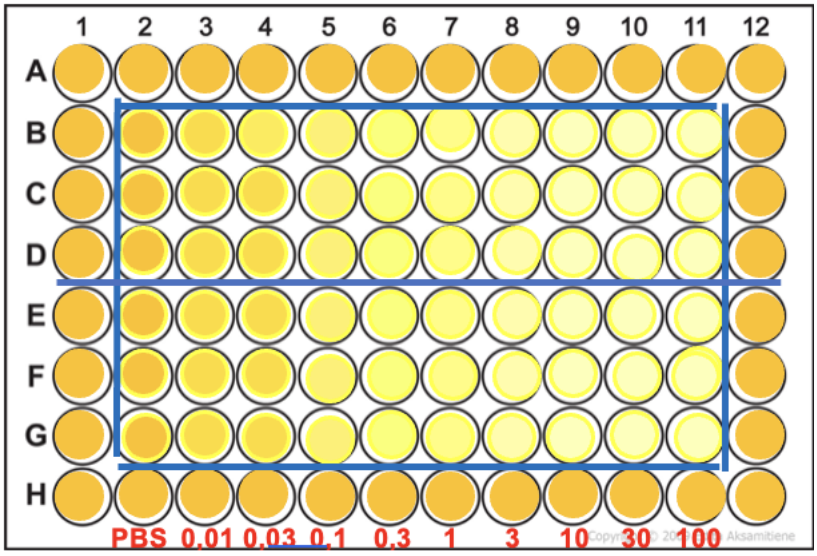


Figure 2.1

Schematic setup of the CellTiter-Glo toxicity assay on a 96 well plate to quantify the vularability of soft tissue sarcoma cell lines towards doxorubicin exposition.
The red labelling of the columns showing doxorubicin concentrations that were added to the wells respectively.
Two cell lines could be tested in every 96 well plate seeding the Rows B,C and D or E,F and G with cells.

3.2.1.3 Production of ACT components from murine 129/SVJaeJ spleenocytes

The protocols for the production of ACT effector cells were adopted from previous work were extensive testing and classification of the cells was carried out (Borchmann et al. 2022).
To cultivate the adoptive cellular therapy, wildtype 129/SVJaeJ mice at the age of 6-12 months were used. After cervical dislocation, the spleens were taken out and mashed through a 40 µm cell strainer. The cells were suspended in PBS and centrifuged at 400 G for 3 min at room temperature. After discarding the supernatant, ACK-lysing buffer was added and the splenocytes were resuspended and centrifuged at room temperature for another 3 min at 400 G. The supernatant was discarded and the cells washed with PBS and resuspended in the nutrient medium for culturing murine effector cells (composition shown above). After counting the cells using the Thoma counting chamber, the cells were transferred into different flasks to cultivate **4 different cell types** separately:

Cytokine-induced Killer cells (CIKs):
10 ml (containing 10^6 splenocytes) were transferred into T 75 cell culture flasks and 1000 U murine IFNγ/ml were added. At the same time 10 cm Petry dishes were covered with 5 ml PBS, containing 10 µg/mg anti-mouse CD3 antibody. The dishes and the flasks were put in the

incubator at 37 °C, CO_2 concentration of 5 % and high humidity for 24 h. The anti-CD3 covered plates were washed 5 times with PBS. The splenocytes were transferred from the flasks into the carefully rinsed petri dishes and incubated for 48 h at 37 °C, CO_2 concentration of 5 % and high humidity after adding 300 U IL2 / ml medium.
The differentiating and proliferating splenocytes were then allocated to T75 or T175 flasks, depending on the number of cells that had developed.
For the next 5 days the cells were observed and as the population grew, they were distributed to several flasks whereas corresponding amounts of medium and IL2 [300U/ml] was added. Every second day 300 U IL2 were added for every ml of medium in the flask.

Tumor-induced T cells (CTLs):
The day before harvesting the splenocytes:
1 x10^6 403460 undifferentiated pleomorphic sarcoma cells in a total volume of 10 ml of FCS-free sarcoma cell nutrient medium were transferred into T75 cell culture flasks and cultured for 24 h at 37°C, CO_2 concentration of 5 % and high humidity with 50g/ml mitomycin.
After harvesting the splenocytes:
The mitomycin containing medium was discarded and the adhesive tumor cells were carefully rinsed twice with PBS and twice with the nutrient medium for murine effector cells, to make sure there was no residual mitomycin in the flasks.
In each flask 10^6 splenocytes, IL2 [10 U/ml], IL7 [10 ng/ml], anti-mouse CD28 mAb [2µg/ml] murine anti-PD-1 Ab [20 µg/ml] and 10 ml of effector cell nutrient medium were added and they were put into the incubator at 37°C, CO_2 concentration of 5 % and high humidity.Over the next 7 days, the cells were observed and new medium and corresponding amounts of IL2 [10 U/ml] was added as needed.
Every second day 10 U IL2 were added for every ml of medium in the flask.

γδ-T cells:
10 cm petri dishes were coated with anti-mouse γδ-TCR mAb [10 µg/ml] in 5 ml PBS and incubated for 2 h at 37 °C, CO_2 concentration of 5 % and high humidity.
The dishes were then carefully rinsed 4 times with PBS and 10^6 splenocytes in 10 ml of nutrient medium were given onto each dish.
After adding IL2 [100 U/ml], the splenocytes were incubated for 72 h at 37°C.
The adhesive cells were then scraped off and transferred into T75 flasks. Over the next 5 days they were then cultured in the incubator at 37°C, CO_2 concentration of 5 % and high humidity. Medium and corresponding amounts of IL2 [100 U/ml] was added as needed. Every second day 100 U IL 2 were added for every ml of medium in the flask.

Lymphokine activated killer cells (LAKs)

10^6 splenocytes in 10 ml of nutrient medium were transferred into T75 flasks.

IL2 [6000 U/ml] was added and the flasks were put into the incubator at 37°C, CO_2 concentration of 5 % and high humidity.

Over the next 8 days, the cells were observed and as the population grew, they were distributed to several flasks whereas corresponding amounts of medium and IL2 [6000 U/ml] was added. Every second day 6000 U of IL2 were added for every ml of medium in the flask.

After 8 days the ACT was ready to inject and therefore harvested using the cell scraper. The cell solutions were centrifuged for 3 min at 800 rpm, the supernatant was discarded and the ACT pellets were resuspended in PBS.

The 4 cell types were counted separately using the Thoma chamber and afterwards mixed together in equal quantities. Every mouse should receive 2,5 Mio cells of every cell type (10^6 ACT cells in total).

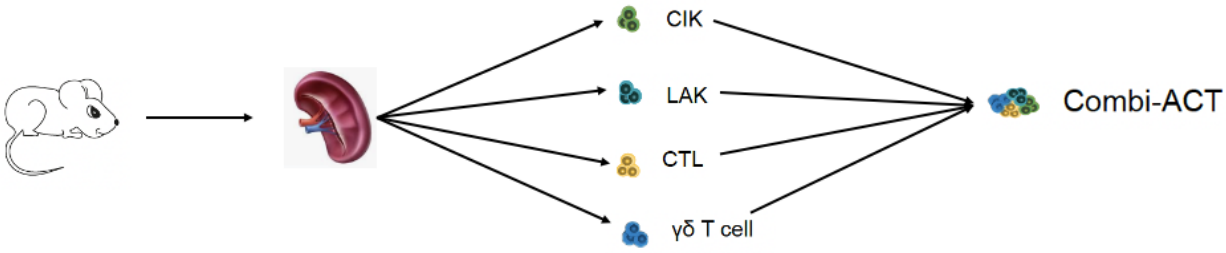


Figure 2.2

Illustration displaying the composition of the combination ACT.
CIKs – Cytokine-Induced Killer cells; CTLs – Cytotoxic T Lymphocytes; LAKs – Lymphokine Activated Killer cells

3.2.1.4 ACT killing assay with 403460 undifferentiated pleomorphic sarcoma cell line

To assess the cytotoxic efficacy of individual components of the adoptive cell therapy (ACT) as well as the complete ACT cell mixture in vitro, a luciferase-based viability assay was conducted. The experimental setup was adapted from the methodology described by Borchmann et al. (Borchmann et al. 2022)

403460 undifferentiated pleomorphic sarcoma cells that were stably transfected with luciferase were used.

The cells were harvested, centrifuged and resuspended in murine effector cell nutrient medium (composition shown above). Luciferin was added to the cell solution to attain a concentration of 400 µg per ml. 100 µl of the luciferase nutrient medium stock, containing 10^4 cells, was pipetted into every well of the 96 well plate. ACT therapy components (γδT cells, CIKs, CTLs and LAKs were added in different concentrations into the wells as single cell ACTs respectively and the complete ACT-1:1:1:1-combination of all four ACT subtypes that was later used to therapy murine undifferentiated pleomorphic sarcoma mouse models (See setup in illustration).

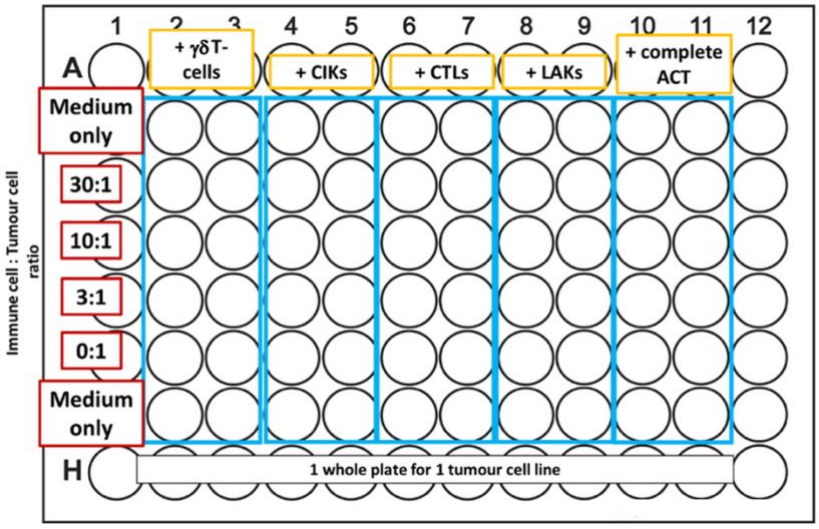


Figure 2.3

Schematic illustration of the luciferase assay to evaluate the killing efficacies of ACT components against undifferentiated pleomorphic sarcoma cells.
The perpendicular labeling on the left shows immune cell : tumor cell relations.
"Medium only" representing wells containing no cells.
CIKs – Cytokine-Induced Killer cells; CTLs – Cytotoxic T Lymphocytes; LAKs – Lymphokine Activated Killer cells

Each well was then filled with effector cell medium to a total volume of exactly 200 µl.
After breeding the 96 well plates in the incubator for 24 h at 37 °C, the luminescence intensity was measured using the Hidex Sense Multiplate Reader.

3.2.2 In Vivo

3.2.2.1 S.c. and i.m. Injection of 403460 undifferentiated pleomorphic sarcoma cells

6-30 week-old male and female 129/SVJaeJ mice were anesthetized intraperitoneally with ketamin/xylazin (ketamin 100mg/kg BW / xylazin 0.5 mg/kg BW). They were weighted and placed on a warming mat.

For the subcutaneous trial the flanks were shaven and 10^5 403460 undifferentiated pleomorphic sarcoma cells within a volume of 100µl PBS were injected subcutaneously into the dorsal flank region on both sides using a 27G canula.

For orthotopic experiments both dorsal thigh regions of the mice were shaven and 10^5 403460 undifferentiated pleomorphic sarcoma cells within a total volume of 50µl were injected intramuscularly into the gastrocnemius muscle on both sides using a 27 G canula.

The mice were put back into their cages after injecting and were observed for 30 min to make sure they overcame the anesthesia properly and nothing was accidently harmed by the injection.

3.2.2.2 Monitoring of s.c. and i.m. tumor growth in 129/SVJaeJ mice

The growth of the subcutaneously injected soft tissue sarcoma in 129/SVJaeJ mice was measured twice a week using a caliper. Therefore, the mice didn't have to be anesthetized. To evaluate the growth of the intramuscularly injected tumors, MRI scans were performed weekly. The animals were anesthetized using a 2,5% Isoflurane supply to minimize stress and prevent movements during the analysis.

Each tumor volume was calculated from MRI data with the formula for the volume of ellipsoid bodies:

$$V = \frac{4}{3} x\ \pi\ x \left(\frac{1}{2}\ x\ l\right) x\ \left(\frac{1}{2}\ x\ w\right) x\ \left(\frac{1}{2}\ x\ h\right)$$

V = Volume

l = length

w = width

h = height

3.2.2.3 Interventions and antitumor treatment in sarcoma bearing 129/SVJaeJ mice

As the tumor reached a size of 0.5 cm in at least one dimension, the treatment was started. Therefore, the mice were randomly divided into 12 groups:

- No therapy (hereafter named Vehicle)
- Doxorubicin monotherapy (hereafter named Doxo)
- TLR agonist mix (3,7,9) (hereafter named TLR)
- Anti-PD-1 monotherapy (hereafter named aPD-1)
- ACT monotherapy (hereafter named ACT)
- Doxorubicin + anti-PD-1 (hereafter named Doxo aPD-1)
- Doxorubicin + TLR agonist mix (hereafter named Doxo TLR)
- Anti-PD-1 + TLR agonist mix (hereafter named aPD-1 TLR)
- ACT + TLR agonist mix (hereafter named ACT TLR)
- ACT + anti-PD-1 (hereafter named ACT aPD-1)
- ACT + anti-PD-1 + TLR agonist mix (hereafter named Triple)
- IL2

Initial tumor diameters and sexes were evenly distributed between the groups.
Doxorubicin was injected i.p. once a week in a dosage of 2mg/kg BW.
10 mg anti-mouse PD-1 / kg BW was injected i.p. twice a week.
100µg of the TLR agonist mix consisting of TLR3 agonist (poly I:C), TLR7 agonist (gardiquimod) and TLR9 agonist (ODN-2395) in equal quantities was injected twice a week intratumorally into the right tumor.
The day before injecting the ACT, a lymphodepletion had to be conducted by intraperitoneal administration of 1mg of Fludarabine and 4mg of Cyclophosphamide.
The 1:1:1:1 adoptive immune cell mix was prepared as shown above in section 2.2.1.3.. 10^6 effector cells were injected intraperitoneally on day 1. Within the first 5 days of the therapeutic regimen all ACT mice were also injected i.p. with 10^5 U of IL2 daily.
The IL2 group also received 10^5 U of IL2 over 5 days to serve as a control group for ACT treated mice.
A mouse was taken out as the tumor reached a size of more than 15 mm within at least one dimension. Humane endpoints were also reached as soon as a mouse showed signs of suffering, the tumor penetrated through the skin or if it started limping (orthotopic model).
After a first cohort, where all therapie regimens were tested on each respective Group in a heterotopiacal mouse model (5 mice each), the ACT, Triple, and Vehicle regimen was tested

in the second, now orthotopic mouse cohort (4 mice per therapy regimen). In the third cohort, three heterotopic mouse model therapy groups were established: ACT, Triple, and Vehicle (again 5 mice each). The aim in this last cohort was to investigate therapy-induced changes in the TME. Therefore, animals showing a good therapeutic response (tumor size smaller than before the start of the therapy) were euthanized at this time point.
The group size planning was calculated from the statistical power needed and feasibility.

