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Synergistic antitumor effect of combined laser thermotherapy and immunomodulators in malignant melanoma: pilot studies

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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ANA CATARINA OLIVEIRA TRIGO

SYNESGISTIC ANTITUMOR EFFECT OF COMBINED LASER THERMOTHERAPY AND IMMUNOMODULATORS IN MALIGNANT MELANOMA: PILOT STUDIES

Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto – Especialização em Oncologia Laboratorial

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Abstract

The field of oncology is important in medical research due to the high mortality rate caused by cancer. The inflammatory tumor microenvironment (TME) and the tumor's ability to evade the immune system are considered a cancer hallmark. The interaction between the immune system and tumor cells is complex, as the immune system can both prevent and facilitate tumor progression. This interaction has led to the development of immunotherapies. Immune-checkpoint inhibitor (ICI)-based immunotherapy has shown promise in certain types of cancer, but its success is limited as it heavily relies on the immunogenicity of the tumor. The hypothesis that hyperthermia can improve the immune status of the TME, enhancing the effectiveness of ICI immunotherapy, has prompted our research group to evaluate the combined effects of laserthermotherapy and ICI therapy in a poorly immunogenic melanoma mouse model.

This dissertation presents the findings of three pilot studies, with the primary objective of optimizing conditions and approaches for subsequent use in a more comprehensive conclusive study, that aims to evaluate the combined antitumor effect of laserthermotherapy with anti-PD-1 and anti-CTLA-4 immunotherapy in a synergistic manner. Pilot study 0 evaluated, by flow cytometry, the distribution of the immune cell populations in the peripheral blood of wild-type C57BL/6 mice and showed that the lymphocytic populations are the most frequent. Pilot study II sought to evaluate the impact of laserthermotherapy treatment, specifically the TRANBERG laser, as a standalone treatment on the distribution of immune cell populations of C57BL/6 poorly immunogenic melanoma mice model, but the limited number of animals hindered conclusive findings. Nevertheless, Pilot study II still held significant value as it allowed team members to familiarize themselves with the TRANBERG laser. Pilot study III was conducted to determine the optimal dosages of anti-PD-1 and anti-CTLA-4 antibodies to be carried forward into the final study. This involved evaluating the effects of two dosages of anti-PD-1 and anti-CTLA-4 (2,5 mg/kg and 5,0 mg/kg), as well as their respective isotype controls, on the distribution of immune cell populations in C57BL/6 poorly immunogenic melanoma model. Flow cytometry analysis revealed an increase in $CD8^+$ T cells in the tumor for mice treated with anti-PD-1 and anti-CTLA-4 at 5,0 mg/kg. However, the differences between the treatment antibodies and their isotype controls were expected to be more pronounced, leading to a suggested dosage of 10 mg/kg of the treatment antibodies for the final study.

Overall, the pilot studies conducted prior to the conclusive study held great significance as they allowed for the optimization of protocols and methodologies.

Keywords: cancer immunotherapy, immune-checkpoint inhibitors, laserthermotherapy

Resumo

O domínio da oncologia é de grande importância na investigação médica devido à elevada taxa de mortalidade causada pelo cancro. O microambiente tumoral inflamatório, assim como a capacidade das células tumorais escaparem ao sistema imunitário, são duas características do cancro. A interação entre as células tumorais e o sistema imunitário é complexa, uma vez que o último pode impedir ou facilitar a progressão tumoral. Esta interação conduziu ao desenvolvimento de imunoterapias. A imunoterapia baseada em inibidores de *checkpoints* imunitários (ICIs) tem-se mostrado muito promissora em certos tipos de cancro. Contudo, o seu sucesso é limitado, uma vez que depende da imunogenicidade do tumor. A hipótese de que a hipertermia pode melhorar o estado imunitário do microambiente tumoral, aumentando a eficácia da imunoterapia com ICIs, levou a equipa de investigação a avaliar a imunomodulação do microambiente tumoral resultante da combinação da laser-termoterapia com terapia com ICIs, num modelo animal de melanoma pouco imunogénico.

Esta dissertação resume os resultados de três estudos-piloto realizados para otimizar as condições para um estudo conclusivo de maior dimensão. O objetivo geral do estudo final é avaliar o efeito antitumoral combinado da laser-termoterapia com a imunoterapia anti-PD-1 e anti-CTLA-4 de uma forma sinérgica. O estudo piloto 0 utilizou a citometria de fluxo para analisar a distribuição das populações de células imunitárias no sangue periférico de animais *wild-type*, e revelou que as populações linfocíticas eram as mais prevalentes. O estudo-piloto II centrou-se no impacto da laser-termoterapia, especificamente utilizando o laser TRANBERG, nas populações de células imunitárias num modelo animal de melanoma. No entanto, o número limitado de animais que terminaram o estudo não permitiu tirar conclusões definitivas. Apesar disso, o segundo estudo-piloto foi valioso para permitir que a equipa se familiarizasse com o laser TRANBERG. O estudo-piloto III foi realizado para determinar as dosagens ótimas de anticorpos ICIs a serem transpostas para o estudo final. Este estudo envolveu a avaliação dos efeitos de duas dosagens de anti-PD-1 e anti-CTLA-4 (2.5 e 5.0 mg/kg) na distribuição das populações de células imunitárias, num modelo pré-clínico animal de melanoma. A análise por citometria de fluxo revelou um aumento das células T $CD8^+$ infiltradas no tumor dos animais tratados com anti-PD-1 e anti-CTLA-4 a 5.0 mg/kg. No entanto, esperava-se que as diferenças entre os anticorpos de tratamento e os respetivos controlos isotípicos fossem mais pronunciadas, o que levou à sugestão uma dose de 10 mg/kg dos anticorpos ICIs para o estudo final.

Em geral, os estudos-piloto realizados antes do estudo conclusivo tiveram grande importância, pois permitiram a otimização de protocolos e metodologias.

Palavras-chave: imunoterapia do cancro, inibidores de checkpoints imunitários, laserterapia

Preface

This work was sponsored by Clinical Laserthermia Systems AB (Medicon Village, Scheelevägen 2, 223 81 Lund, Sweden), and the research work was developed in the Experimental Pathology and Therapeutics Group, IPO Porto Research Center (CI-IPOP), in collaboration with the Cellular Immunology Department, IPO-Porto - Instituto Português Oncologia do Porto Francisco Gentil, EPE (Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal) and i3S – Instituto de Investigação e Inovação em Saúde da Universidade do Porto (Rua Alfredo Allen, 208 4200-135 Porto, Portugal).

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The investigation conducted in this dissertation gave rise to the following presentations:

- Trigo, A.C.; Rodrigues, C.A.; Gaiteiro, C.; Mendes, N.; et al. “Synergistic antitumor effect of combined laser thermotherapy and immunomodulators in malignant melanoma – a flow cytometry analysis” – 5th Symposium of the MSc in Oncology, ICBAS-UP, Porto, Portugal (February, 2021) (Oral communication presented by Trigo, A.C.)

- Palmeira, C.; Rodrigues, C.A.; Trigo, A.C.; Gaiteiro, C.; Mendes, N.; Godinho, I.; Sousa, M.E.; et al. “The application of Flow Cytometry in testing new therapeutic strategies in preclinical in vivo models” – XVII Congress of the Iberian Society of Cytometry, Porto, Portugal (June, 2021) (Oral communication presented by Palmeira, C.)

- Trigo, A.C.; Rodrigues, C.A.; Gaiteiro, C.; Mendes, N.; Santos, L.; et al. "Estudo das populações celulares do sistema imunológico no sangue periférico de animais C57BL/6 wild type por citometria de fluxo” – II International LatinFlow 2021 – Online Meeting (November, 2021) (Poster presented by Trigo, A.C.)

- Rodrigues, C.A.; Maia, P.; Gaiteiro, C.; Mendes, N.; Ferreira, D.; Trigo, A.C.; et al. " Imunofenotipagem do microambiente tumoral de um modelo singénico de melanoma pouco imunogénico submetido a Imunoterapia – um estudo preliminar" – 4rd National Meeting of Young Oncology Researchers (ENJIO), Porto, Portugal (September, 2022) (Poster considered one of the ten best posters, selected for a "speed talk" and presented by Rodrigues, C.A. – awarded as the "Best poster presentation" of the Meeting, 500€ prize)

- Rodrigues, C.A.; Gaiteiro, C.; Mendes, N.; Maia, P.; Ferreira, D.; Trigo, A.C.; et al. "Synergistic antitumoral effect of combined laser thermotherapy and Immunotherapy: an exploratory in vivo study” – 1st IMMUNO-model COST Action Conference, Barcelona, Spain (June, 2023) (Oral communication presented by Rodrigues, C.A.)

- Rodrigues, C.A.; Maia, P.; Gaiteiro, C.; Mendes, N.; Ferreira, D.; Trigo, A.C.; et al. " The Antitumoral Effect Of Interstitial Laser Thermotherapy Combined With A PD-1 Inhibitor: An Exploratory Study” – Animal Models of Cancer Conference, Torres Vedras, Portugal (July, 2023) (Poster presented by Rodrigues, C.A. – awarded as one of the four best posters of the Conference, 220€ prize)

- Maia, P; Rodrigues, C.A.; Gaiteiro, C.; Mendes, N.; Ferreira, D.; Trigo, A.C.; et al. “Estudo do efeito antitumoral sinérgico da combinação de termoterapia e anti-PD-1 num

modelo in vivo" – 5th National Meeting of Young Oncology Researchers (ENJIO), Porto, Portugal (September, 2023) (Poster considered one of the ten best posters, selected for a "speed talk" and presented by Maia, P.)

- Maia, P; Rodrigues, C.A.; Gaiteiro, C.; Mendes, N.; Ferreira, D.; Trigo, A.C.; et al. "Explroring the antitumoral Effect of combining laserthermotherapy and anti-PD1 in a poorly immunogenic mice model" - 1 st Young Researchers Day (CI-IPO Porto), Porto, Portugal (to be presented November 2023)

Abbreviations

ACT - adoptive cell transfer

ADCC - antibody-dependent complement cytotoxicity

AIDS - acquired immunodeficiency syndrome

ALK - anaplastic lymphoma kinase

APC - antigen-presenting cell

ATP - adenosine triphosphate

B2M - β-2 microglobulin

BCR – B cell receptor

CAR - chimeric-antigen receptor

CHOP - CCAAT/enhancer-binding protein homologous protein

CLP - lymphoid progenitor cells

CRT – calreticulin

CSCC - cutaneous squamous cell carcinoma

CSR - class switch recombination

CTC - circulating tumor cell

ctDNA - circulating tumor DNA

CTL – cytotoxic T lymphocyte

CTLA-4 - cytotoxic T lymphocyte-associated molecule-4

CXCL - C-X-C motif chemokine

DAMPs - danger associated molecular patterns

DC - dendritic cell

dMMR – deficient MMR

EBF - early B-cell factor

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

EGFR - epidermal growth factor receptor

EMA - European Medicines Agency

ER - endoplasmic reticulum

Fab - fragment antibody binding

Fc - fragment crystallizable

FCSB - flow cytometry staining buffer

FDA - Food and Drug Administration

FGL-1 - fibrinogen-like protein 1

GM-CSF - granulocyte macrophage colony-stimulating factor

HCC - hepatocellular carcinoma

HLA - human leukocyte antigen

HMGB1 - high mobility group box 1

HNSCC - head and neck squamous cell carcinoma

HPV - human papilloma virus

HSC - hematopoietic stem cell

HSP - heat shock protein

IC – immune checkpoint

ICAM-1 - intercellular adhesion molecule 1

ICD - immunogenic cell death

ICI - immune checkpoint inhibitor

ICOS- inducible T-cell costimulatory

IDO 1 - indoleamine2,3-dioxygenase 1

IFN - interferon

Ig – immunoglobulin

IHC – immunohistochemistry

IL – interleukin

ImILT - Immune stimulating interstitial laser thermotherapy

IrAEs - immune-related adverse effects

Lag-3 - lymphocyte activation gene-3

mAb – monoclonal antibody

MAC - membrane attack complex

MAGE - melanoma-associated antigen gene

MCC - Merkel cell carcinoma

MDSCs - myeloid-derived suppressor cells

MHC - major histocompatibility complex

MMR - mismatch repair

MPM - malignant pleural mesothelioma

MSI - microsatellite instability

MSI-H - highly unstable MSI

MSI-L - lowly unstable MSI

NGS - next-generation sequencing

NK – natural killer cells

NSAIDs - nonsteroidal anti-inflammatory drugs

NSCLC - non-small cell lung cancer

OS - overall survival

PAMPs - pathogen associated molecular patterns

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PD-1 - programmed cell death receptor-1

PD-L1 - programmed cell death ligand-1

PFS - progression-free survival

pMMR – proficient state MMR

PRR - pattern recognition receptor

RCC - renal cell carcinoma

rhIL-2 - recombinant human IL-2

ROS - reactive oxygen species

SCLC - squamous cell lung cancer

SHM - somatic hypermutation

TAA - tumor associated antigen

TAMs - tumor-associated macrophages

TCR - T cell receptor

Tfh - follicular helper T cell

TGF- β - transforming growth factor beta

TIGIT - T cell immunoglobulin and ITIM domain

TIL - tumor infiltrating lymphocyte

TLR- toll-like receptor

TMB - tumor mutational burden

TME - tumor microenvironment

TNF-β - tumor necrosis factor-beta

T_{Reg} – Regulatory T cell

T-VEC - talimogene laherparepvec

UPR - unfolded protein response

VCAM-1 - vascular cell adhesion protein 1

VEGF - vascular endothelial growth factor

VISTA - V-domain Ig suppressor of T cell activation

VSIG-3 - V-Set and Immunoglobulin domain containing-3

WES - whole-exome sequencing

WT – wild-type

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I. Background

1. Cancer and cancer immunology

Cancer is largely considered a genetic disease accepted to arise due to a number of reasons, including activation of oncogenes, malfunction of tumor suppressor genes or mutagenesis due to external factors [1]. Although it is usually referred to as a single condition, in the broader sense, cancer refers to more than 100 different types of cancer disease. It is characterized by the excessive growth of abnormal cells with the capability of invading and spreading to other tissues of the body [2, 3]. Cancer diseases usually form a subset of neoplasms, but they can also be distributed diffusely. Even though the tumorigenesis mechanism is complex, it is highly accepted that it is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal cells into highly malignant derivatives [4]. Some of these genetic alterations include alterations of glycosylation patterns and gain, loss or translocation of chromosomes [5]. Altered glycosylation patterns greatly contribute to cancer heterogeneity, as they regulate cancer cell's growth by glycosylation of certain growth factor receptors. These alterations occur either in oncogenes, known to promote tumor growth, or in tumor suppressor genes, that inhibit tumor growth [5]. Notably, DNA methylation also plays a critical role in cancer pathogenesis. On one hand, hypermethylation causes silencing of tumor suppressor genes, while on the other hand hypomethylation of mobile DNAs is a mechanism of oncogene activation [6]. These epigenetic mutations can affect a broad range of tissues or organs resulting in distinct cancer types [5, 6].

Evidence of distinct and more complex cancer paradigms is growing and researchers now believe that there is much more than the genetic causes of cancers to be directly implicated in the development and maintenance of the cancer phenotype [7]. In order to acquire a neoplastic state, cells must have some specific characteristics which distinguishes them from normal cells, also known as cancer hallmarks. These hallmarks consist of distinctive and complementary capabilities that enable tumor growth and metastatic dissemination and constitute an organizing principle that provides a logical framework for understanding the diversity of neoplastic diseases. Since cells evolve progressively to a neoplastic state, they acquire a succession of these hallmark capabilities. Hanahan and Weinberg have established six cancer hallmarks, namely the cell's capability to: sustain chronic proliferation by disrupting cell growth signals; suppress processes that negatively control cell proliferation; regulate downstream "apoptotic triggers" and programed cell death; proliferate indefinitely; increase angiogenesis to sustain the tumor needs; and invade surrounding tissues and cause metastasis at distance [4]. More recently, they admitted that

other factors should be taken into consideration, given that the genomic instability of the cells and their inflammatory microenvironment contribute to the tumorigenic process by altering cancer cells' genome and releasing oxygen reactive species, respectively. Moreover, cancer cells also have the capability to change their metabolism (Warburg's effect) and the ability to escape the immune system that otherwise would destroy them and prevent the establishment of a neoplasia [8].

In the oncology field, it is crucial to understand the terminology used to describe different types of cancer: primary cancers are the ones that remain localized to their site of origin, whereas metastatic cancers are those that have migrated to other locations of the body [9]. Whereas metastatic cancers still contain cells from the primary cancer, they must acquire distinct traits over time that enable them to become metastatic. The metastasis mechanism involves the ability of a certain group of transformed cells to detach themselves from the primary cancer and migrate to other sites via lymphatic or blood circulation. These transformed cells secrete enzymes, such as matrix metalloproteinases, to degrade extracellular matrix proteins, and utilize chemotaxis to allow them to migrate to other locations. The metastatic cancer cells that survive this mechanism can attach themselves to endothelial cell lining of the capillary or blood vessels and migrate to the secondary tissue using complex signaling pathways [10]. Despite being traditionally considered to occur in advanced cancer stages, accumulating evidence has described metastatic dissemination during early tumor formation [11]. Both primary and metastatic tumor development is dependent on complex mechanisms that comprise cancer cells, extracellular matrix (ECM), and "accessory" non-cancer cells, consisting of resident mesenchymal support cells, endothelial cells, and infiltrated immune cells. It is the crosstalk between these components that shapes tumor development and progression [12].