3.2.2.4 Analysis of murine tissues

If a humane endpoint was reached or the tumor became large enough, the mice were killed by cervical dislocation.
The spleen, liver, colon and kidneys were taken out, fixed in PFA for 24 h and stored in sterile PBS at 4 °C.
Blood was taken by cardiac punction and the centrifuged plasma was stored at -20 °C.
The tumors were prepared from their subcutaneous or intramuscular location (recording to the model) and partly fixed in PFA for 24 h for histological analysis likewise the other organs.

3.2.2.4.1 Immunohistochemistry

Embedding of the histological specimen, as well as cutting and staining of the immunohistological slices was kindly performed by the institute of pathology at the University of Cologne.
The tumor samples were stained with anti-mouse CD3 Ab, anti-mouse CD4 Ab, anti-mouse CD8 Ab, anti-mouse CD31 Ab, anti-mouse CD45R/B220 Ab respectively.
The slides were scanned using the Nanozoomer S360 digital slide scanner by Hamamatsu. To analyze and qualitatively evaluate the scans, the
NDP viewer 2.0 by Hamamatsu was used.

3.2.2.4.2 Flow cytometry

Tumor tissue as well as the spleens were processed for FACS analysis:
First the tissue was mashed through a 40 µm cell strainer. The cells were then suspended in 1000 µl PBS and transferred into an eppi. After centrifuging at 350 G for 4 min the supernatant was discarded, the pellet was resuspended within 1000µl of ACK lysis buffer and incubated for 5 min at room temperature to get rid of any Erythrocytes. Subsequently, the suspension was centrifuged at 350 G for another 4 min. The cells were washed with PBS after discarding the supernatant and another centrifuging step, the cells were counted and every specimen was divided into three different staining groups (for another analysis panel each):
The antibodies that were used for the 3 analysis panels respectively are shown below:

1. T cell panel: CD45, CD49b, CD3ε, TIM-3, γδTCR, CD8, PD-1, CD4, CD69, intracellular marker: FOXP3, IFNγ, Ki67
2. B cell panel: CD80, CD45, Galectin-9, CD40, PD-L1, CD86, CD45R/B220, CD24, EpCam, I-A/I-E (MHC II), CD19
3. Macrophage panel: CD80, CD45, CD163, CD11c, CD206, CD86, F4/80, Gr-1 (Ly-6G/Ly-6C), CCR2, , I-A/I-E (MHC II), CD11b, CX3CR1

In every panel Zombie UV fixable viability kit was used for life-death differentiation.

The staining mixes (prepared within FACS buffer and containing the extracellular antibodies only) were applied to the cell pallets respectively and resuspended.

The incubation time for the staining step was 30 min at 4 °C in the dark.

After washing the cells with FACS buffer, 500 µl of fixation buffer was added, resuspended and incubated for 15 min at RT in the dark.

Another washing step with FACS buffer followed and the cells were permeabilized using 500 µl of Triton permeabilization buffer and incubating the suspension for 20 min at 4 °C in the dark.

The cells were again washed with FACS buffer and stained with the intracellular antibodies (only staining panel 1) incubating for 30 min at 4 °C in the dark.

After washing and resuspending in FACS buffer the stained cells were pipetted into U-bottom 96-well plates and analyzed by the Cytoflex^{TM} flow cytometer from Beckman Coulter.

Kaluza Flow Cytometry Analyzing software by Beckman Coulter was used to evaluate the data:

Gating strategy:

Panel 1:

First, cells were identifies by gating on FSC-A vs. SSC-A. From all gated cells, singlets were identified by gating on SSC-A vs. SSC-H to exclude cell doublets. Next, viable cells were selected by excluding dead cells using Aqua Zombie viability dye. CD45 was used to distinguish between cancer cells from immune cells within the population of living cells. T cells were identified by gating on $CD3^+$ events. Within those T cells, T helper cells were distinguished from cytotoxic T cells and gd T cells by CD4-, CD8- and γδTCR staining respectively. T cells were further analyzed by CD69, and IFNγ expression within the CD4+ or CD8+ compartments.

Viable tumor cells were analyzed on reproduction rate by staining on Ki 67.

Panel 2:

Viable immune cells were identified as shown in panel 1.

CD45R/B220 was used to identifiy B cells. $CD45R^-MHCII^+$ immune cells were defined as non B cells APCs. Activation state was analyzed staining with CD80, CD86 and CD19.

Panel 3:
Viable immune cells were identified as shown in panel 1.
Macrophages were identified as F4/80^{+}, DCs as CD11c^{+} F4/80^{-} immune cells. The macrophages were defined as tumor promoting by CD206, CD163 double positivity. Macrophages that show low counts of CD206 and/ or CD163 were labeled rather tumor suppressive.
Granulocates were distinguished from Monocytes by Gr-1 and CD11b staining. Granulocytes are high in Gr-1 and CD11b, whereas monocytes are defined as CD11b high and Gr-1 low.

In each panel compensation was performed using single-stained controls, and doublet discrimination was applied throughout the gating hierarchy.
A minimum of 100,000 total events were collected per sample to ensure statistical robustness.

3.3 Statistics

The results of the experiments were presented as arithmetic mean or median values ± SD. The significance between was calculated using the paired or unpaired t-test. A difference was considered significant / highly significant if the probability of error were $p < 0.05$ / $p < 0.01$.
All data sets were processed and evaluated with Excel. Flow cytometrie data was previously edited and analyzed via Kaluza
Statistic values were calculated and figures were created using Prism 8 by Graphpad Software inc..

4 RESULTS

4.1 Killing assays

4.1.1 Soft tissue sarcoma cell lines show similar resilience to doxorubicin exposition in cell viability assay

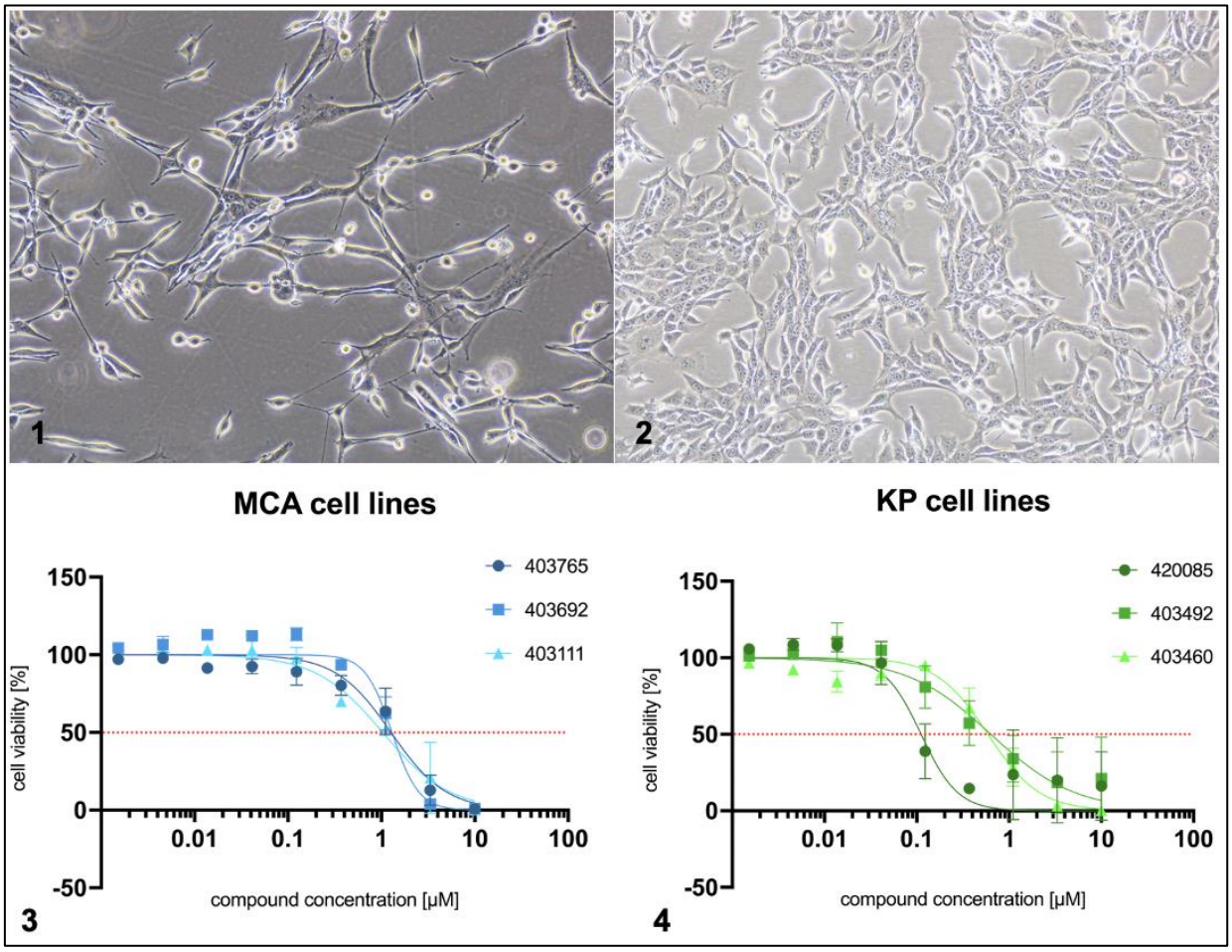


Figure 3.1

*Microscopic picture of MCA soft tissue sarcoma cell line 403411 (**1**) and KP soft tissue sarcoma cell line 403460 (**2**) in proliferating state (recognizable from the sprawled-out cell bodies). The figures below display cell viability rates in 3 different MCA (**3**) and KP (**4**) cell lines respectively (n=2). The percentage specifications show cell viability proportions of soft tissue sarcoma cell lines cultured in different concentrations of doxorubicin in relation to cells cultures in nutrient medium only.*

Doxorubicin based chemotherapeutic treatments display the current standard of care treatment against unresectable and advanced soft tissue sarcoma (Tap et al. 2016; 2020; Gronchi et al. 2021).

To evaluate the resilience of soft tissue sarcoma cell lines to the exposition of doxorubicin in different concentrations cell titer glo assays were performed.
The implementation and conditions under which 3 KRAS G12D / p53 mutated cell lines (403460, 403492 and 420085) (hereafter referred to as KP cell lines), and 3 MCA induced/ p53 mutated cell lines (403765, 403692 and 403111) (hereafter referred to as MCA cell lines) were exposed to doxorubicin are shown above.
MCA soft tissue sarcoma cell populations show no inhibition in growth up to doxorubicin concentrations of up to 0,1 µM while the reproduction of KP soft tissue sarcoma cell lines is already affected at doxorubicin concentrations higher than 0.03µM. At doxorubicin concentrations of 10 µM almost all tumor cells are dead.
Even though KP soft tissue sarcoma cells are already affected by slightly lower concentrations of doxorubicin, both MCA and KP soft tissue sarcoma cell lines show similar sensitivity towards doxorubicin exposure.
Overall, the conclusion can be drawn, that doxorubicin concentrations of about 1µM lead to a decrease in soft tissue sarcoma cell viability by about 50 % in most cell lines (Figure 3.1).

4.1.2 The combination of adoptive immune cell therapy components leads to inceased in vitro killing effects compared to mono-immune cell therapy on soft tissue sarcoma cells

Petitprez et al. suggested that a rich immune cell infiltration in the TME leads to particularly favorable response rates to immunotherapies and advantageous effects on survival (Petitprez et al. 2020).
Previous work in my working group let to the conclusion that it is the specific composition of immune cells penetrating the TME including γδT cells, T cells and NK cells that can effectively tackle tumor cells (Borchmann et al. 2022)
Suggesting, that those favorable cell infiltrates could possibly be inducible in soft tissue sarcoma, the killing efficacies of the ACT combination and of its individual components were tested against 403460 KP mutated undifferentiated pleomorphic sarcoma cells using a Luciferase toxicity assay.

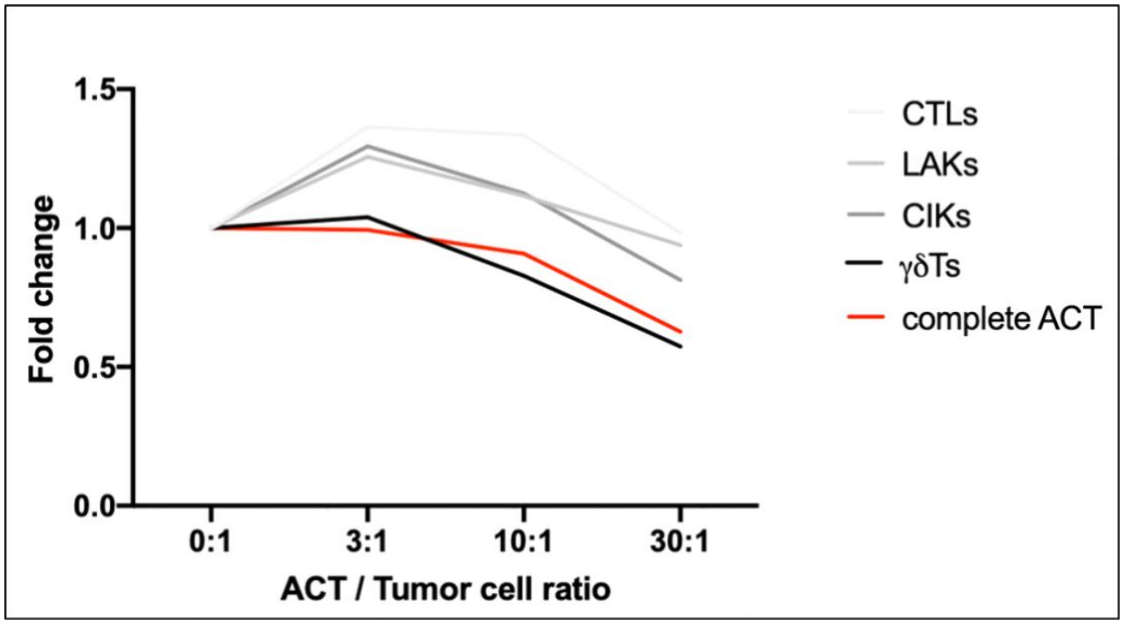


Figure 3.2

This graph shows cell viability rates of luciferase transfected 403460 undifferentiated pleomorphic sarcoma cells in co- culture with different proportions of adoptive immune cells displayed as the fold change of the cell culture without any ACT treatment (n=2).
"complete ACT" representing the 1:1:1:1 combination of all 4 ACT components
ACT cell types are indicated by color code as to be seen in the figure
CIKs – Cytokine-Induced Killer cells; CTLs – Cytotoxic T Lymphocytes; LAKs – Lymphokine Activated Killer cells

Based on data of the luciferase viability assay, the in vitro killing success of CTLs, LAKs and CIKs seems to be limited, and the tumor cell growth within these co-cultures seems to be even triggered in 3:1 or 10:1 ACT : tumor cell ratios compared to tumor cells cultured in medium only. γδ-T cells and the ACT combination led to unaffected growth in 3:1 and a reduction of tumor cell counts in 10:1 and 30:1 ACT : tumor cell ratios (Figure 3.2).