The relationship between cancer and the immune system consists of three basic principles on how the immune system acts to protect the host: it identifies "non-self" antigens present in pathogens or malignant cells; it is able to develop effector functions to specifically target the pathogen or malignant cell, while still protecting the individual; and it creates an immunological memory, via the adaptative immune system, for subsequent defense mechanisms when the host is attacked again by the same antigen [13]. As a result of this process, the immune system acquires characteristics that give rise to the immunoediting paradigm, that provides a balance between immune surveillance and tumor development. This complex mechanism consist of three of three main phases, them being the elimination, equilibrium and escape phases (Figure 1.) [14]. The elimination phase often occurs in early stages of carcinogenesis, in which the immune system destroys newly formed cancer cells, through the recognition of neoantigens and cancer testis antigens expressed on the surface

of these cancer cells, that arose from mutations and genetic alterations. If the elimination of pre-cancerous and cancerous cells is effective, there will be no clinically detectable tumors. However, if the immune system fails to destroy all cancer cells, this could result in the establishment of an equilibrium phase, where the immune system is capable of controlling tumor growth but is unable to fully eliminate it. In this phase, there is a selective pressure on cells to suffer mutations in order to evade immune surveillance, whether it is by becoming less immunogenic or by inducing an immunosuppressive sate in the tumor microenvironment (TME). If successful, these evading mechanisms can lead to the escape phase, allowing out-of-control cancer cell growth, resulting in the appearance of clinically visible tumors [14-16].

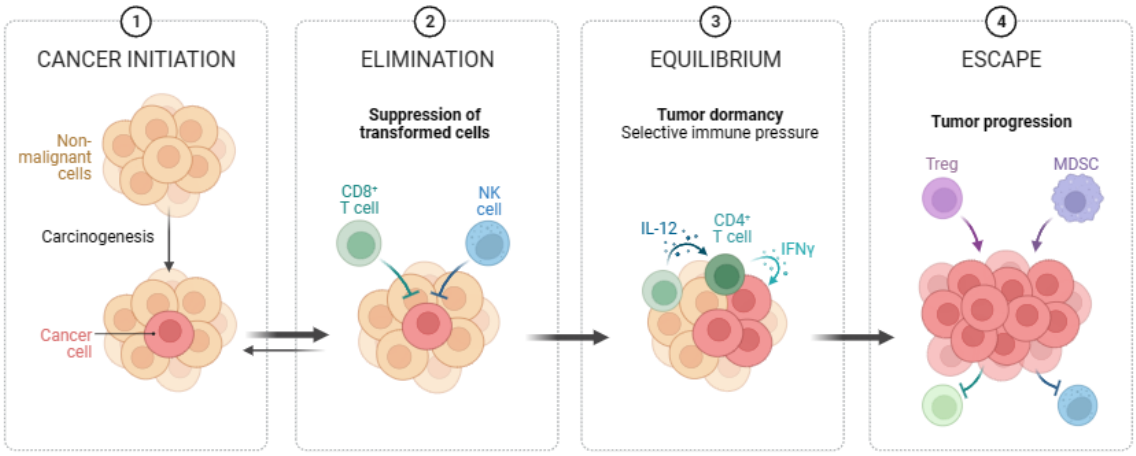

Figure 1. Phases of cancer immunoediting.
The immunoediting process is divided in the elimination, equilibrium and escape phases. During the elimination phase, both innate and adaptive immune systems identify and eliminate immunogenic tumor cells. The immune pressure gives rise to the selection of poorly immunogenic tumor cells and to a static phase, the equilibrium, in which the growth and elimination of tumor cells is balanced, and the quiescence state is promoted. Finally, tumor evolution favors the induction and selection of immunoevasive features on tumor cells, thus driving tumor survival and growth. (Adapted from BioRender.com)

Over the last decades, studies have demonstrated that immune cells are key players when it comes to cancer-related inflammation. There have been efforts to understand how cells of the immune system affect tumor fate in the different cancer stages: early neoplastic transformation, clinically detectable tumors, and metastatic tumors. The role of the immune system in cancer development and progression goes as early as 1863, when Rudolph Virchow first hypothesized that cancer development is a product of unsolved inflammation [17]. Today it is known that at least 25% of all cancers are associated with chronic inflammation [18, 19], and it may arise due to microbial infections, autoimmunity, and immune deregulation. For example, human papilloma viruses (HPVs) induce inflammation and are responsible for 90%-100% of all cervical cancers [20]. Additionally, chronic infection

with *Helicobacter pylori* elevates the risk of gastric cancer [21]. Interestingly, obesity and tobacco, both inflammation-inducing factors, are also associated with an elevated risk of cancer [22]. This evidence suggests that some types of cancer are associated with unresolved inflammation.

While the role of chronic inflammation in tumor development is wildly accepted, less is known about the influence of acute inflammation in cancer progression. There are studies that show the locally induction of acute inflammation with an attenuated strain of *Mycobacterium bovis* in the bladder treats squamous bladder cancer [23]. Thus, there is a dual role of inflammation in cancer development, as it can both initiate, and in certain cases, prevent or eliminate tumor development. This dual role is evident in the clinic, as cancer is more often diagnosed in immunodeficient patients [24]. On the other hand, a lower risk of cancer has been linked to a long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), which are known to suppress the immune system [25].

The increased research and knowledge on cancer biology have led to important improvements, not only on cancer treatment options, but on cancer patients' survival as well. Yet, cancer remains one of the main causes of mortality all over the world, affecting millions of people every year [26]. Due to the complex nature and heterogeneity of cancer biology, it is difficult to develop a treatment for one particular type of cancer. Recent advances are targeting mutations and symptoms associated with cancer hallmarks. Nevertheless, it would be more advantageous to understand and focus on a common denominator present in cancer, such as the immune system [27].

1.1 The innate and adaptive immune system

The immune system consists of a network of biochemical processes involving cells, bioactive molecules, cytokines and proteins, with the main goal of distinguishing self from non-self antigens, this way protecting and defending the host, maintaining its normal state of homeostasis [13]. It can act in one of two forms of immune responses: innate or adaptive.

Innate immune responses are nonspecific and immediate responses against foreign antigens, such as pathogens, allergens and non-self proteins. Even though it is short-lived and not able to develop immunological memory, the innate immune system is still able to distinguish self from non-self antigens and different groups of pathogens through receptor like toll-like receptors (TLRs) and danger or pathogen associated molecular patterns (DAMPS and PAMPs, respectively) [13, 27]. Moreover, the innate immune system can still provide immediate protection to the host by making use of soluble bioactive molecules and cytokines. Interestingly, the function of cytokines majorly depends on the microenvironment

in which they are secreted in, the location of the receptor it binds to, and the signaling pathways that are activated upon their binding to the receptor [28]. Comparatively, even though the complement proteins can be activated by the classical, alternative or lectin pathways, all these result in the activation of these proteins. Complement proteins may act in opsonization, as a chemoattractant for immune cells and can also mediate pathogen death thought the establishment of the membrane attack complex (MAC) [29]. The main players in cell-mediated innate immune response are phagocytes, namely neutrophils, monocytes and macrophages, and natural killer (NK) cells. Phagocytes confer immune protection by engulfing non-self antigens and killing them with lysosomal enzymes, while NK cells induce the apoptosis of cells that have an abnormal or altered major histocompatibility complex (MHC) class I expression, by secreting perforin or granzyme [30, 31]. Eosinophils, basophils and mast cells are also cell components of the innate immune system that recruit more immune cells to the inflammation site by releasing chemotactic inflammatory mediators [27].

In contrast with the innate immune system, adaptive immune responses involve the development of immunological memory. Thus, adaptive immune responses occur over time and are characterized by a slower response, when comparing to innate immune responses, given that naïve lymphocytes, namely T and B cells, must gain the ability to differentiate and mature in effector T cells or plasma cells, respectively [32].

In their lifetime, B or T cells may or may not encounter an antigen. If they do, they can be activated and multiplied into many identical cells, identified as a clone. This process is known as clonal selection and is one of the most important processes of immunology [33]. Each member of a given clone carries the same antigen receptor, therefore having the same antigen specificity as the original lymphocyte. During clonal selection, cells can be produced as either effector or memory cells. Effector cells are relatively short-lived cells responsible for defending the host in an immune response [33]. Effector B cells are called plasma cells and their main function is to secrete antibodies, while effector T cells can be cytotoxic or helper T cells and are engaged in cell-mediated responses. The production of effector cells upon encounter with an antigen for the first time is called primary immune response. Memory cells can also be produced during primary immune response, but don't become activated at this point. These long-lasting memory cells are activated when the organism is re-exposed to the same antigen that gave rise to their formation, resulting in a second immune response. This process is called immunological memory and is responsible for lifetime immunities to diseases [34, 35].

1.1.1 T cell activation

When it comes to their T cell receptor (TCR) type, T cells can be broken down into two subsets: $\alpha\beta$ T cells and $\gamma\delta$ T cells. $\gamma\delta$ T cells are only a small subset of T cells, and do not require MHC-mediated antigen presentation, as they can recognize non-self antigens by pattern recognition [36]. On the other hand, $\alpha\beta$ T cells can be further divided into two other subsets: $CD4^+$ and $CD8^+$ T cells. The differentiation of naïve $CD4^+$ T cells into effector $CD4^+$ T cells involves the co-stimulation between the TCR and the MHC-II, which is only present in antigen-presenting cells (APC), namely B cells, dendritic cells (DC) and macrophages [34].

Primary T cell activation is tightly regulated and involves the integration of three signals delivered in sequence: (1) antigen recognition, (2) costimulation, and (3) cytokine-mediated differentiation and expansion (Figure 2.). The first step of T cell activation is triggered when the T cell interacts with an APC, such as macrophages or DCs, that presents the antigen on its surface in association with MHC-II molecules. The TCR on both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) then binds the antigen held in the MHC complex, followed by CD4 and CD8 molecules, stabilizing the whole structure. This initial binding usually takes place in secondary lymphoid organs and is the crucial step to set the whole response in motion [37].

In addition to this first step, both helper and CTLs require several secondary signals to become activated. For helper T cells, the first signal involves the binding of the signaling proteins CD80 (B7.1) or CD86 (B7.2), present on the surface of APCs, to their receptor protein CD28, on the surface of T cells. If both signals are received, the helper T cells start proliferating, leading to the production of millions of T cells that are able to recognize the antigen. In order to control the response, the binding of the B7 ligands to their CD28 receptor leads to the production of cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), also known as CD152. This is a molecule that competes with CD28 for the B7 molecules, therefore reducing the activation signals and winding down the immune response [34, 37]. As for CTLs, they are less dependent on CD28 to become activated, but still require signals from other co-stimulatory molecules, such as CD70 or CD137. Contrary to $CD4^+$ T cells, the differentiation of naïve $CD8^+$ T cells into effector $CD8^+$ T cells is dependent on MHC-I, present in virtually all nucleated cells. MHC-I molecules recognize peptides derived from non-self antigens and abnormal antigens. Through their specific TCR, $CD8^+$ T cells bind to the MHC-I - antigen complex. $CD8^+$ T cells then release granzyme and perforin, resulting in the death of the target cell [38].

Once activated, T cells are given survival signals by several molecules, namely inducible T-cell costimulator (ICOS), 4-1BB and OX40, found on the T cell surface. These molecules are stimulated by their respective ligand, normally found on APCs. This is a critical mechanism to regulate immune responses, since it ensures that T cells are only activated by the APCs that have responded to a pathogen [34].

Once the T cells have recognized the antigen in the context of MHC and received the costimulatory signals, they receive a third activation signal in the form of cytokines to instruct and direct T cell differentiation and expansion. These determine the type of functional role and differentiation that the cell will have [37]. In the case of helper T cells, they are not a uniform group and can be pushed into two general subpopulations, depending on the type of cytokines and transcription factors they are exposed to: Th1, cells exposed to interleukin (IL)-12, and Th2 cells, exposed to IL-4. Th1 cells mainly synthesize gamma interferon (IFN-γ), tumor necrosis factor-beta (TNF-β), and IL-2, while Th2 cells primarily produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Each one of these cells performs a specific task in the tissue and in developing further immune responses: Th1 cells are mainly involved in cell-mediated responses, whereas Th2 cells are associated to responses involving extracellular pathogens and allergic diseases and assist in stimulating B cells to produce antibodies [34]. In addition to that, T cells can also differentiate into regulatory T (T_{Reg}) cells, known to help reduce inflammation by production of anti-inflammatory cytokines like transforming growth factor beta (TGF- β), IL-35, and IL-10 [39]. Other cells at the inflammatory site, such as neutrophils, mast cells and epithelial cells, can also secrete cytokines, chemokines, and other molecules to further induce activation and proliferation of T cells [37].

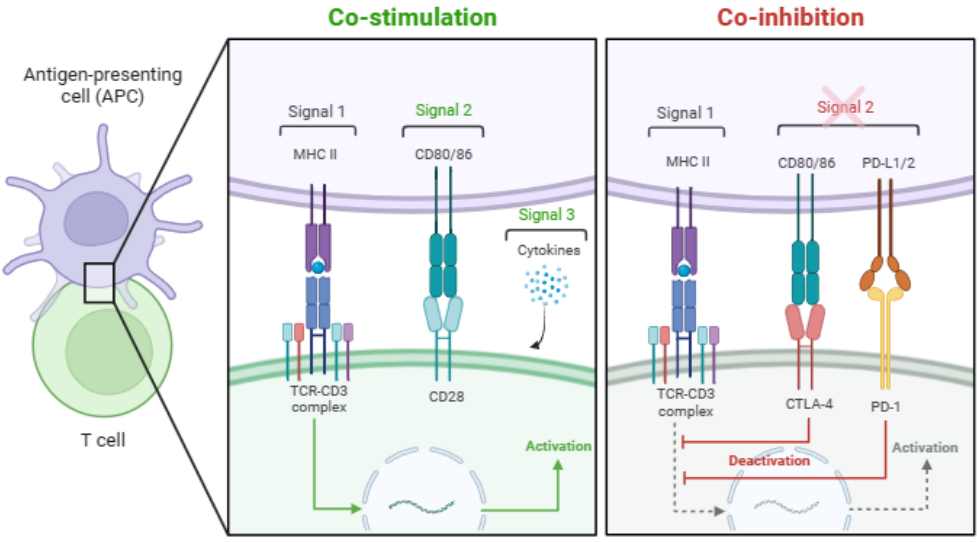

Figure 2. CD4⁺ T cell co-stimulation and co-inhibition.
CD4⁺ T cell activation requires Signal 1 in the form of TCR interacting with antigen-MHC complex which is key in innating downward signaling. Interaction of co-stimulatory molecules form part of Signal 2 as they work in tandem with TCR-antigen-MHC complex to enhance TCR signaling and initiate T cell proliferation. Signal 3 comes from APCs in the form of cytokines, and in CD4⁺ T cells it is key in dictating which subset it will differentiate into. Co-inhibitory molecules also form part of the second signal but unlike co-stimulatory molecules, they inhibit TCR signaling, thus dampening immune responses. (Adapted from BioRender.com)

1.1.2 B cell activation and maturation

The process of B cell development starts in the fetal liver and progresses to the bone marrow, specifically in hematopoietic stem cells (HSCs). In the bone marrow, stromal cells play a crucial role by providing cytokines and chemokines, such as C-X-C motif chemokine 12 (CXCL12) and IL-7, which aid in the early stages of B cell development [40]. These signals transmitted by stromal cells enable HSCs to differentiate into common lymphoid progenitor cells (CLPs), which express c-kit and IL-7 receptors. These receptors are responsible for providing the necessary signals for the survival and proliferation of CLPs upon encountering the respective ligands. With the expression of transcription factors E2A and early B-cell factor (EBF), CLPs undergo further development and transform into pro-B cells. Subsequently, B cells within the bone marrow undergo a sequential genetic rearrangement of heavy-chain and light-chain immunoglobulin genes, known as V(D)J recombination. This process ultimately leads to the generation of immature B cells that express immunoglobulin (Ig) M. The immature B cells migrate from the bone marrow to the spleen, where they undergo further differentiation into T1 and T2 stages. Subsequently, the B cells reach a state of maturity wherein they co-express IgD and IgM. At this stage, they await activation by foreign antigens [35].

In order for mature B cells located in the peripheral lymphoid organs to activate and differentiate into antibody-secreting plasma cells, they require two signals. The first signal is obtained from antigen-coupled B cell receptors (BCRs), while the second signal can be obtained either in a T cell-dependent or T cell-independent manner [35]. T cell-independent antigens, such as lipopolysaccharides and glycolipids, predominantly elicit the formation of transient plasma cells that produce antibodies with low affinity. T cell-dependent responses, initiated upon antigen recognition and interaction with follicular helper T (Tfh) cells, enable B cells to promptly differentiate into short-lived plasma cells or enter the germinal center for further maturation into plasma cells or memory B cells that exhibit a greater affinity for the antigens [41]. The germinal center can be divided into two distinct zones: the dark zone and the light zone. In the dark zone, B cells experience somatic hypermutation (SHM) at the variable regions of the BCR genes and undergo clonal expansion. In the light zone, B cells engage in affinity maturation by interacting with Tfh cells and follicular dendritic cells, ultimately selecting B cell clones that possess high affinity BCRs. Tfh cells play a crucial role in this process by producing the CD40 ligand to ensure B cell survival, as well as IL-21 to enhance cell proliferation and differentiation [42]. Class switch recombination (CSR) also occurs in germinal center B cells and consists of altering the constant region of the immunoglobulin from one isotype to another. Germinal center B cells that fail to be positively selected by follicular dendritic cells undergo apoptosis, while those who are selected have the opportunity to re-enter the dark zone and enhance their BCRs, ultimately resulting in improved affinity. It is within the germinal center that B cells possessing receptors of high affinity are able to undergo further differentiation, either into plasma cells or memory B cells [41, 43]. These germinal center-derived plasma cells travel through the circulatory system, reaching the bone marrow where they proceed to secrete antibodies that are specific to particular antigens. Over time, these antibody-secreting cells transform into long-lived plasma cells, thus providing long-term protection against specific antigens [44].