4.2 Tumor growth and survival

4.2.1 ACT therapy improves overall survival rates in s.c. undifferentiated pleomorphic sarcoma mouse model

Previous studies and the in vitro experiments with our adoptive immune cells led to the assumption that immunotherapeutic combination regimens might provide improved survival margins and inhibitory effects on tumor growth (Dufresne and Brahmi 2020). To test these suggested effects, subcutaneous undifferentiated pleomorphic sarcoma mouse models were

established and treated with different therapeutic regimens using PD-1 inhibitors, a mix of TLR-3, -7 and -9 agonists, and our adoptive cellular immunotherapy (see details in section 2.2.2.3)
Untreated mice showed better survival rates in our heterotopic undifferentiated pleomorphic sarcoma mouse model compared to mice treated with doxorubicin. (Fig.3.3 (1))
Also, aPD-1 inhibition or treatment with TLR agonists did not lead to improvement but rather deterioration of survival rates compared to untreated mice. (Fig 3.3 (2))
Mice treated with ACT mono therapy showed better survival rates than non-treated mice. This effect was not seen in mice that received IL2 at the same doses as those in the ACT group (Figure 3.4).

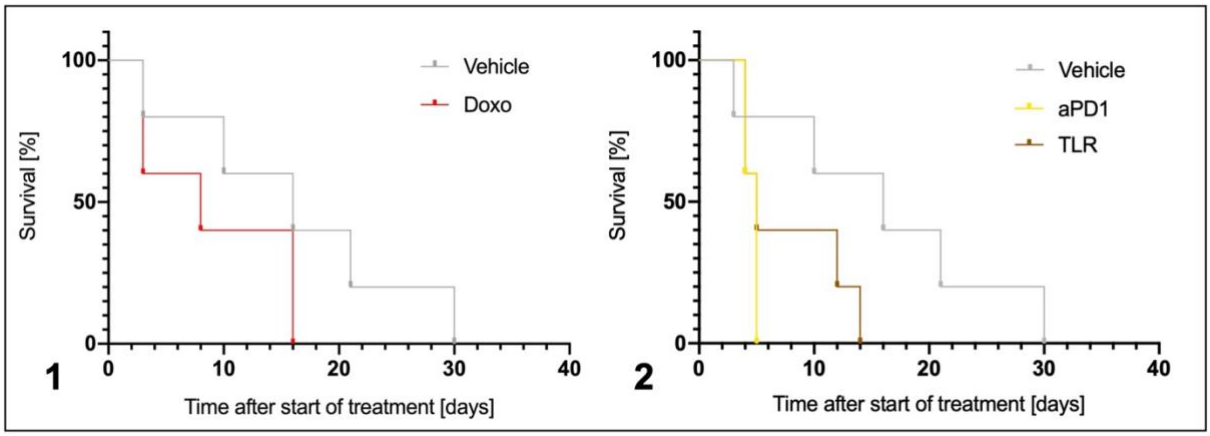


Figure 3.3

These Kaplan Meier plots compare mice bearing subcutaneous undifferentiated pleomorphic sarcoma, treated with different therapeutic regimens. Intervention groups are marked by color code as to be seen in the figures (Vehicle [n=5] MOS:16 d / Doxo [n=5] MOS:9,2 d / aPD-1 [n=5] MOS:4,6 d / TLR agonists [n=5] MOS:7,8 d) respectively. Both plots use the same Vehicle data.

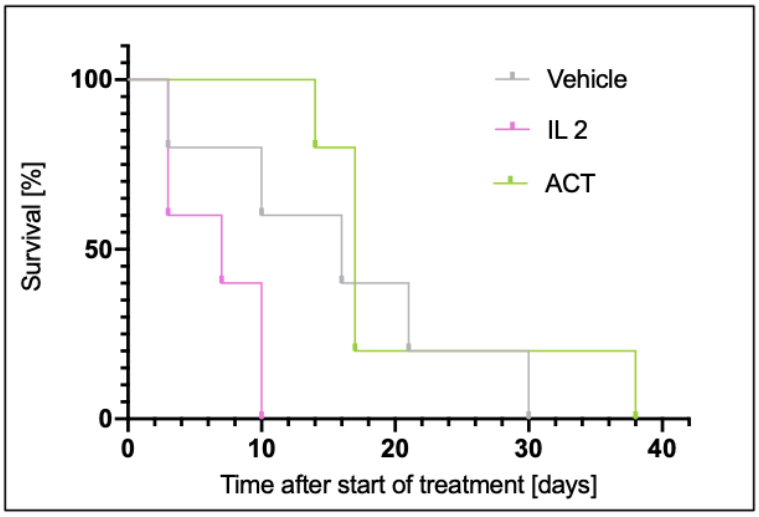


Figure 3.4

These Kaplan Meier plots compare mice bearing subcutaneous undifferentiated pleomorphic sarcoma, treated with different therapeutic regimens. Intervention groups are marked by color code as to be seen in the figure (Vehicle [n=5]) MOS:16 d / IL2 [n=5] MOS:6,6 d / ACT [n=5] MOS:20,6 d).
The Vehicle data are the same as in figure 3.3.

4.2.2 aPD-1 therapy needs to be combined with other therapeutics to mediate beneficial effects regarding therapy response

As mentioned in 3.2.1, aPD-1 monotherapy does not provide any beneficial effects on survival of s.c. undifferentiated pleomorphic sarcoma bearing mice compared to untreated mice.
If other therapeutics are included in the therapy regimen, the MOS is improved to more than doubled. Starting from a MOS of 4,6 days in aPD-1 mono therapy group, the MOS of aPD-1combination therapies is reached after 12,3 (aPD-1+TLR) to 22,4 days (aPD-1+ACT)
According to the survival curves, ACT combinations seem to surpass the therapeutic effects of non-cellular immunotherapies.
Moreover, combining ACT with aPD-1 seems to be superior to a combination of ACT and TLR, in terms of survival rates (Figure 3.5).

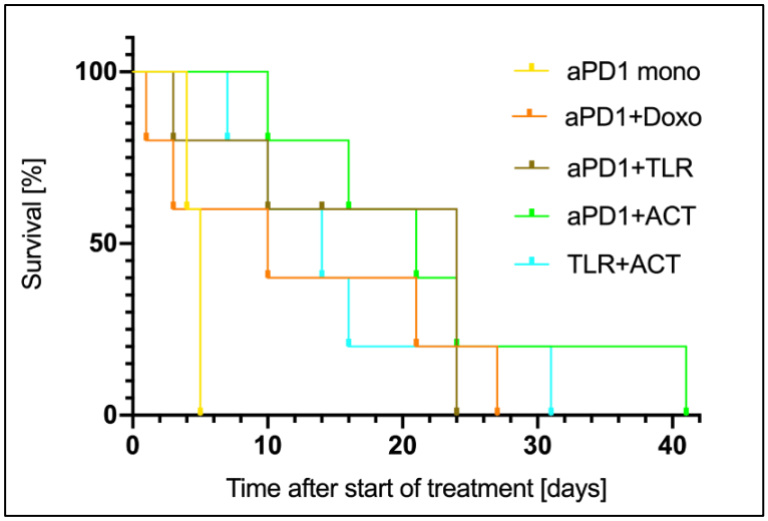


Figure 3.5

These Kaplan Meier plots compare mice bearing subcutaneous undifferentiated pleomorphic sarcoma, treated with different therapeutic regimens. Intervention groups are marked by color code as to be seen in the figure (aPD-1 mono [n=5] MOS:4,6 d / aPD-1+Doxo [n=5] MOS:12,4 d / aPD-1+TLR agonists [n=5] MOS:12,3 d / aPD-1+ACT [n=5] MOS:22,4 d / TLR agonists +ACT [n=5] MOS:15,6 d)

The aPD-1 mono data are the same as in figure 3.3.

4.2.3 Triple therapy treated s.c. undifferentiated pleomorphic sarcoma mice show best survival rates and tumor growth inhibition

ACT and Triple treated s.c. undifferentiated pleomorphic sarcoma mouse models showed improved survival rates compared to doxorubicin or vehicle mice (Fig.3.6)

The Triple regimen seems to be even superior to ACT monotherapy in both enhancing the MOS (Fig.3.6. (1)) and the induction of tumor shrinkage (Fig.3.7).

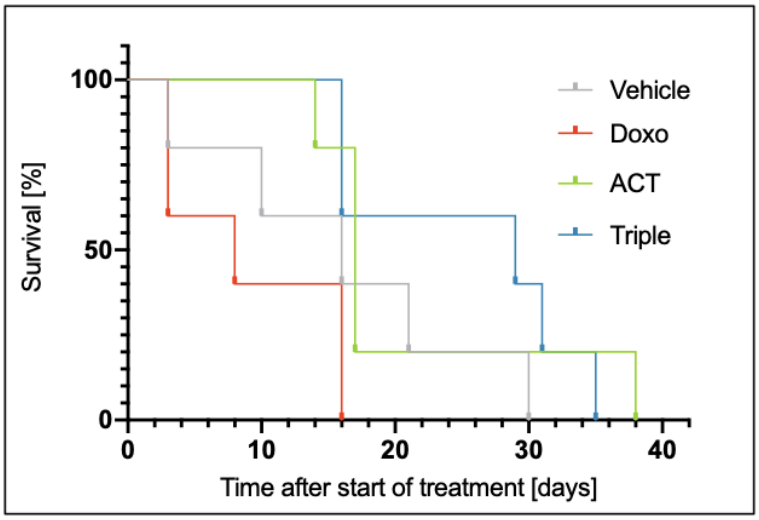


Figure 3.6

Kaplan Meier plot comparing subcutaneous undifferentiated pleomorphic sarcoma bearing mice treated with different therapeutic regimens. Intervention groups are marked by color code as to be seen in the figure (Vehicle [n=5] MOS:16 d / Doxo [n=5] MOS:9,2 d / ACT [n=5] MOS:20,6 d / Triple (ACT+aPD-1+TLR agonists) [n=5] MOS:25,4 d). Vehicle and Doxo data were taken from figure 3.3. ACT data were taken from figure 3.4.

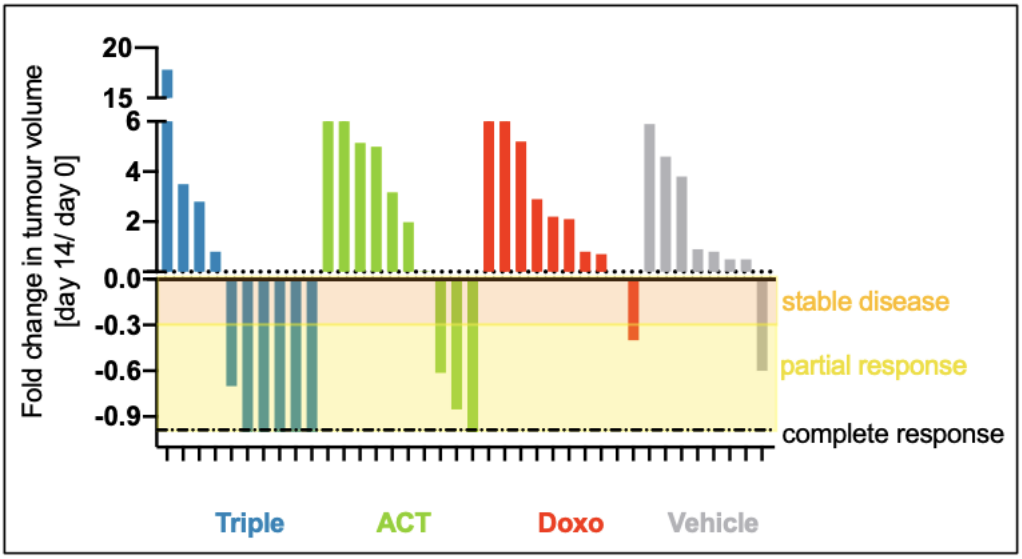


Figure 3.7

Waterfall plot showing fold change of tumor volume on day 14 after start of the treatment. The displayed data refers to s.c. undifferentiated pleomorphic sarcoma murine tumors on day 0 of the therapy.

A loss of up to 30 % in tumor volume was defined as stable disease, loss of more than 30 % as partial response and no detectable residual tumor as complete response.