Antibodies, also known as Igs, are globular plasma proteins weighing approximately 150 kDa, that exhibit a uniform basic structure. Each antibody consists of four polypeptide chains: two identical heavy chains and two identical light chains that are connected through disulfide bonds. The light chain is comprised of polypeptides with a molecular weight of approximately 22 kDa, while the heavy chain consists of larger polypeptides weighing around 50 kDa or more. In mammals, the Ig heavy chain can be classified into five types denoted by the Greek letters α (IgA), δ (IgD), ε (IgE), γ (IgG), and μ (IgM). Similarly, there are two types of Ig light chain in mammals, namely lambda (λ) and kappa (κ) [45]. All antibodies possess a fragment antibody binding (Fab) domain, which is capable of binding to various antigens. In addition, they have a domain known as fragment crystallizable (Fc),

which can bind to its corresponding Fc receptors on effector cells. This binding allows them to perform effector functions (Figure 3.). While all naïve B cells express IgD and IgM on their cell membranes, other antibody isotypes, including IgA, IgG, and IgE can be produced by both immediate and long-term memory plasma cells through the processes of immunoglobulin class switching, affinity maturation, and SHM [45].

The distinct functions carried out by the various isotypes and subsets of antibodies in different conditions have been well-documented. However, in general, they all encompass the following effector functions:

- Neutralization: Antibodies have the ability to bind to pathogens, thereby hindering their entry into cells.

- Opsonization: Antibodies can effectively tag pathogens for phagocytosis by macrophages or neutrophils.

- Complement activation: Pathogen-bound antibodies initiate the complement cascade, which ultimately results in the lysis or phagocytosis of the pathogen.

- ADCC: Antibodies possess the capability of binding to infected cells and recruit natural killer cells to eliminate them.

- Allergic reactions: IgE antibodies specifically bind to allergens, triggering the release of histamine and consequently inducing allergic symptoms.

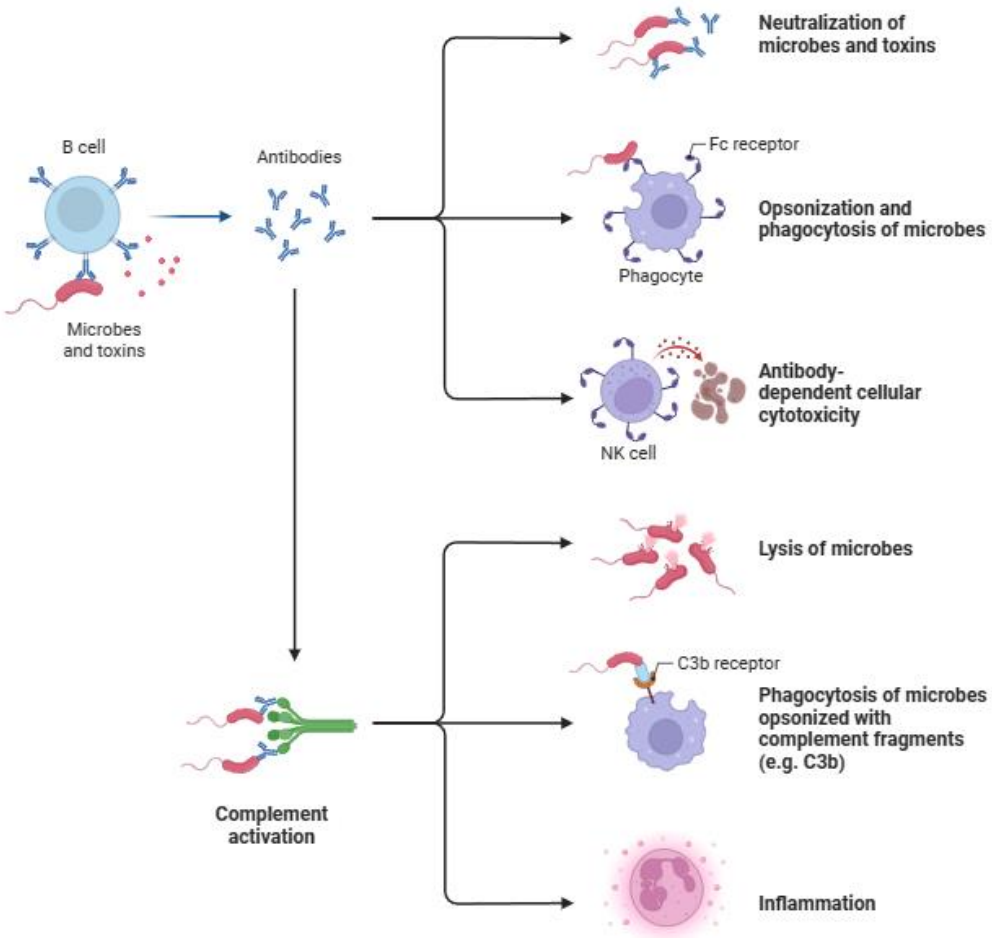

Figure 3. Effector functions of antibodies.
Antibodies have an active role in neutralization of microbes and toxins, opsonization and phagocytosis of microbes, ADCC, and activation of complement, which in turn will result in lysis of microbes, phagocytosis of microbes opsonized with complement fragments, and inflammation. (Adapted from BioRender.com)

1.2 Immune surveillance and cancer escape mechanisms

The interaction between the immune system and tumor cells in the host is a complex process that both hinders and facilitates tumor development and progression. This phenomenon is referred to as cancer immunoediting and was first proposed by Schreiber *et al.*, who suggest that the immune system engages with the tumor in three distinct stages: elimination, equilibrium, and escape [16]. Additionally, the cancer immunosurveillance hypothesis, originally formulated by Burnet and Thomas, is now considered as part of the broader concept of cancer immunoediting [46].

Immunosurveillance occurs in the elimination phase, followed by the selection of tumor variants in the subsequent phase. Eventually, this leads to immune escape, wherein tumor cells are able to grow without restrictions, resulting in the manifestation of clinical symptoms [47]. Various mechanisms contribute to immune escape, including the preferential selection

of tumor cells that are resistant to immune responses, abnormalities in immune cells like DCs and T cells that are involved in fighting against tumors, hindrances in the movement of T cells, and the creation of a TME that suppresses the immune system. All these factors play different roles at different stages of the cancer immunity cycle [48].

The immune system requires a series of sequential events to effectively eliminate tumor cells. Initially, cancer antigens within the TME are captured by APCs. These APCs then travel to the nearby lymph nodes and present the antigens to naïve T-cells using MHC-I and MHC-II molecules. This presentation primes and activates immature T-cells, transforming them into effector T-cells. Subsequently, the effector T-cells enter the bloodstream and migrate towards the site of the tumor. Once there, they can specifically recognize tumor cells through interactions between their TCRs and MHC molecules. Each step in the cancer immune cycle plays a critical role in effectively killing cancer cells. Any disruption or obstruction in these steps can undermine the immune response against cancer or even allow cancer cells to evade the immune system [48, 49].

The major mechanisms by which tumors suppress the immune system and evade destruction are described bellow.

Low tumor mutational burden: The association between the tumor mutational burden (TMB) and the effectiveness of immunotherapy showed that tumor cells with a low mutational burden have a lower production of neoantigens on MHC-I molecules. Consequently, these cells are less likely to be identified as foreign antigens, which ultimately hinders the activation of T cells and their ability to initiate cytotoxic killing [50].

Antigen loss: Immunotherapy is often hindered by the acquired resistance developed by tumors during immunoediting. This process involves the loss of the most effective antigens that stimulate the immune system, as less immunogenic cells are selectively favored. Consequently, the immune system becomes unable to recognize cancer cells, leading to resistance against immunotherapy. Nevertheless, the precise mechanisms responsible for the loss of these important neoantigens remain unclear, necessitating further investigation [48, 51].

DCs maturation dysfunction: Immature DCs undergo maturation when exposed to tumor antigens. This maturation process involves increased expression of costimulatory molecules, MHC/peptide complexes, and cytokines. However, any disruptions during DC maturation can undermine their functionality, leading to dysfunction. Numerous studies have shown that various factors like IL-35, lipids and tumor exosomes inhibit the maturation of DCs, allowing tumors to evade detection by the immune system [52-54].

Immune checkpoint proteins upregulation: Immune checkpoints (ICs) play a crucial role in regulating immune responses. They consist of receptor and ligand pairs that help maintain a balance between inhibitory and costimulatory signals after T cells recognize and activate antigens. The primary function of ICs is to ensure that the immune response remains controlled, preventing the development of autoimmunity. However, cancer cells can exploit these checkpoints to evade the immune system's attacks by rendering ineffective the tumor infiltrating lymphocytes (TILs), whose role is to target cancer cells [55, 56]. For instance, when programmed cell death ligand-1 (PD-L1), an IC ligand expressed by cancer cells, binds to its IC receptor programmed cell death receptor-1 (PD-1) on the surface of activated T cells, the T cells exhibit an "exhausted" state and lose their effectiveness [55]. Another example of an IC is lymphocyte activation gene-3 (Lag-3), a co-inhibitory receptor that is overexpressed on activated $CD4^+$ T cells, $CD8^+$ T cells, and T_{Reg} cells in tumors. The interaction between Lag-3 and its ligands, including MHC-II, galectin-3, LSECtin, and fibrinogen-like protein 1 (FGL-1) on tumor cells, inhibits T cell activity, reduces cytokine production, and decreases T cell proliferation [57, 58]. T cell immunoglobulin and ITIM domain (TIGIT) is another co-inhibitory molecule found on effector T cells, T_{Reg} cells, and NK cells. TIGIT binds to two ligands, CD155 and CD112, which are present on various cell types, including tumor cells, resulting in the suppression of the antitumor immune response. TIGIT exerts its immunosuppressive effect by repressing $CD8^+$ T cell activation, proliferation, cytokine production, and metabolism, enhancing the suppressive activity of T_{Reg} cells, and inducing NK cell exhaustion. TIGIT is upregulated on T cells across various types of tumors and is associated with a poor prognosis [59, 60]. V-domain Ig suppressor of T cell activation (VISTA) is a suppressor of immune response expressed on various immune cells including T_{Reg} cells, effector T cells, DCs, macrophages, and tumor cells. The interaction between VISTA and its ligand V-Set and Immunoglobulin domain containing-3 (VSIG-3) hampers T cell proliferation and inhibits the production of immune-signaling molecules such as IFN-γ, IL-2, and IL-17. VISTA expression has also been observed in various malignancies and is associated with acquired resistance to anti-PD-1 therapy [61].

Overexpression of Vascular Endothelial Growth Factor: To penetrate into the TME, effector T cells must enter the blood vessels surrounding the tumor, interact with adhesion molecules on the endothelium, and pass through the vessel wall. However, increased levels of proangiogenic factors, particularly vascular endothelial growth factor (VEGF), promote the development of abnormal blood vessels, consequently aiding in immune evasion. These vessels pose a physical barrier that hampers the movement and entry of effector T cells from the bloodstream into the tumors. Moreover, by downregulating the expression of adhesion molecules like vascular cell adhesion protein 1 (VCAM-1) and intercellular

adhesion molecule 1 (ICAM-1), VEGF diminishes the adhesion of effector T cells to the endothelial cells [48, 62].

Defects in antigen presentation pathway: Once the effector T cells infiltrate the tumor site, they can identify tumor cells that display the peptide-MHC-I complexes on their surface. This recognition by CTLs is crucial for effectively eliminating the tumor cells. However, various scientific findings have revealed that abnormalities in the antigen presentation pathway within tumor cells can lead to a downregulation or loss of peptide-MHC-I complexes on the cancer cell surface. Consequently, this impairs the ability of T cells to recognize and mount an immune response against the tumor. For instance, it is established that defects in β-2 microglobulin (B2M), which is an essential constituent of MHC-I, can confer resistance to immunotherapies, enabling the tumor to evade immune destruction [63, 64].

Upregulation of immunosuppressive enzymes: Indoleamine2,3-dioxygenase 1 (IDO 1) is frequently expressed in tumors, where it acts as a pivotal enzyme in the conversion of L-Tryptophan into kynurenine [65]. Enhanced expression of IDO 1 in tumors results in L-Tryptophan depletion, and generation of kynurenine and its subsequent downstream catabolites. These molecular changes contribute to the suppression of the immune response against tumors through various mechanisms, including the induction of cell cycle arrest and impairment of T cell function, promotion of T_{Reg} cell differentiation, and stimulation of proliferation and activation of myeloid-derived suppressor cells (MDSCs) [66, 67].

Immune suppressive cells present in the TME: The presence of immunosuppressive tumor-associated immune cells, including T_{Reg} cells, tumor-associated macrophages (TAMs) and MDSCs, in the TME also constitute a mechanism by which cancer cells can evade the immune system and destruction. There are multiple mechanisms through which T_{Reg} cells exert immunosuppressive functions. These include the depletion of IL-2 by T_{Reg} cells, thereby restricting the activity of $CD8^+$ T cells, the secretion of inhibitory substances like IL-10, TGF-β, and adenosine, the expression of IC molecules such as CTLA-4, PD-1 and LAG-3, and the production of granzyme and/or perforin to eliminate effector T cells. Collectively, T_{Reg} cells play a crucial role in facilitating immune evasion in cancer and pose obstacles to cancer immunotherapy [68, 69]. In the TME, TAMs serve as crucial immune-suppressive cells, impeding effective antitumor immune responses. TAMs express PD-1 ligands, including PD-L1 and PD-L2, thus hindering T cell functions. Additionally, TAMs release immunosuppressive cytokines like IL-10 and TGF-β, which inhibit the activity of $CD4^+$ and $CD8^+$ T cells and enhance the expansion of T_{Reg} cells. Moreover, TAMs secrete chemokines such as CXCL8, stimulating PD-L1 expression on macrophages. Furthermore, TAMs produce arginase and IDO, inducing metabolic deprivation in T cells [70]. MDSCs

comprise a group of immature myeloid cells that exhibit potent immunosuppressive attributes. MDSCs release immunosuppressive cytokines like IL-10 and TGF-β, impairing the antitumor effects of T cells and recruiting T_{Reg} cells. Additionally, MDSCs overexpress IDO and arginase-1, resulting in the degradation of L-tryptophan and L-arginine, respectively, leading to T cell dysfunction. Similarly, MDSCs also upregulate inducible NO synthase to convert L-arginine into NO, once again inducing T cell anergy. Furthermore, the expression of PD-L1 on MDSCs is closely associated with immune suppression in the TME [71].

2. Immunotherapy

The field of immuno-oncology has made a significant impact on the treatment of individuals with cancer. William B. Coley, who is now widely recognized as the pioneer of immunotherapy, initially endeavored to utilize the immune system to combat cancer in the late 1800s [72]. As an orthopedic surgeon specializing in bone sarcomas, Coley observed that certain patients experienced spontaneous regression of their unremoved tumors after suffering from notable postoperative wound infections, prevalent at that point due to suboptimal aseptic practices during that era. Starting from 1891, Coley administered injections of live and inactive bacteria, such as *Streptococcus pyogenes* and *Serratia marcescens*, to over a thousand patients with the intention of inducing sepsis and eliciting powerful immune and antitumor responses. This combination of bacteria, known as "Coley's toxin," became widely recognized as the first recorded active intervention for cancer immunotherapy [72]. Coley achieved impressive long-term complete remissions in various types of cancers, including sarcoma, lymphoma, and testicular carcinoma. However, due to the absence of a known mechanism of action for Coley's toxin and the risks associated with intentionally infecting cancer patients with pathogenic bacteria, surgeons and oncologists in the early 1900s began favoring surgery and radiotherapy as standard alternative treatments [73, 74].

It would require over fifty years before gaining a better comprehension of the primary agents involved in sepsis, which would provide some insight into the workings of Coley's toxin. These agents belong to a group of signaling molecules known as cytokines, which include interleukins, interferons, and chemokines [28]. Once again, a race ensued to apply these groundbreaking discoveries to the treatment of cancer [75]. Physicians and researchers experienced moderate success in employing this innovative approach, occasionally achieving clinical remission in metastatic renal cell carcinoma (RCC) through the administration of high-dose IL-2 [76], while facing questionable outcomes with IFN in

stages III and IV melanoma [77]. These moderate achievements often came with significant adverse effects. Although novel delivery methods like pegylation helped reduce some of the toxicities, the irregular and unpredictable immune responses associated with these therapies meant that only a small, carefully chosen subset of cancer patients could reap the benefits [78].

The subsequent groundbreaking leap in cancer immunotherapy emerged from a deeper comprehension of the mechanism of immune surveillance, so much that Science acclaimed cancer immunotherapy as the "breakthrough of the year" in 2013, transforming the realm of oncology [79].

Currently, there are six main approved immunotherapy methods that can be administered to patients, each yielding different rates of response: (1) targeted monoclonal antibodies (mAbs), (2) oncolytic virus therapy, (3) cytokines, (4) cancer vaccines, (5) cell-based immunotherapy, and (6) IC therapies (Figure 4.) [46, 55]. These approaches can be further categorized into two broad groups: active and passive immunotherapies. The active approach involves guiding the immune system of the patient towards identifying tumor associated antigens (TAA) present on the tumor's surface. These antigens may involve specific proteins or carbohydrates that are either exclusively or abundantly expressed in tumor cells. In contrast, passive immunotherapy aims to enhance the natural anticancer response of the immune system by implementing monoclonal antibodies, lymphocytes, and cytokines [80]. It is important to note that the effectiveness and administration of immunotherapy significantly rely on factors such as cancer type, grade, predictive response rate, and the presence of crucial biomarkers [81]; nevertheless, patient response rates may still vary.