4.2.4 ACT based immunotherapy shows compelling antitumor effects in orthotopic undifferentiated pleomorphic sarcoma mouse model

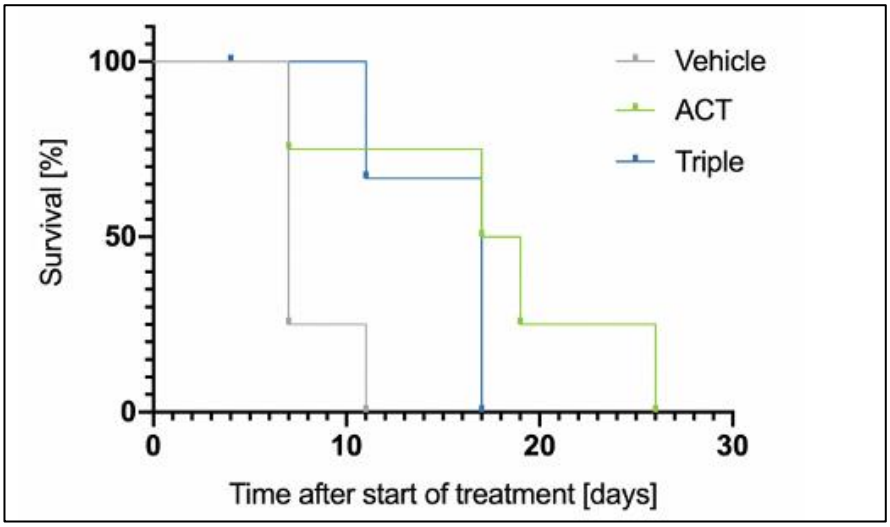


Figure 3.8

Kaplan Meier plot, comparing orthotopic undifferentiated pleomorphic sarcoma bearing mice treated with different therapeutic regimens (Vehicle [n=4] MOS:8 d / ACT [n=4] MOS:17,25 d / Triple [n=4] MOS:11,25 d.

In order to show the immune modulating effect and therapy response orthotopically, we decided to establish an orthotopic sarcoma model within the murine musculature. For this purpose, we i.m. injected 10^5 undifferentiated pleomorphic sarcoma cells in the gastrocnemius muscle on both sides. Serial MRI measurements revealed successfull induction and tumor progression after about 6 days. In a next step we applied different therapeutic regimens comprising ACT mono therapie and Triple therapy regimen. To establish reference values, an untretated vehicle group was also conducted. Tumor volumes were measured weekly by in vivo MRI measurements. The orthotopic model worked out well and showed reliable tumor accretion. The analysis of tumor growth and survival rates revealed that mice treated with ACT mono or Triple therapy regimen do not only show enhanced survival rates compared to non treated mice as to be seen in figure 3.8, but also the inhibition of tumor growth was noticeable in the intervention groups. Interestingly some ACT mono treated tumors could be observed even shrinking after initial steady growth. This effect did not last for more than a view days and was not found in all specimens of this therapy group.

4.3 Modifying effects of ACT combination therapies on the murine immune system and the undifferentiated pleomorphic sarcoma TME

The antitumor effects of immune modulating therapies are based on many different direct or indirect changes in the immune system.

Petitprez et al. already identified the TME immune infiltration in soft tissue sarcoma as a predictive marker to therapy response and survival rates.

Since the overall survival and growth kinetics are beneficially influenced by the administered immune therapies in our mouse models, changes within the spleen (representing the systemic immune response) and the TME were explored using multi-color FACS analysis.

To collect data of responding tumors, ACT and Triple treated s.c. undifferentiated pleomorphic sarcoma mouse models were killed during therapy-induced tumor shrinkage to smaller volumes than on day 0 of the therapy regimen.

The data for progressive tumors of ACT, Triple or Vehicle mice was summarized from heterotopic and orthotopic mouse models as they showed similar results in FACS analysis.

4.3.1 ACT leads to increased presence and activation of APCs in secondary lymphoid organs

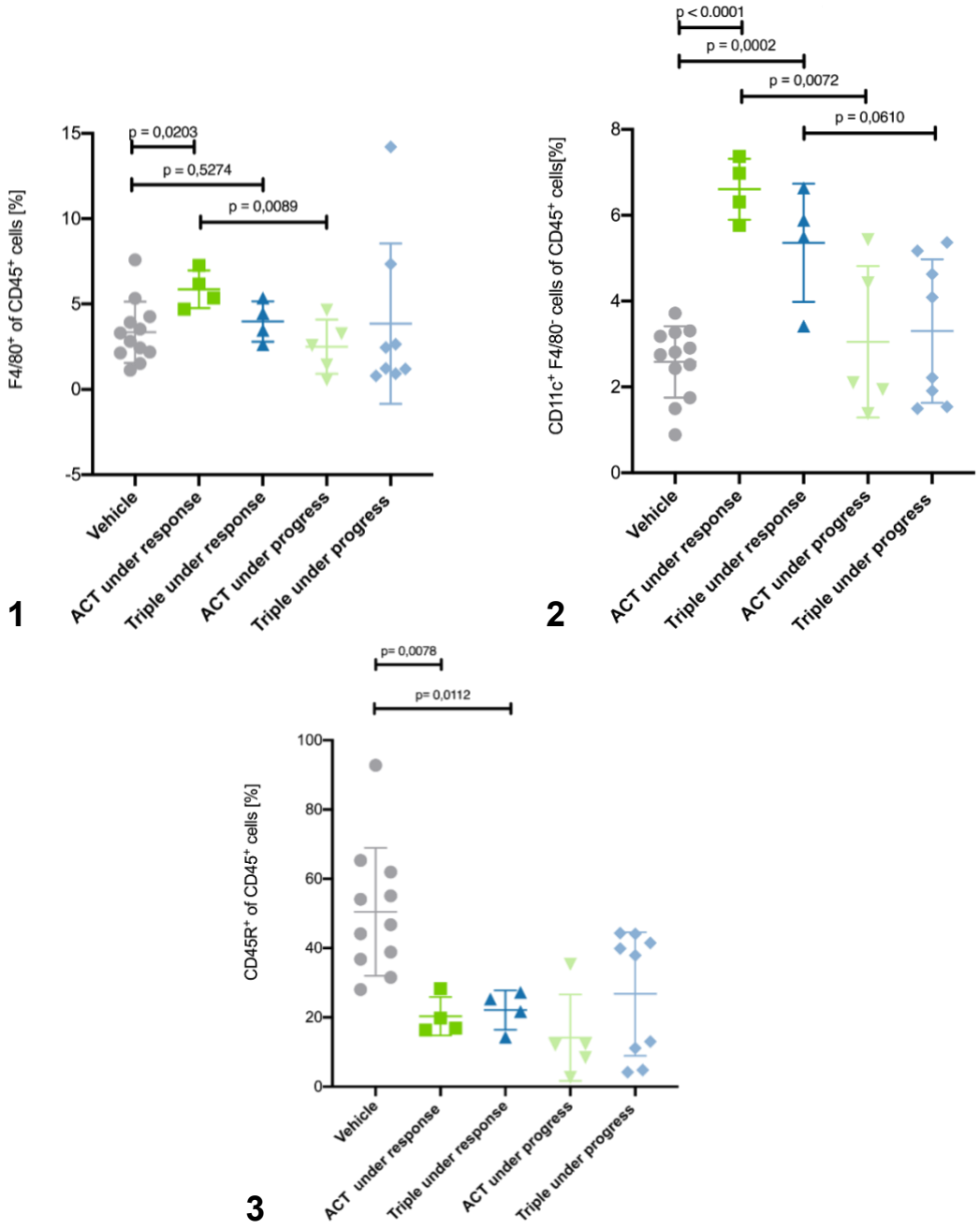


Figure 3.9

FACS data from responding vs. progressing undifferentiated pleomorphic sarcoma mouse models. These diagrams compare untreated "Vehicle", ACT mono- and Triple combination therapy treated mice as they show proportions of APCs within the spleen tissue that represents the systemic immune response:

1 *the percentage of macrophages (defined as F4/80 expressing CD45⁺ cells) among CD45⁺ cells*

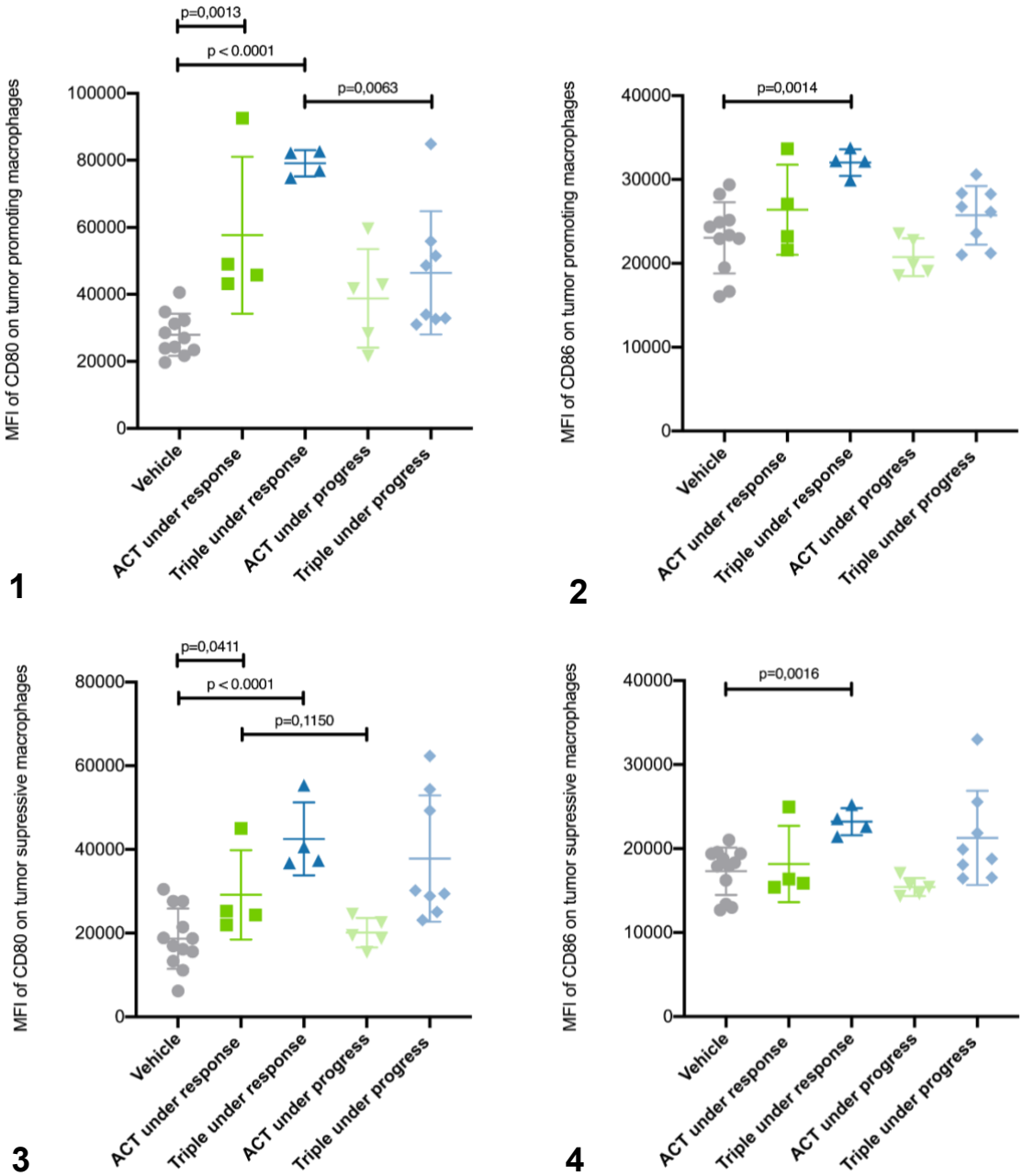
2 *the percentage of DCs (defined as F4/80⁻, CD11c expressing CD45⁺ cells) among CD45⁺ cells*

3 *the percentage of B cells (defined as CD45R expressing CD45⁺ cells) among CD45⁺ cells.*

Statistical analysis data was gained performing the unpaired t-test.

Figure 3.9 displays the systemic immune response, represented by specimens of the spleen as a secondary lymphoid organ. As to be seen in (1) and (2) both the infiltration with macrophages and DCs is enhanced within therapy responding mice under ACT and Triple therapy regimen compared to untreated mice. ACT mono treatment showed the most favorable impact on macrophages- and DC-infiltration. This effect vanishes as the mice showed tumor progression.

In comparison to that, the numbers of B cells is lowered within the spleens of all immunotherapeutically treated mice both during therapy response and under progressive disease (3).



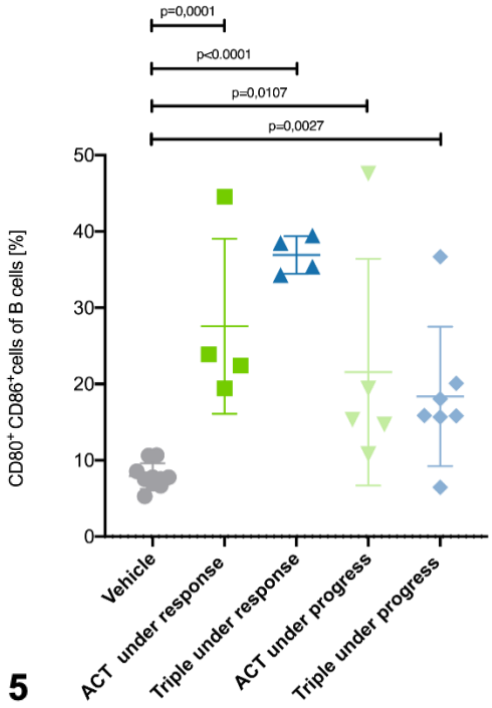


Figure 3.10

FACS data from responding vs. progressing undifferentiated pleomorphic sarcoma mouse models. These diagrams compare untreated "Vehicle", ACT mono- and Triple combination therapy treated mice, displaying proportions of activation markers expressed on APCs within the spleen tissue that represents the systemic immune response:

1 *the MFI of CD80 on tumor-promoting macrophages (defined as $CD206^+$, $CD163^+$ macrophages)*

2 *the MFI of CD86 on tumor-promoting macrophages (defined as $CD206^+$, $CD163^+$ macrophages)*

3 *the MFI of CD80 on tumor suppressive macrophages (defined as $CD206^-$, $CD163^-$ macrophages or those expressing only CD206 or CD163)*

4 *the MFI of CD86 on tumor suppressive macrophages (defined as $CD206^-$, $CD163^-$ macrophages or those expressing only CD206 or CD163)*

5 *the percentage of activated B cells (defined as CD80 and CD86 expressing B cells) among all B cells*

Statistical analysis data was gained performing the unpaired t-test.

Since high expression of CD80 and CD86 is associated with the activation of APCs such as B cells and a high stimulating interaction potential with T cells, this indicates a positive response of the murine adoptive immune system to the administered ACT therapy. Thus, an effect of the same beyond the direct anti-tumour effects has been proven. Macrophages were subdivided into tumor-promoting macrophages (TPM) and tumor suppressive macrophages (TSM) by their expression of CD206 and CD163.