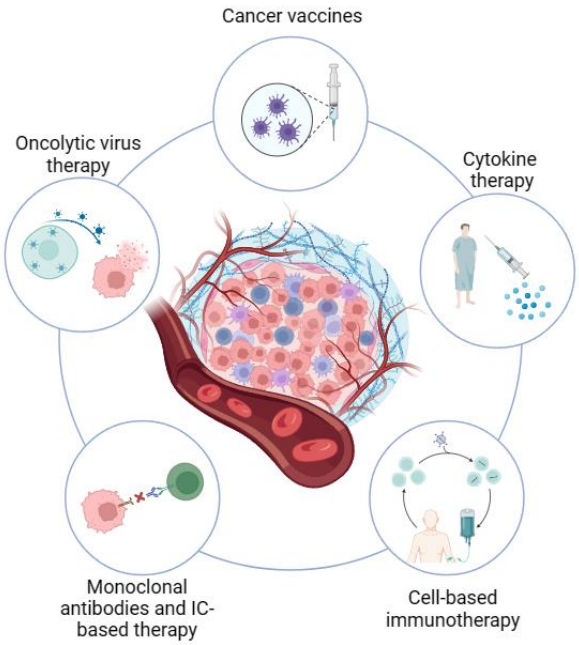

Figure 4. Approved immunotherapy methods: cancer vaccines, cytokine therapy, cell-based immunotherapy, targeted monoclonal antibodies and IC-based immunotherapy, and oncolytic virus therapy (created with Biorender.com)

2.1 Monoclonal antibodies

Over the past two decades, mAbs have emerged as a significant therapeutic option for various types of cancer, such as breast cancer, lymphoma, and colorectal malignancies [82]. These mAbs are artificial copies of large proteins generated by specific B-cell clones, possessing distinctive antigen specificity which enables them to attach to epitopes found on cancer cells or within their plasma. Typically belonging to the IgG class, therapeutic mAbs consist of a Fab and a Fc [83]. mAbs can be administered either unconjugated or conjugated with therapeutic drugs, such as chemotherapy drugs, radioactive particles, or toxins that would produce a cytotoxic effect on cancer cells [83].

The primary actions of most unconjugated mAbs involve ADCC and complement-dependent cytotoxicity. Additional mechanisms of mAbs include inducing direct cell death or blocking signals that promote cell survival, angiogenesis, or ICs. On the other hand, conjugated mAbs play a vital role in targeted delivery systems, including mAb-drug conjugates, mAb-radionuclide conjugates, or even mAbs linked to nanoparticles, liposomes, or biodegradable polymers. However, the anticancer effectiveness of these mAb conjugates no longer relies on ADCC and complement-dependent cytotoxicity actions, but rather on radionuclides, toxins, or other anticancer agents that are specifically aimed at the tumor or malignant cells - a strategy that limits toxicity in normal tissue [55, 83].

Considering the mechanism through which mAbs exert their therapeutic effects, it is not surprising that the rates of response can differ. Essentially, this approach focuses on targeting the epitope of an antigen that is exclusively found on tumors, with the intention of inducing cell death. One primary factor contributing to the variability in response rates is the high specificity of these mAbs. As the term "mono-" implies, these antibodies specifically recognize just one epitope [12]. Consequently, if there are any alternative forms of the epitope resulting from mutations, mAbs would be unable to identify and bind to the antigen under investigation. Collectively, the specificity of mAbs is a significant factor contributing to the varied rates of response, rendering immunotherapy less effective [80].

The Food and Drug Administration (FDA) has authorized a multitude of therapeutic mAbs to effectively treat various forms of cancers. In 1997, rituximab (Rituxan, Genentech) emerged as the first mAb given the green light for clinical usage, specifically indicated for patients diagnosed with certain B-cell malignancies [83]. Since then, numerous other mAbs have obtained approval, including ipilimumab (Yervoy, Bristol-Myers Squibb), nivolumab (Opdivo, Bristol-Myers Squibb), cetuximab (Erbitux, Lilly), among others. Many other mAbs are currently under assessment by the FDA or are advancing through phase 3 clinical trials [55, 83].

2.2 Oncolytic virus therapy

Oncolytic virus therapy emerged when it was discovered that viruses can destroy tumor cells by causing cytopathic effects. These infectious agents can infect living cells and take control of their genetic machinery, enabling them to replicate inside the cells. The therapy involves using genetically modified viruses to infect tumor cells. The cytopathic effects of the viruses lead to destruction of the infected tumor cells, creating a pro-inflammatory environment that enhances systemic antitumor immunity. Additionally, viral infections resulting in cell death in tumor tissues promote inherent tumor immunity in cancer patients. Various DNA and RNA viruses have been identified as potential candidates for this treatment, based on studies conducted in laboratory settings (*in vitro*), animal models (*in vivo*), and clinical trials. These viruses are referred to as "oncolytic viruses" because they are specifically engineered to target tumor cells [46, 84].

In 2015, the FDA approved the first oncolytic virus therapy known as talimogene laherparepvec (T-VEC) for the treatment of melanoma. T-VEC is a genetically modified form of the herpes simplex virus that has shown remarkable clinical benefits in patients with advanced melanoma. It has been approved to treat metastatic melanoma that cannot be surgically removed [85].

2.3 Cytokine therapies

Cytokines play a vital role as messengers in coordinating cellular interactions and communications within the immune system. They are released by both immune and nonimmune cells in response to various stress factors like infection, inflammation, and tumorigenesis. By being secreted, cytokines facilitate the rapid transmission of immune signals in a highly efficient and complex manner, allowing for potent and coordinated immune responses against specific antigens. These signaling molecules regulate processes such as leukocyte differentiation, migration, activation, and suppression [75, 86]. In the field of cancer immunotherapy, certain cytokines have the ability to directly enhance or suppress T-cell response against cancer cells. Hence, it is not surprising that the initial approaches to cancer immunotherapy involved the systemic administration of cytokines, particularly interferons and interleukins. The development of recombinant DNA technology using genetically engineered *Escherichia coli* strains enabled the large-scale production of purified recombinant human cytokines suitable for systemic administration to patients [83]. While IFN-α and IL-2 have been extensively studied and utilized for cancer treatment, ongoing research is exploring the potential use of other cytokines for cancer immunotherapy [75, 86].

Interferons are cytokines that have diverse effects on the immune system, such as modulating immune responses, inhibiting viral replication, and suppressing cell proliferation. IFN-α, a member of the I IFN family, was discovered decades ago and is known for its immune-boosting properties [84, 87]. It activates and matures DCs, facilitating the presentation of antigens to immune cells and enhancing antitumor immunity. Furthermore, IFN-α promotes a specific type of immune response called Th1 response, which increases the activity of CTLs involved in destroying tumor cells, and it also strengthens the cytotoxic abilities of NK cells. On top of that, IFN-α directly hampers tumor cell growth and oncogene expression [87]. Moreover, it should be noted that IFNs can also increase MHC expression, making them valuable adjuvants in various cancer immunotherapy approaches, including cancer vaccines [55, 84].

Recombinant technology enabled the production of IFN-α, making it the first cytokine to be synthesized in this manner. Back in 1986, it was granted approval for treating hairy cell leukemia, and later, it gained recognition for treating chronic myelogenous leukemia as well [88, 89]. Among the many subtypes of IFN, IFN-α, specifically IFN-α2b, is widely utilized in cancer immunotherapy. Regulatory bodies have approved IFN-α2b for managing metastatic melanoma, follicular lymphoma, and acquired immunodeficiency syndrome (AIDS)-related Kaposi sarcoma [55, 83, 89].

Interleukins are mainly produced by specific subsets of $CD4^+$ helper T-cells that play a role in the growth, activation, and suppression of $CD8^+$ cytotoxic T-cells, macrophages, and NK cells. The discovery of IL-2 in 1976 has contributed to its potential application in cancer treatment [84, 90]. IL-2 acts as a critical cofactor in activating cytotoxic TILs and enhancing the antitumor activity of NK cells. It also induces lymphokine-activated killer cells, which mediate antitumor effects [55, 83]. One significant aspect of IL-2 is its dose-dependency, particularly in activating different subsets of immune cells. At higher doses, IL-2 triggers a Th1 immune response and promotes CTL antitumor activity. At lower doses, IL-2 exhibits both immune-enhancing and immune-suppressing activities by stimulating T_{Reg} cells [89]. To harness the broad effects of IL-2, recombinant human IL-2 (rhIL-2) was developed. Similar to native IL-2, rhIL-2 broadly stimulates the immune system by promoting the proliferation and differentiation of T-cells, B-cells, and NK cells. Additionally, rhIL-2 enhances the cytolytic activity of lymphocyte subsets and fosters interaction between malignant cells and the immune system [91]. Approval for treating RCC was granted to rhIL-2 (aldesleukin [Proleukin, Prometheus Laboratories]) in 1992 [92], with an added indication for metastatic melanoma in 1998 [93].

While both of these cytokines types have been employed to boost the immune system, it is important to note that these treatments are most effective when the patient's immune system is relatively robust [80]. This requirement could contribute to the varying response rates observed. Consequently, cytokines are typically used in conjunction with other forms of immunotherapy due to their limited tolerability and potential for severe toxicity. Researchers are actively studying the combination of cytokines with other immunotherapies, such as adoptive cell transfer (ACT) therapy, to overcome these challenges [80, 84].