Within spleen tissue of ACT and Triple treated mice, both TPM (Fig.3.10 (1+2) and TSM (Fig.3.10 (3+4)) expressed higher amounts of CD80 and CD86 on their surface compared to untreated mice. Triple therapy leads to even higher expression of those surface proteins, than ACT mono therapy in comparison.

Also, higher amounts of activated B cells within spleen tissue can be found in mice treated with immunotherapy compared to untreated mice (5).

The data shown in chapter 3.3.1 suggest that the use of ACT, as well as Triple therapy does have significant impact on the immune system and moreover, leads to a pertinent generalized activation of the adaptive immune system.

4.3.2 Tumor progression correlates with decreased immune infiltration in immunotherapeutically treated STS mouse models

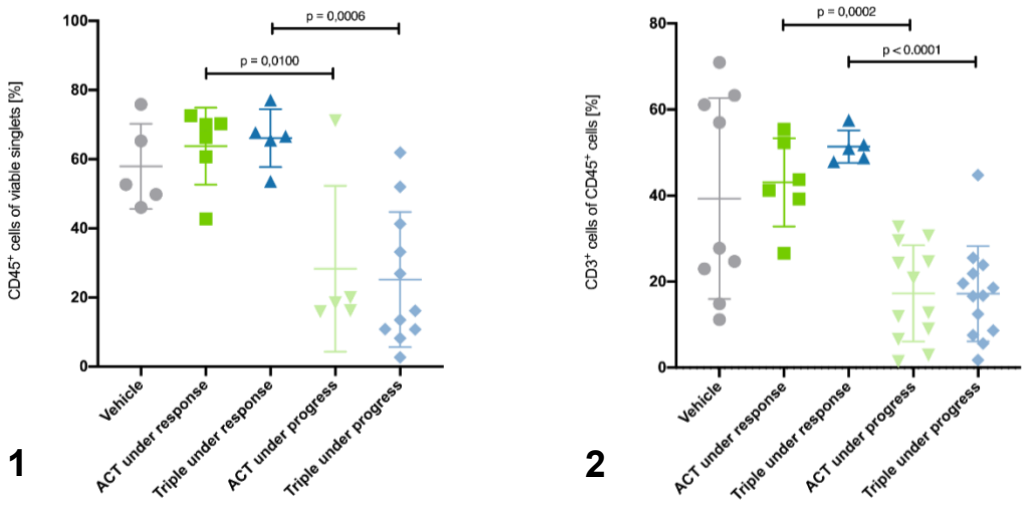


Figure 3.11

FACS data from responding vs. progressing undifferentiated pleomorphic sarcoma mouse models. These diagrams compare untreated "Vehicle", ACT mono- and Triple combination therapy treated mice, displaying percentages of immune cells within the tumor specimen **(1)** *and the percentages of T cells ($CD3^+$) among all $CD45^+$ cells* **(2)**
Statistical analysis data was gained performing the unpaired t-test.

When evaluating the immune infiltration into the tumor site, the total amounts of immune cells (1) and especially the amounts of T cells (2) in progressive undifferentiated pleomorphic sarcomas, that are treated with ACT or Triple therapy stand out as their counts are drastically decreased compared to therapy responsive tumors.

4.3.3 Increased levels of activation markers are expressed on T cells in response to ACT and Triple therapy

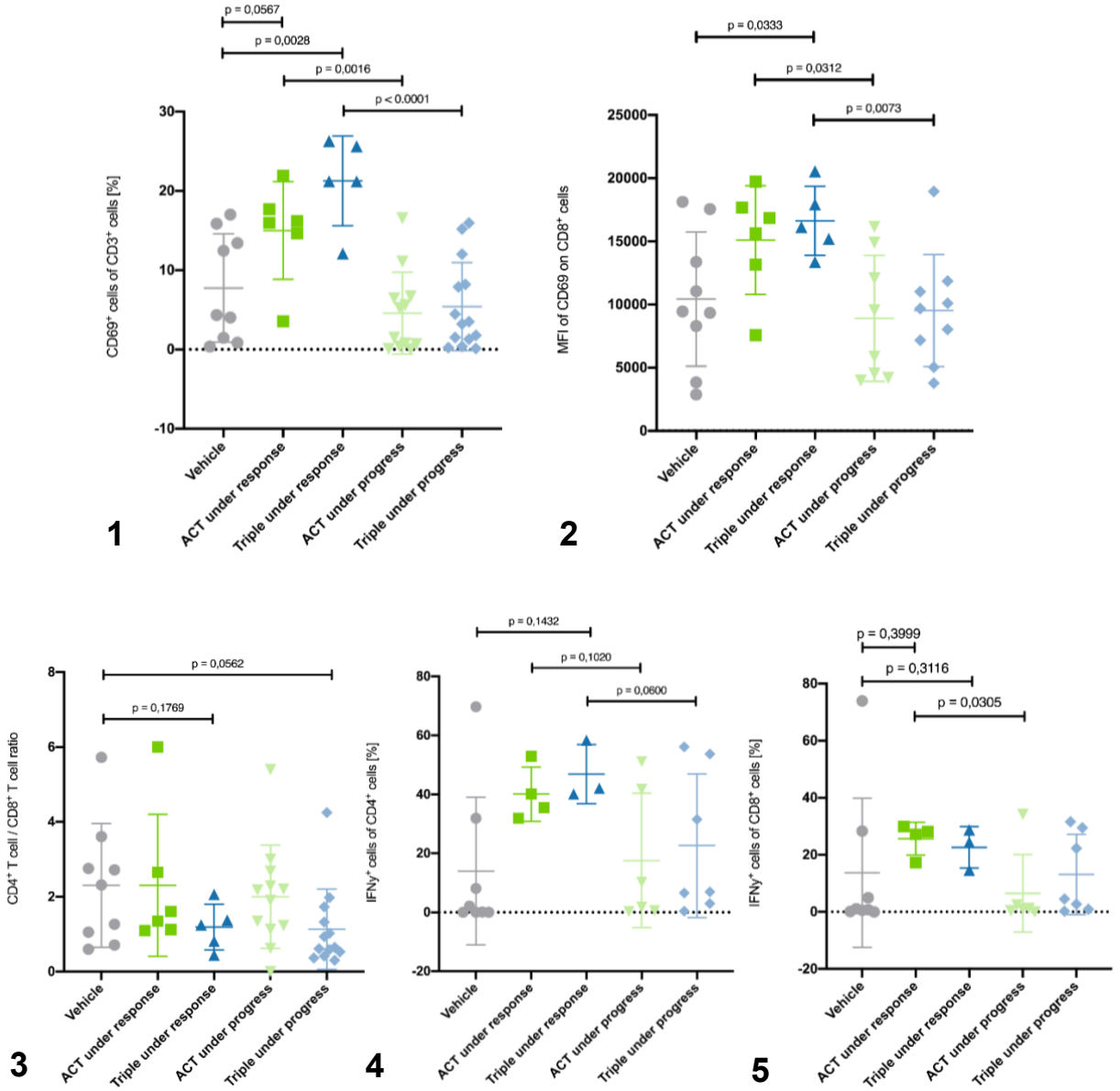


Figure 3.12

FACS data from responding vs. progressing undifferentiated pleomorphic sarcoma mouse models. These diagrams compare tumor specimen of untreated "Vehicle", ACT mono- and Triple combination therapy treated mice.

1 displays the percentage of CD69 expressing T cells among all T cells

2 displays the MFI of the early activation marker CD69 on $CD8^+$ T cells

3 *shows a comparison of the CD4/ CD8 ratio that calculated for the differently treated tumors respectively*

4 displays the percentage of IFNγ expressing $CD4^+$ T cells among $CD4^+$ T cells

5 displays the percentage of IFNγ expressing $CD8^+$ T cells among $CD8^+$ T cells

Statistical analysis data was gained performing the unpaired t-test.

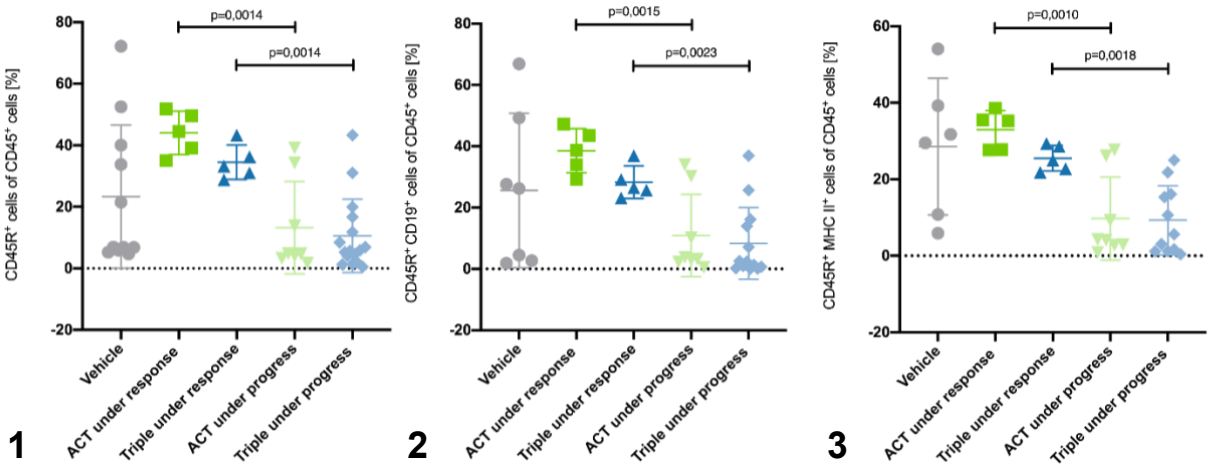
As to be seen in figure 3.12 (1), the amount of CD69$^+$ T cells infiltrating the undifferentiated pleomorphic sarcoma TME is significantly upregulated in therapy responding ACT and Triple treated mice. The same intervention groups also show high amounts of CD69 expressed particularly on CD8$^+$ T cells (Fig.3.12(2)).

While the CD4/ CD8 ratios within the displayed groups are just slightly shifted towards relatively higher proportions of CD8$^+$ T cells within responding Triple treated tumors (Fig.3.12(3)), both CD4$^+$ and CD8$^+$ T cells show higher activation in the TME of responding intervention groups. This can be seen in the distinct upregulation of IFNγ in these groups, depicted in CD4$^+$ T cells (Fig.3.12(4)) and CD8$^+$ T cells (Fig.3.12(5)).

4.3.4 ACT intervention triggers adoptive immune cell invasion into the murine undifferentiated pleomorphic sarcoma tumor microenvironment

The initial increase in B-cell infiltration within the TME of therapy-responsive mice declines as the undifferentiated pleomorphic sarcoma develops mechanisms to evade immunotherapy, leading to renewed tumor growth (1) (2) (3).

Not only that the infiltration into the TME of B cells is upregulated in tumors responsive to the immunotherapeutic interventions, but also the percentage of activated B cells is significantly upregulated upon ACT and Triple treatment (4). This distinct upregulation of CD80 and CD86 on the surface of tumor infiltrating B cells within responsive tumors, that dwindles away in progressing tumors, leads to the conclusion, that B cell infiltration and activation plays a crucial role in the beneficial antitumor effects of the ACT and ACT based immune therapies.



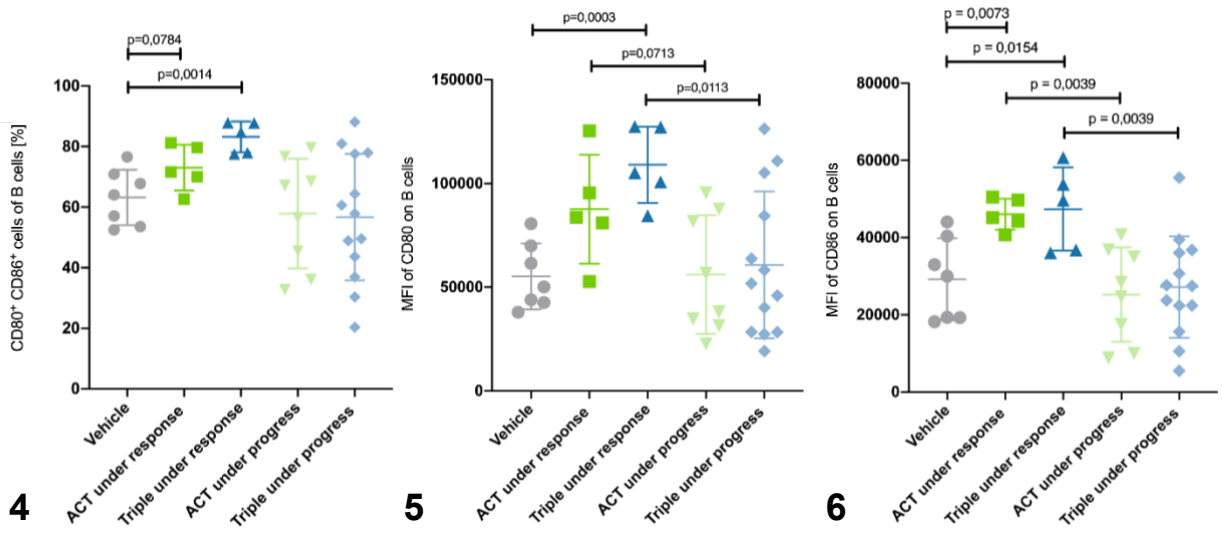


Figure 3.13

FACS data from responding vs. progressing undifferentiated pleomorphic sarcoma mouse models. These diagrams compare tumor specimen of untreated "Vehicle", ACT mono- and Triple combination therapy treated mice showing B cells and activation markers expressed on their surface:

1 displays the percentage of B cells (defined as $CD45R^+$ cells of $CD45^+$ cells) among all $CD\ 45^+$ cells

2 displays the percentage of $CD19^+$ B cells among all B cells

3 displays the percentage of MHC class II^+ B cells among all B cells

4 displays the percentage of activated B cells (defined as $CD80^+$,$CD86^+$ B cells) among B cells

5 displays the MFI of CD80 expressed on B cells

6 displays the MFI of CD86 expressed on B cells

Statistical analysis data was gained performing the unpaired t-test.

4.3.5 Summary of FACS analysis data results

Cellular marker	Significance	Results	Interpretation
CD 45	General immune cell marker	• Increased percentage of $CD45^+$ cells of all viable singlets in responding ACT- and Triple treated tumors compared to progressing tumors. • Significantly decreased percentage of $CD45^+$ cells of all viable singlets in progressing tumors	• The ACT- and Triple immunotherapy leads to an altered immune infiltration. • Immune escape mechanisms reverse those effects
CD 45 R	Murine B cell marker	• Increased percentages of $CD45R^+$ of immune cells in responding ACT and Triple	• The ACT- and Triple immunotherapy leads to a massive

		treated tumors, compared to vehicle or progressing tumors • Significantly decreased percentages of $CD45R^+$ of immune cells in secondary lymphoid organs of immunotheraputically treated mice	B cell infiltration in the tumor tissue. • Those B cells infiltrate from secondary lymphoid tissue • Immune escape mechanisms reverse those effects
CD19	General B cell marker	• Significantly increased percentages of $CD45R^+$ $CD19^+$ cells in responding ACT and Triple treated tumors, compared to progressing tumors	• The ACT- and Triple immunotherapy leads to a massive B cell infiltration in the tumor tissue. • Immune escape mechanisms reverse those effects
F4/80	Murine macrophage and macrophage activation marker	• Significantly increased percentages of $F4/80^+$ cells of immune cells in secondary lymphoid organs of ACT treated, responding mice, compared to mice with progressive tumors under ACT therapy or Vehicle	• The ACT therapy leads to an improved antigen presentation by macrophgages in secondary lymphoid organs • Immune escape mechanisms reverse those effects
CD 11c	DC marker	• Increased percentages of $CD11c^+$ cells of immune cells in secondary lymphoid organs of ACT and Triple treated, responding mice, compared to mice with progressive tumors under immunotherapy or Vehicle.	• The ACT- and Triple therapy leads to an improved antigen presentation by dendritic cells in secondary lymphoid organs • Immune escape mechanisms reverse those effects
CD80; CD86	APC activation marker and marker of APC induced T cell activation	• Significantly increased MFI of $CD80^+$ and $CD86^+$ in macrophages and B cells in responding ACT and Triple treated tumors, compared to mice with progressive tumors under immunotherapy or Vehicle	• The ACT- and Triple therapy leads to an improved activation of macrophages and B cells • Immune escape mechanisms reverse those effects
MHC 2	APC activation marker	• Significantly increased percentages of $CD45R^+$ $MHCII^+$ cells of immune cells in responding ACT and Triple	• The ACT- and Triple therapy leads to an improved rate of activated B cells within the TME

		treated tumors, compared to progressing tumors	
CD3	General T cell marker	• Increased percentage of $CD3^+$ cells of all viable singlets in responding ACT and Triple treated tumors. • Significantly decreased percentage of $CD3^+$ cells of all viable singlets in progressing tumors	• After an initial alteration of the immune reaction against tumor tissue as an effect of the therapy, tumor escape mechanisms counteract those effects
CD69	Early activation marker on T cells	• Significantly increased percentages of $CD69^+$ T cells in therapy responding tumors of ACT and Triple treated mice, compared to mice with progressive tumors under immunotherapy or Vehicle • Significantly increased percentages of $CD69^+$ of $CD8^+$cells in therapy responding tumors of ACT and Triple treated mice, compared to mice with progressive tumors under immunotherapy	• The ACT- and Triple therapy leads to an improved activation of T cells • Immune escape mechanisms reverse those effects
IFNγ	T cell activation marker	• Increased percentages of $IFN\gamma^+$ $CD8^+$ and $CD4^+$ cells cells in therapy responding tumors of ACT and Triple treated mice, compared to mice with progressive tumors under immunotherapy or Vehicle • Some progressive tumors still show high percentage of $IFN\gamma^+$ $CD8^+$ and $CD4^+$ cells	• The ACT- and Triple therapy leads to an improved activation of T cells • Immune escape mechanisms often reverse those effects
CD4/CD8 ratio	Ratio of $CD4^+$ T cells to $CD8^+$ T cells	• Triple treated, responding and progressive tumors show lower CD4/CD8 ratios compared to untreated tumors	• The additional administration of TLR agonsists and aPD 1 antagonists leads to a shift in T cell differentiation towards $CD8^+$ T cell

4.3.6 TLR -3, -7, and -9 agonist injections into the tumor inhibits tumor cell reproduction rate

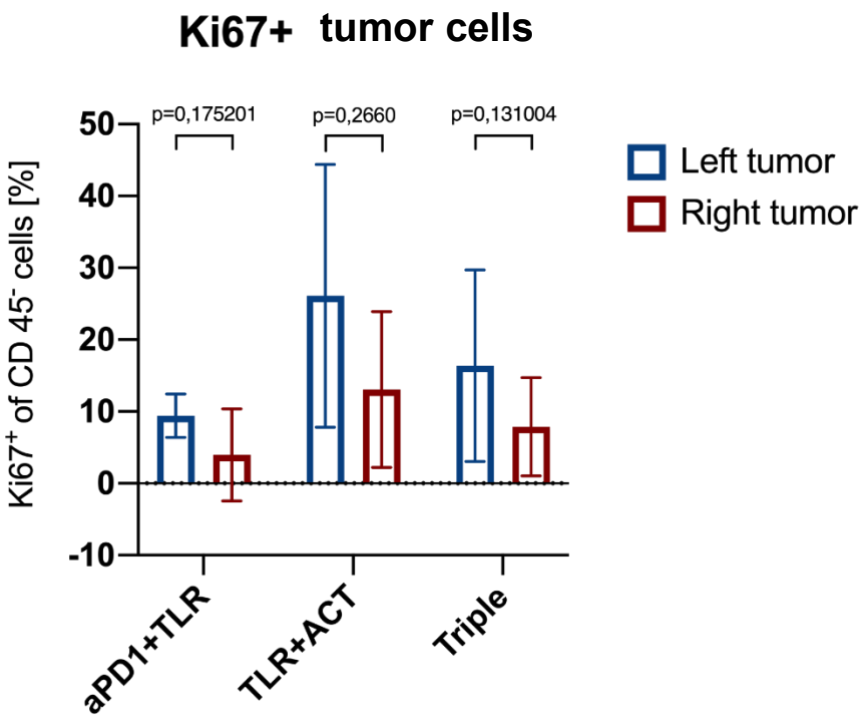


Figure 3.14

This column diagram compares the reproductive activity of undifferentiated pleomorphic sarcoma cells (defined as CD45⁻ viable cells) in the left vs. the right tumor.
As shown in 2.2.2.3 the right tumor was directly injected with TLR agonist mix (TLR 3 agonist - poly I:C / TLR 7 agonist - Gardiquimod / TLR 9 agonist – ODN-2395)
Statistical analysis data was gained performing the unpaired t-test.
Mean and SD are given.

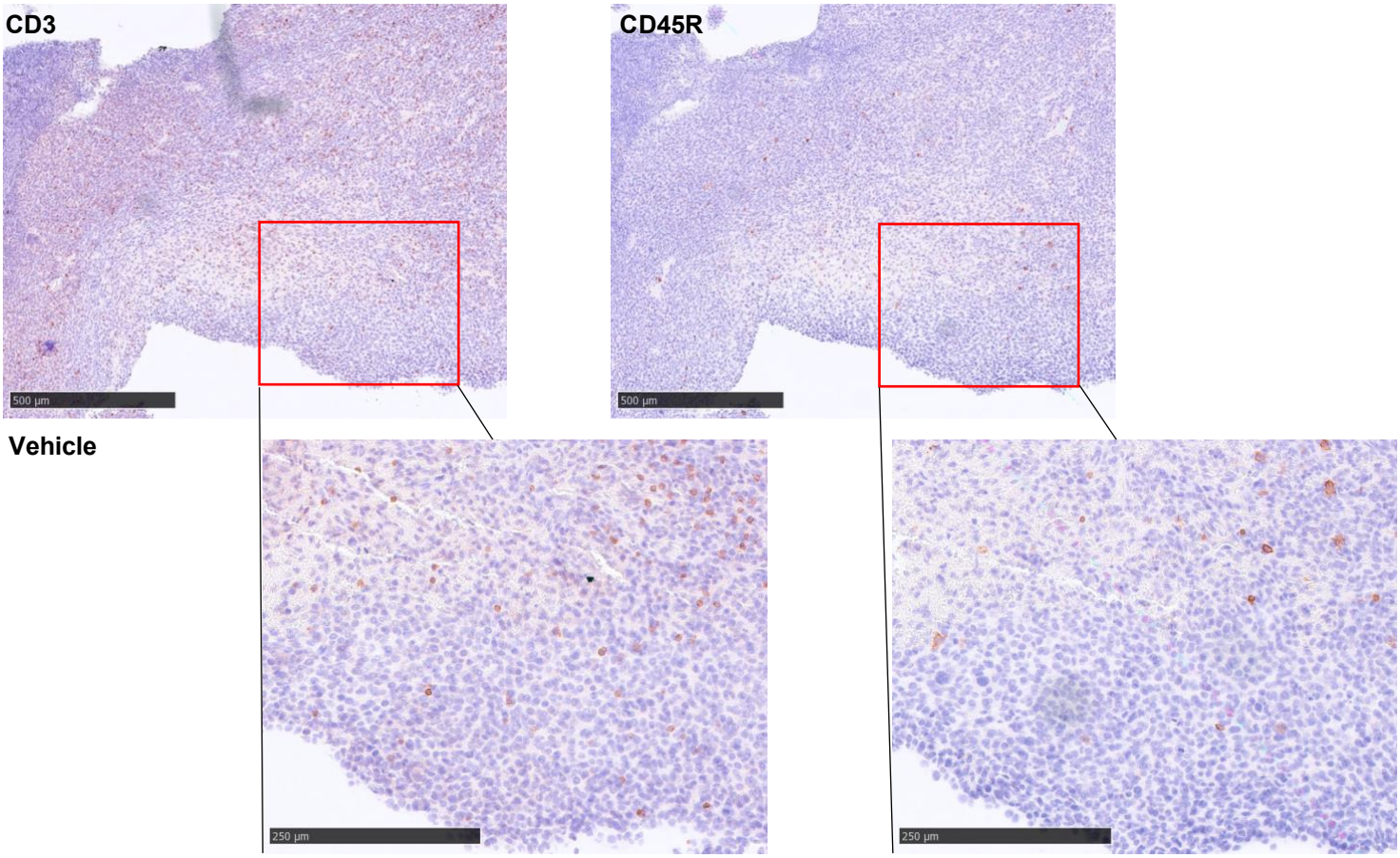
Ki67 is commonly known as a marker for cellular reproduction rate, being expressed to greater extend in rapidly growing tissue (Yang et al. 2018). The reproductivity of undifferentiated sarcoma cells was therefore determined by the expression of Ki67.

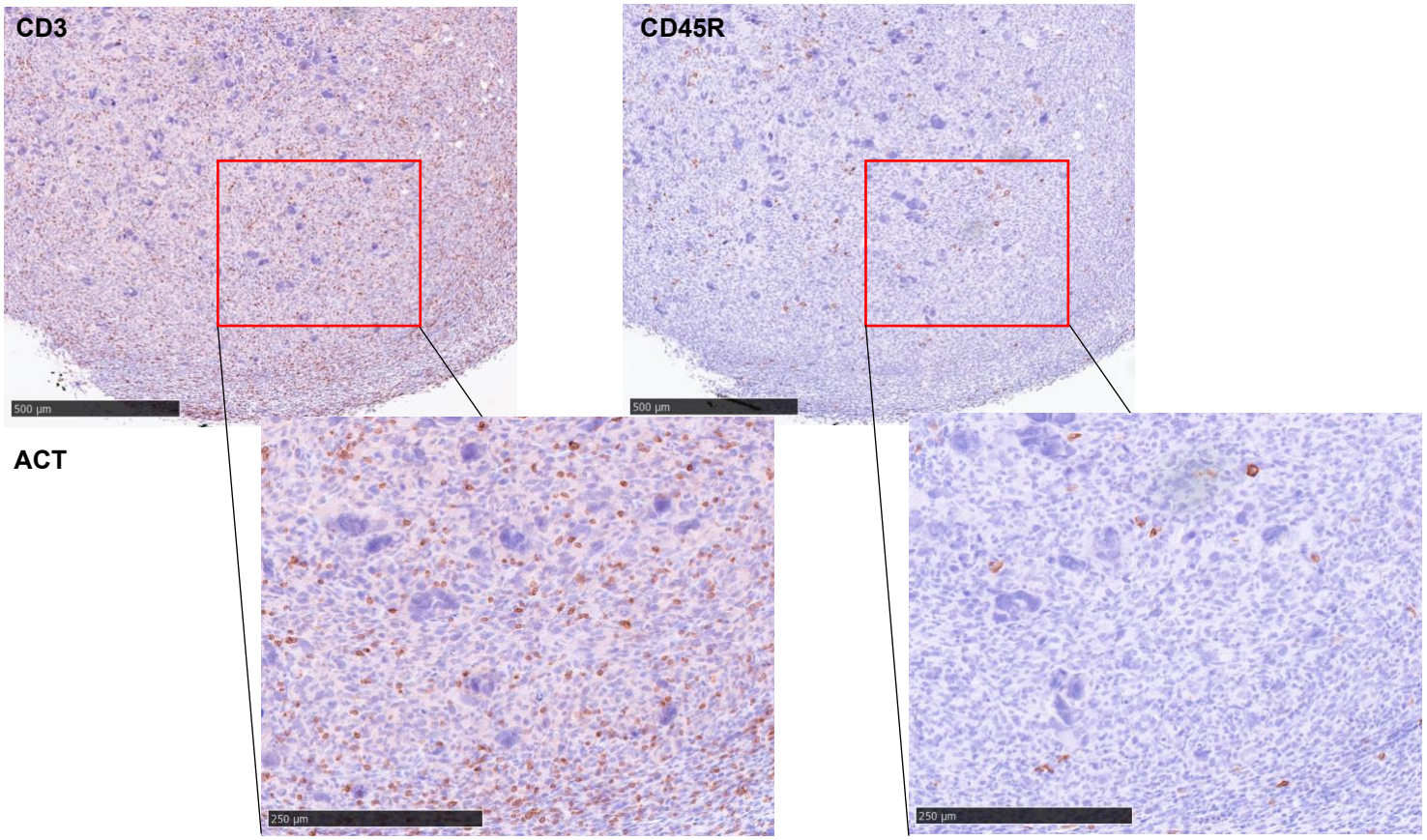
As to be seen in figure 3.14, the cell reproduction rate and thus the tumor growth rate, is lower in the right tumors that were regularly injected with TLR-3, -7 and -9 agonist mix.

Suggesting that this part of the applied immunotherapy has effects at the injection site triggering significantly no or substantially lower systemic effect. This local effect is found with TLR agonist combination therapies, regardless of whether they are used with ACT or aPD-1 only, or in triple combination.

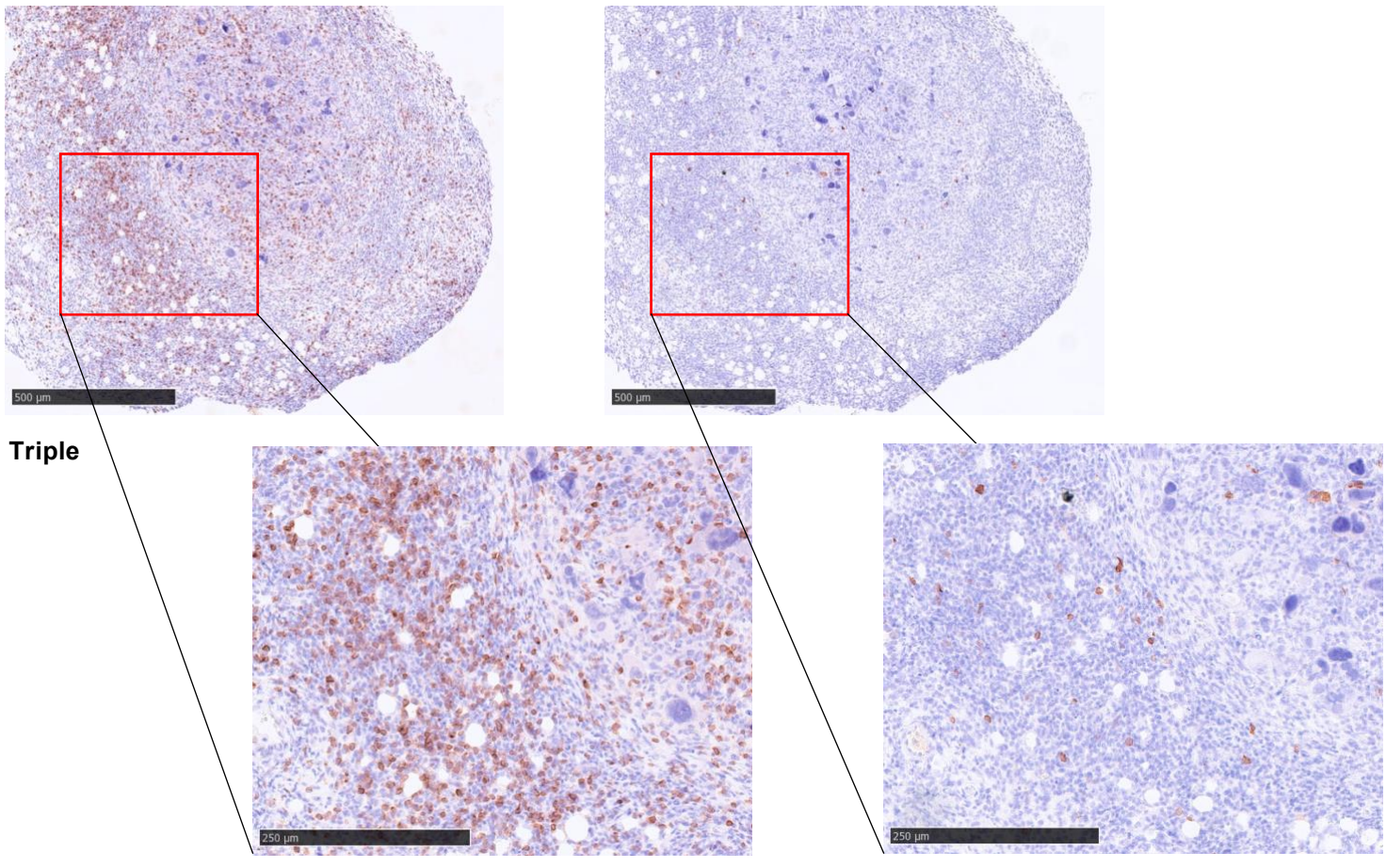
4.3.7 Immunohistochemistry of therapy responding undifferentiated pleomorphic sarcoma mouse models underlines FACS data results

Analyzing the immunohistochemical staining of ACT and Triple treated tumors under response and comparing those to untreated, growing tumors, it is noticeable that therapy responding tumors show slightly higher infiltration with B cells and particularly T cells. These finding correlate to the FACS data presented in 3.3.2 and 3.3.4.
What is even more eye catching is the changes within the tumor tissue.
Whereas the untreated vehicle tumors show homogenous size and arrangement of the tumor cells, both ACT and Triple therapy responding tumors show fields of altered tumor cells with an impressive anisocytosis, containing large, inhomogeneous nuclei and multinuclear cells. In more periphery areas of those samples, homogenous tumor cells arrange like those in untreated specimens.





ACT



Triple

Figure 3.14

Immunohistochemically stained tumor specimens of untreated (vehicle) ACT treated (ACT) and ACT, aPD-1 and TLR-3,-7, and -9 agonist mix treated (Triple) subcutaneous undifferentiated pleomorphic sarcoma mouse models.
ACT and Triple specimens origin from tumors during therapy response.
The left microscopic pictures show CD3 expression on T cells (brown). The right pictures show a CD45R staining that highlights murine B cells (brown).

5 DISCUSSION

Options for adequate and successful non-interventional therapeutic regimens against unresectable soft tissue sarcomas are limited and have barely improved within the last years. In general, soft tissue sarcomas are considered to be immunologically poorly active tumors, showing low mutational burdens (The Cancer Genome Atlas Research Network 2017). This might make them less vulnerable to immunotherapeutic interventions and thus more difficult to combat and may be one reason for poor improvement in treatment. Also, lower incidence rates of soft tissue sarcoma compared to other malignancies lead to limited social interest for investigation in improved therapy regimens.

In this thesis, the effects of an innovative adoptive cellular immunotherapy on murine undifferentiated pleomorphic sarcoma cells in vitro were explored.

The antitumor efficacy of this adoptive cellular immunotherapy was intended to be enhanced by combination with aPD-1 antibodies and TLR agonists.

Furthermore, using these approaches it was intended to induce changes in the TME immune infiltration in soft tissue sarcoma mouse models beneficial to the overall survival. The characteristics and the significance of the induced immunological changes were investigated. So far, it has not yet been described in detail whether and how exactly the TME immune infiltration of undifferentiated pleomorphic sarcoma is amenable to the influence of immunotherapy combinations such as the innovative ACT combination of 4 different effector cell types and the combination with aPD-1 and TLR 3, 7 and 9 agonists.

This work has not been submitted for publication.

The following hypotheses were to be evaluated:

> **Hypothesis 1:** *Doxorubicin significantly affects undifferentiated pleomorphic sarcoma cell line proliferation in vitro*

This hypothesesis can be seen confirmed by the results that were generated with the performed cell viability assay (Fig.3.1). But it was notably that different undifferentiated pleomorphic sarcoma cell lines showed different resilience against the toxicity of doxorubicin. All MCA cell lines showed a 50 % cell viability at a compound concentration of about 1 µM, while KP cell lines seemed to be a little more susceptible to the cytotoxicity of doxorubicin reaching a 50 % viability even at lower concentrations.

One KP cell line (420085) showed remarkably lower resilience towards doxorubicin.

The increased sensitivity of KP cell lines to doxorubicin is likely due to increased proliferation and production of reactive oxygen species driven by KRAS mutation.

In DNA with heightened replication stress, doxorubicin is more likely to induce substantial DNA damage.
The more heterogenous genetics in MCA deriving sarcoma cells may induce resistance mechanisms against doxorubicin such as efflux pumps, apoptotic response alteration and enhanced DNA repair.
For future experiments it is an interesting task to find out what mechanisms are underlying the increased sensitivity of that one cell line 420085 to doxorubicin. Furthermore it is interesting to find out what is the reason for those changes and whether they could potentially be externally induced to aim for a better therapy response.

Hypothesis 2: *ACT components show antitumor response against undifferentiated pleomorphic sarcoma cells in vitro and there is an additional effect if the effector cells types are combined*

The luciferase viability assay (Fig. 3.2) shows, that the in vitro killing capabilities of CTLs, LAKs and CIKs are quite limited. Just γδ-T cells and the complete ACT combination showed relevant killing effects in higher concentrations.
As γδ-T cells bypass MHC restriction and recognize tumor cells via a variety of tumor ligants, their superior killing in vitro can be explained by non antigen specific cytotoxic capabilities (Chan et al. 2022; C. Q. Wang, Lim, and Tan 2023).
In the ACT combination, complementary mechanisms of tumor cell recognition and killing might lead to superior direct killing effects. This underlines findings in previous results of our working group, while this exact experimental setup has not been executed so far.

Hypothesis 3: *The ACT treatment can improve survival in our soft tissue sarcoma mouse models in comparison to standard chemotherapy regimens*

Figure 3.3 and figure 3.4 show enhanced MOS in ACT treated mice, compared to mice that were treated with doxorubicin only (20,6 d (ACT) vs 9,2 d (Doxo)).
Doxorubicin on the other hand led to better survival than aPD-1 or TLR treatment (MOS 4,6 d (aPD-1) an MOS 7,8 d (TLR agonists))
Interestingly the Vehicle group outperformed the doxorubicin group in terms of survival rates (MOS 16 d (Vehicle) vs. MOS 9,2 d (Doxo)). That finding might indicate severe toxicity of doxorubicin treatment in our mouse models. The toxicity might have led those mice to earlier hit breakup criterias and therefore bias these survival results.
Survival in doxorubicin or ACT treatment has not been tested in soft tissue sarcoma mouse models in previous works. Therefore there is no comparison for this data.

Hypothesis 4: *The ACT combination therapy with antiPD-1 antibodies and TLR agonsists shows additional effects on improving survival and growth kinetics in comparison to ACT monotherapy*

In fact the Triple therapy regimen showed the best results in terms of overall survival and tumor growth. The MOS of Triple treated mice (25,4 d) surpasses all other treatment regimens. aPD-1+ACT- and TLR+ACT treatment could also show decent survival rates of 22,4 d (aPD-1+ACT) and 15,6 d (TLR+ACT) respectively.
Figure 3.7 displays the favourable growth kinetics that are induced by the Triple treatment and surpass the growth kinetics of ACT-, Doxo- or non treated mice.
As expected and predicted by preliminary results, those effects could be overcome by the tumor and after initial response all tumors showed progression.
These findings align with preliminary results from Borchman et al who could show similar growth kinetics in mouse models with other solid cancer entities.

Hypothesis 5: *Mice, responding to our combination immunotherapy, show an initial increase in general immune infiltration and -activation. Tumor escape mechanisms counteract those effects over time.*

This hypothesis can be seen confirmed as soft tissue sarcoma bearing mice that were Triple treated, showed significantly higher infiltration with B cells, T cells and immune cells in general as they were under therapy response compared to progressive tumors (Fig. 3.11 and Fig. 3.13).
Also B cells and T cells in Triple treated, responding tumors showed significantly elevated activation markers, compared to vehicle tumors or progressing tumors under therapy (Fig. 3.12 and Fig. 3.13).
The comparison of therapy responding Triple treated tumors to untreated ones, also showed a tendency to higher immune infiltration with B cells, T cells and immune cells in general.
Unfortunately in these cases the increases in immune infiltration were not significant.
Interestingly and aligning with survival and growth data, ACT mono treated tumors showed comparable immune infiltarion and -activation patterns. This shows the significant role of the ACT therapy within the Triple therapy regimen.
In previous work, Borchmann et al. could already show significantly increased immune infiltration and -activation in Triple treated and ACT mono treated KP lung cancer, and B16F10 melanoma mouse models.

He thus proved an induction of rich and viable immune cell infiltration in generally poorly immunogenic tumors (Borchmann et al. 2022).

Some soft tissue sarcoma entities like myxofibrosarcoma or undifferentiated pleomorphic sarcoma show higher mutational burden than other soft tissue sarcoma and might thus be rather receptable towards immunomodulating therapies (Grünewald et al. 2020)
Pollack and colleagues could show, that comparing different entities of soft tissue sarcomas, undifferentiated pleomorphic sarcoma and myxofibrosarcomas express particularly high amounts of PD-1, PDL-1 and rich T cell infiltration (Pollack et al. 2017).
This of course makes UPS a potential target for checkpoint inhibition and immunotherapeutic approaches in general.
D'Angelo and colleagues performed phase 2 trials to further investigate in the effects of nivolumab with or without ipilimumab on different advanced soft tissue sarcoma entities.
They concluded that checkpoint inhibition in undifferentiated pleomorphic sarcoma might be beneficial to the median overall survival and progression free survival when combined with other therapeutic approaches (D'Angelo et al. 2018).
In the experimental setups shown in 3.2.2., the use of aPD-1 antibody alone did not lead to an improvement of survival or inhibition of tumor growth. However, the combination with our new ACT combination clearly enhanced the MOS which in turn fits to suggestions of D'Angelo that the combination with other immunotherapeutic interventions is important to unfold the antitumor effects of aPD-1 in soft tissue sarcoma.
The effect of adoptive cellular immunotherapy has been explored successfully on soft tissue sarcoma mouse models (Sangiolo et al. 2014) and there are rising numbers of clinical trials and scientific works to test the effects of in vitro expanded autologous TILs in patients suffering from progressive sarcoma (NCT03449108 and NCT03935893) (Rosenberg, Parkhurst, and Robbins 2023; König et al. 2024).
Borchmann and colleagues found that especially the concurrent presence of γδ T cells, NK cells, and T cells, led to significantly improved overall survival rates in several solid malignancies (Borchmann et al. 2022). To mimic this infiltration patterns, the particularly effective ACT combination therapy containing CIKs, LAKs, γδ-T cells and CTL was developed, generating potent synergistic antitumor properties.
The effectiveness of this combination therapy could be further improved by pre-treatment lymphodepletion, the additional gavage of aPD-1 antibodies and the administration of TLR-3,-7, and -9 agonists in the tumor site.
With this setup, they were able to tackle poorly immunogenic tumors such as lung cancer, non-Hodgkin lymphomas and others successfully and induce sustainable antitumor responses (Borchmann et al. 2022).

Despite the fact, that the significance of shown findings in 3.1.2 is admittedly limited by the single repetition of the experiment, the killing assay data displays the synergistic antitumor effects of this innovative ACT also against undifferentiated pleomorphic sarcoma in vitro.
The displayed survival data of undifferentiated pleomorphic sarcoma bearing mice support the Borchmann et. al. data even though long-term response could not be achieved.
Firstly, the data show that in our mouse models the ACT mono treatment successfully alters the MOS in comparison to no or doxorubicin treatment.
Beyond that, the additional administration of aPD-1 and the TLR mix as part of the Triple treatment regimen showed additional, presumably synergistic effects on MOS (3.2.3. Fig.3.6)
Subsuming, the ACT mix seems to play the most substantial role for the efficacy of the therapeutical manipulation of the TME.
In the orthotopic mouse model, the ACT therapy group seems to have a better overall survival than the Triple treated mice.
Hypothetical explanations for this observation may be a pseudoprogression in the first days after the start of the therapy. Due to massive immuneinvasion in the tumorsite, accelerated tumorgrowth led the tumors to quickly exceeded the permitted size. Thus the mice had to be sacrificed before the actual therapeutic effect of the Triple therapy regimen could have unfold its full tumor killing capacities.
The correlation of immune infiltration into the TME and the response to immune modulating therapies in soft tissue sarcomas is well known (Ayodele and Razak 2020; Sousa et al. 2021). Petitprez et.al. established a sarcoma immune classification, according to TME, immune infiltration and vascularization pattern. This stratification enables the prediction of response to immunotherapies such as checkpoint inhibition and consequently an improvement in the overall survival rate in soft tissue sarcomas. Besides a generally high immune invasion in the TME, they identified especially a high expression of a B cell lineage signature and the presence of dendritic cells and tertiary lymphoid structures as particularly favorable for overall survival and response to immunotherapy (Petitprez et al. 2020). This evidence led to the question if by the use of our immunotherapeutic regimens, changes in the immune infiltration compositions in the TME could be induced towards a rather beneficial immune classification, according to the data from Petitprez et al..
Analyzing the FACS data, shown in chapter 3.3 ff, we can certainly speak of a modification of the TME, being associated with tumor remission.
Portraying the systemic immune responses by analyzing spleen tissue, a distinct increase of macrophages and DCs in therapy responding mice catches an eye. Especially DCs are well known to positively influence the immunological antitumor response as they are potent APCs, activating the secondary and also the primary immune responses (Petitprez et al. 2020; Kirchner et al. 2024; Jiang et al. 2024). The higher proportion of these cells found in a

secondary lymphoid organ of therapy responding mice might suggest intensified generalized antigen presentation activity and immune cell stimulation.

Both general and local B cell activation within sarcoma tissue was enhanced in therapy responsive mice compared to mice under progressive disease. Also lower B cell proportions in spleens and simultaneously risen proportions of B cells in tumorous tissue during therapy response compared to progressive diseases might suggest immune emigration from secondary lymphoid organs into tumor tissue. This might happen in the context of a therapy induced enhanced immune reaction within the tumor site.

The enhanced expression of activation marker such as CD80 and CD86 on tumor suppressive- and tumor-promoting macrophages, as well as on B cells within the spleen tissue of therapy responding mice, underlines the assumption of a deep stimulation of the entire immune system. It is known that TLR agonists and checkpoint inhibitors lead to increased CD80/CD86 expression (Sagiv-Barfi et al. 2022). With our experiments it could be proven, that the administration of our ACT alone triggers an activation of macrophages and B cells just alike.

During tumor progression under ongoing immunotherapuetical treatment , the immune infiltration vanishes. This observation, shown especially for the entirety of $CD45^+$ cells and T lymphocytes, indicates that an immune escape must have emerged in all sarcomas after initial therapy response.

Borchmann et al. could show a long lasting anti-cancer immunity in melanoma mouse models could be induced by a repeated ACT injection on day 27 after tumor injection (Borchmann et al. 2022).

CD69 is an early marker of T cell activation, and IFNγ indicates a high activation state in T cells. Figure 3.12 shows these markers on CD4+ and CD8+ T cells especially on responding tumors which suggests, that those play a crucial role in the cellular acquired antitumor efficacy of the ACT containing therapy regimens (Cibrián and Sánchez-Madrid 2017; Castro et al. 2018).

IFNγ expression does not only indicate T cell activation in general, but also enhances MHC I and II activity on APCs and leads to a differentiation towards Th1 phenotype T cells. This differentiation stimulates marcrophage activation and helps a cytotoxic T cell activation to take action. Also, enhanced IFNγ expression has proinflammatory effects on other lymphocytes like NK, NKT and antigen-presenting cells and it supports proapoptotic and antiproliferative effects on tumor cells (Cha et al. 2019; Castro et al. 2018; S. Chen, Zhu, and Jounaidi 2024).

The CD4/ CD8 ratio being pushed towards higher counts of $CD8^+$ T cells in responding Triple treated tumors compared to ACT mono treated ones, potentially indicates enhanced direct cytotoxic antitumoral activity of the additionaly administered TLR agonists and aPD-1 antibodies.

The most interesting finding of the experiments was, that the adaptive B cell immune response was activated and triggered to infiltrate the TME by the ACT which consists of T cells (CIKs / CTLs), NK cells (CIKs / LAKs) and γδT cells. This means, that the potent effects of the ACT do not only rely on direct effects of the administered effector cells, but beyond that, lead to a general activation of the immune system (Fig.3.8, Fig.3.11, Fig 3.12.) Also, the relatively weak killing effects displayed in the luciferase assays contrast with the distinct antitumor effect found in vivo. This observation further supports the assumption that the most important in vivo effects of the ACT result from the interactions with the living, responding immune system.
The FACS data suggest that an effective collaboration of the innate and acquired immune system can be nudged by the ACT regimen. This insight closes a gap between the discoveries of Petitprez et. al. and Borchmann et al.
The innovative adoptive cellular immunotherapy, that induces the TME infiltration of γδT cells, NK cells and T cells found to be very favorable for the outcome of solid malignancies, apparently induces changes in the immune response that lead to the especially advantageous B cell immune infiltration described by Petitprez et al. (Borchmann et al. 2022; Petitprez et al. 2020).
The fact that Triple treated murine undifferentiated pleomorphic sarcomas did not only show even higher B cell and T cell activation but also further enhanced survival rates, underlines the significance of this interaction and the additional positive impact of aPD-1 and TLR-3,-7 and -9 agonist mix.
In the small numbers of cases in which immunohistochemical probing was achievable, increased amounts of T cell and B cell infiltration could have been identified in therapy responding tumors.
Analyzing the immunohistochemical staining it is unmissable that the ACT and the Triple treated undifferentiated pleomorphic sarcoma show different appearances of the tumor cells compared to the vehicle tumors. Whereas vehicle tumor specimen present themselves with uniform tumor cell distribution and heterogeneous appearance of the cells, the responding sarcomas showed nests of exceptionally pronounced anisocytosis and nuclear pleocytosis.
An exact grading after the sarcoma immune classification to further categorize the induced changes within the tumors was unluckily impossible to conceive as the Microenvironment Cell Populations-counter method (Becht et al. 2016) was performed by Petitprez et al. to categorize the examined specimen.

The growth data of our orthotopic mouse models could unluckily not be more detailed as the intervals between the measurements could not be shortened due to animal savety regulations and restricted scanning capacities.

The absence of growth data in between the weekly MRI measurements leave substancial phases of the fast growth kinetics unclear.
In the experimental in vivo setups it was particularly difficult to gain histological samples of responding tumors because after an initial response to the therapies, the tumor vanished within 1-2 days leaving very little quantity of tissue to analyze.
Therefore the relatively small amount of data in some FACS analysis figures like Fig. 3.5 or 3.8, where only 4 to 5 data points are displayed for Triple and ACT progressive tumors, can be explained by the small amounts of tumor mass that could be harvested in those mice. Thus, a cut-off count of events that was determined at 10^4 to further proceed with the gating protocol, was not reached for certain cellular subsets. This was done to guarantee statistically valuable findings. Even though lower numbers of specimen automatically compromise the statistical meaning to a certain extend, the created data can still be an indicator of how infiltration patterns change during therapy response.
Since there is no sufficient data for macrophage and DC infiltration in tumor, it is unfortunately not possible to make any further statements about this.
Again, a lack of analyzable tumor volume in responding (thus shrinking) tumors, prohibited the analysis of further cell lines such as macrophages and DCs.
This is also the reason why there was just very small quantities of tumorous tissue for immunohistochemical staining.
For the reasons mentioned above, quantitative evaluation of the immunohistological specimen was not feasible and thus more data could not be generated.
To overcome those limited numbers of analyzable material, larger mouse cohorts need to be treated with our immunotherapies to generate more therapy responding tumor tissue.
The use of one undifferentiated pleomorphic sarcoma cell strain in all mouse setups was purposefully chosen as it allows a good comparison between the tumors. On the other hand it doesn't display the mutational variety within the group of undifferentiated pleomorphic sarcomas and other soft tissue sarcomas that show up in clinical everyday life.
The applicability of data from murine heterotopic undifferentiated pleomorphic sarcoma mouse models on other murine or even human soft tissue sarcoma cell lines cannot be seen as granted. Therefore, experiments should be performed with other entities of soft tissue sarcomas. Furthermore, PDX derived, humanized sarcoma mouse models should be established to take the next step towards clinical studies. Similar and confirming results could subsequently lead to the next phase on the way to a potential clinical application of a Triple regimen.
Checkpoint inhibitors such as anti-PD-1 inhibitors play an important role in modern antitumor therapy having found their way into various therapy regimens against several malignancies

such as melanomas, non-small cell lung cancer, Hodgkin lymphomas and many others (European Medicines Agency (EMA) 2021).

Also several TLR agonists are approved for human medical use already or are under clinical investigation in clinical trials (Luchner, Reinke, and Milicic 2021).

Investigations on ACTs as a therapeutic approach in cancer patients have been performed since the late 20th century and clinical trials of different approaches were performed (Creelan et al. 2021; Mata and Gottschalk 2015; König et al. 2024; Subhi-Issa et al. 2025).

New approaches to combat STS are tested in ongoing trials as the scientific research process is boosted by a growing understanding of microbiological principles, innovative implementations of genetical engineering and AI driven research. This allows to reconsider perceived barriers . (M. Husain et al. 2023; Wood et al. 2024; Chawla et al. 2025) The clinical application of top notch therapeutic approaches such as genetically engineered effector cells or cancer vaccinations are the next step towards improving survival and quality of life for patients with hard-to-treat cancers.

To proceed with our research results, further investigations should evaluate the efficacy of a second adoptive cell transfer in the orthotopic sarcoma mouse models employed in the current study.

Considering further augmentation of the studied Triple regimen to overcome tumor immune escape, more distinctive analysis of immune cells within the TME of responsive and progressing tumors must be put to consideration. For example, T and B cell subsets, as well as tumor associated macrophages, the expression of immune suppressing receptors on tumor cells and the presence of compromising cytokines should be examined.

After generating data about effects of the Triple therapy against human soft tissue sarcoma entities in vitro and in mouse models, the implementation of the immunotherapeutic combination regimen in clinical trials should be considered the following step.

As there are many examples of the application of ACTs in human, the scalability and technical realization seems not to be a relevant hurdle.

Tumor samples can be obtained through biopsy and preserved, while stem cells for the production of ACT components can be mobilized and isolated from PBMCs, then further processed and expanded at large scale using closed-system bioreactors.

TLRs and aPD-1 antibodies are already approved for clinical use in humans.

In summary, our results show the striking antitumor effects of the immunotherapy combination regimens combating soft tissue sarcoma, most notably the triple regimen. Therapeutic effects could be revealed at a cellular level using FACS analysis. Also associations between induced TME modifications and beneficial effects on tumor growth and survival rates could be made.

The treatment regimens have shown their effectiveness and should be further investigated to strive for clinical implementation and thus potentially create a better alternative to existing, outdated therapies which unfortunately still reflect clinical reality. This dissertation is intended to be a silver lining for soft tissue sarcoma patients and a modest contribution to upcoming discoveries that have the potential to improve or even save human lifes.

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Jonathan Bochtler
Wolfbachstrasse 9 | 8032 Zürich | Schweiz
mail: jonathan.bochtler@web.de | fon: +41 76 696 0072

Berufserfahrung

03 / **2024-2026** **Assistenzarzt in der Allgemeinchirurgie, Seespital Horgen, Schweiz**

Akademia

08 / **2020-2026** **Promotion an der Universität zu Köln (Disputation ausstehend)**
Promotion mit Forschungsschwerpunkt Immuntherapie in Weichteilsarkomen in der Forschungsgruppe Krebstherapie und molekulare Bildgebung bei Professor Roland Ullrich

04 / **2017**-10/ **2023** **Universität zu Köln**
Studium der Humanmedizin

2010-2012 **Zinzendorf Gymnasium Königsfeld**
Allgemeine Hochschulreife

2004-2010 **Fürstenberg Gymnasium Donaueschingen**

Publikationen

Hochprozessierte Lebensmittel und Krebsrisiko
Bochtler, J., Hartmann, M. & Alakus, H.
Hochprozessierte Lebensmittel und Krebsrisiko.
Onkologie **29**, 642–644 (2023). https://doi.org/10.1007/s00761-023-01356-3

Einfluss der COVID-19-Pandemie auf Krebsvorsorgeuntersuchungen
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Einfluss der COVID-19-Pandemie auf Krebsvorsorgeuntersuchungen.
Onkologie **29**, 455–456 (2023). https://doi.org/10.1007/s00761-023-01340-x

Praktisches Jahr

07 / **2023**-10 / **2023** **Stadtspital Triemli, Universität Zürich, Schweiz**
Klinik für Chirurgie

03 / **2023**-07 / **2023** **Universitätsklinikum Köln, Deutschland**
Klinik für Innere Medizin

11 / **2022**-03 / **2023** **Universitätsklinikum Köln, Deutschland**
Abteilung für Kinderchirurgie

Sonstige praktische Erfahrungen

10 / **2014**-03 / **2017** **Ausbildung zum Gesundheits- und Krankenpfleger**
St.Vincentius Klinikum, Karlsruhe (Heute: Vidia Kliniken)

11 / **2013**-09 / **2014** **Hauptamtliche Anstellung als Rettungssanitäter und Erste-Hilfe-Ausbilder**
Deutsches Rotes Kreuz, Kreisverband Donaueschingen

10 / **2012-** **Freiwilliges soziales Jahr im Rettungsdienst**
10 / **2013** Deutsches Rotes Kreuz, Kreisverband Donaueschingen

Sonstiges

Sprachen	Deutsch (Muttersprache), Englisch (Flüssig in Schrift und Wort)
EDV	Microsoft Office (Word, Excel, PowerPoint), Prism, Kaluza Flow Cytometry
Förderung	Stipendiat der Mildred Scheel School of Oncology Stipendiat “Hellste Köpfe” der Deutschen Röntgengesellschaft