2.4 Cancer vaccines

Another currently available immunotherapeutic treatment for cancer involves the use of cancer vaccines, that utilize specific antigens found in tumors. These antigens stimulate T-cells to initiate an immune response against the tumor [80, 94]. Cancer antigens are often released into the bloodstream and TME due to necrotic events that occur as a result of surgeries, radiation, or chemotherapy [83]. However, effective T-cell responses against these antigens can be compromised by low affinity to self-antigens, immunosuppressive therapy, tumor-induced immune response inhibition caused by cytokine secretion (such as IL-10 and TGF- β), or manipulation of immune cells by immunosuppressive factors like T_{Reg}

cells, TAMs, and MDSCs. One of the main goals of cancer vaccines is to stimulate the immune system to recognize and eliminate cancer cells [55, 83].

One key factor that influences the variation in patient response rates to cancer vaccines is the specificity of the vaccine and whether the commercially available form of the vaccine contains the same antigen found in the patient's tumor. This is crucial for the immune system to effectively recognize and target the tumor within the body [80]. Cancer vaccines can be peptide-based, immune cell- or DC-based, or tumor cell-based, each offering distinct advantages and disadvantages [55].

The crucial breakthroughs in the cancer vaccines field emerged when scientists discovered MZ2-E and MZ2-D. These are antigens found in melanoma cells, specifically encoded by the melanoma-associated antigen gene (MAGE) family. They can activate CTLs, triggering an immune response against the tumor [95]. At the same time, gpl00, another melanoma antigen, was found to induce immune responses mediated by TILs, leading to rejection of the tumor *in vivo* [96]. These significant findings opened up possibilities for using tumor antigens as vaccines in cancer immunotherapy [84].

Peptide-based vaccines trigger an immune response against a specific tumor antigen that is present alongside human leukocyte antigen (HLA) molecules on the surface of tumor cells. These vaccines are less likely to cause harm to normal cells and tissues, although they do have limitations including the requirement for accurate identification of the tumor antigen peptide and the patient's HLA type. Nonetheless, due to their ability to stimulate immune responses targeted at specific antigens while ensuring safety, there is considerable interest in further exploring the potential of these vaccines [89].

Besides tumor antigens, DC-based vaccination has also demonstrated notable clinical outcomes. DCs have a superior capability as APCs and play a vital role in stimulating antitumor immunity. Specifically, once activated by tumor antigens, DCs can internalize, process, and subsequently present the processed epitopes to T cells, thereby inducing immune responses mediated by CTLs [97]. Leveraging their proficiency in antigen presentation, DCs are utilized in DC-based vaccines, which involve reintroducing isolated DCs loaded with tumor antigens or tumor cell lysates and stimulated with a defined maturation cocktail in a laboratory setting [98]. One example of such a vaccine is sipuleucel-T (Provenge, Dendreon Corp.), an approved DC-based immunotherapy for castration-resistant prostate cancer treatment. Preparing this vaccine requires the patient to undergo leukapheresis to obtain blood enriched with APCs, including DCs, which are crucial to this vaccine protocol. These collected APCs are then activated through exposure to the recombinant prostate tumor antigen PA2024 and granulocyte macrophage colony-

stimulating factor (GM-CSF), a potent immune activator. As a result, these activated APCs, when administered to the patient as a vaccine, can activate the patient's T cells to specifically target prostate cancer cells expressing the PA2024 antigen [99].

Second-generation DC-based vaccines employ innovative *in vitro* culturing techniques using specialized media supplemented with essential cytokines, enhancing immunogenicity and improving DC function. Furthermore, advancements in recombinant technology have enabled the generation of genetically engineered DCs capable of secreting growth factors or interleukins that activate T cells and NK cells, consequently significantly improving the anticancer immune response produced by these cancer vaccines [83].

Moreover, whole tumor cells can also be employed to trigger immune responses. Tumor-cell–based vaccines utilize whole tumor cells as a source of immunogenic material. Unlike peptide-based vaccines, tumor-cell–based vaccines are not restricted by HLA type, allowing them to present a wide variety of epitopes to activate the immune system and initiate the defense mechanism. These vaccines can be either autologous, utilizing tumor cells from the recipient, or allogeneic, utilizing tumor cells from another patient. After obtaining tumor cells, they can be prepared for immunization via irradiation. Subsequently, they are administered either alone or in conjunction with an adjuvant, such as GM-CSF [55].

2.5 Cell-based immunotherapy

Immune cells possess the ability to detect and eliminate tumors. By stimulating immune cells and harnessing the body's own immune response tailored to combat tumors, immune cells can once again contribute to the detection and eradication of tumors. While cell immunotherapy has proven effective in treating blood-related tumors, its efficacy is not as desired when applied to solid tumors, due to the diversity within these tumors and the influence of the surrounding microenvironment [100].

Cell-based immunotherapy refers to a treatment modality that utilizes natural T cells or genetically modified T cells, either allogenic or autologous, that have been expanded outside the body to specifically target tumor antigens. These tumor antigens may encompass various forms such as mutated proteins, tissue differentiation antigens, vascular antigens, among others [94]. The success of cell-based immunotherapy, specifically ACT, was first reported by Rosenberg *et al.*, who demonstrated that high doses of IL-2, in combination with autologous lymphokine-activated killer cells exhibited effectiveness in patients with metastatic cancers [101]. Following this initial successful trial of adoptive immunotherapy, the team further refined their approach by utilizing TILs and found that the adoptive transfer of TILs expanded in IL-2 showed enhanced therapeutic potential [102].

Even though TILs respond to epitopes as well as antigens and neoantigens that arise from specific mutations within the tumor, the identification of the tumor antigen is not a requirement for TIL cell-based treatment since the TILs that infiltrate the tumor are already specific to the antigen. However, TIL-based therapy does require the extraction of a tumor sample to isolate an adequate amount of TILs, so that they can be cultured outside of the body [83]. These studies provide a basis for utilizing TILs in the treatment of advanced human cancers.

The success of allogenic immune cell-based therapy has led to the emergence of new genetic engineering approaches aimed at optimizing effectiveness and minimizing toxicity. A personalized approach has been devised for TIL immunotherapy, which involves analyzing the mutations found in tumors. This requires scanning tumor samples using exome sequencing to identify all the genes that have undergone mutation. Subsequently, the surrounding amino acid sequences near the mutation sites are entered into a prediction software to determine all potential epitopes. These peptide sequences are then utilized to expand TILs in the laboratory, ensuring they are precisely targeted [55].

Investigations have explored genetic engineering-based approaches to cell-based cancer immunotherapy in order to overcome the challenges associated with generating autologous TILs. Among these strategies, the development of chimeric-antigen receptor (CAR) therapy shows great promise. CAR-T cell therapies utilize fragments of antibodies to identify particular antigens that are present on the surface of cancer cells [84]. By modifying the TCR, its antigen-binding component is linked to an artificial signaling molecule that triggers activation signals in T cells upon binding to the antigen/MHC complex. Following modification, the expanded CAR-T cells are reintroduced into the patient's body, where they can specifically target and eliminate cancerous cells. The signaling provided by CAR can effectively replace natural TCR signaling, resulting in a focused immune response [83]. Numerous CARs targeting various tumors have been developed and have demonstrated impressive clinical outcomes in the treatment of patients with relapsed or refractory B-cell malignancies, such as acute and chronic lymphocytic leukemia [103]. However, the efficacy of CAR-T cell therapy in targeting solid tumors has been limited, with only modest results observed thus far. At present, the primary challenge for CAR treatment lies in the scarcity of tumor surface antigens that exhibit sufficient specificity [83, 104].

2.6 Immune checkpoint inhibitors

Despite the significant progress made in cell-based immunotherapies, a novel group of mAbs, known as immune checkpoint inhibitors (ICIs), are now being introduced into medical

practice and have become a crucial form of immunotherapy [105]. The IC is found on the surface of T cells or tumor cells and serves as the target for inhibiting the excessive activation of T cells. Normally, inhibitory checkpoint proteins prevent damage from autoimmune diseases, but when facing a tumor, they hinder T cells from approaching the tumor, thereby weakening the immune system's ability to recognize and eliminate cancer cells [106]. Through the use of ICIs, the immune response of T cells can be greatly enhanced, restoring the immune system's antitumor capabilities. Numerous studies have utilized IC inhibition as a means of immunotherapy, and while there are already a vast number of effective ICs for this purpose, there are still further specific sites being explored [107]. The primary focuses of ICIs are molecules such as CTLA-4, PD-1, and PD-L1. The effectiveness of ICIs is linked to the expression of biomarkers such as PD - L1, TMB and microsatellite instability (MSI) [107].

2.6.1 CTLA-4

Several research studies have demonstrated the efficacy of antibodies that inhibit the CTLA-4, which leads to increased immune response against tumor cells. The positive results obtained from clinical trials of ipilimumab formed the basis for its approval by the FDA in 2011 for the treatment of patients with advanced melanoma [108].

CTLA-4, a protein found in the immunoglobulin superfamily, is exclusively expressed on T cells where its main role is to control the intensity of early T cell activation. Essentially, CTLA-4 counteracts the function of CD28, a T cell co-stimulatory receptor. CD28 only influences T cell activation when the TCR binds to a specific antigen. At that point, CD28 signaling greatly enhances TCR signaling and triggers T cell activation. Both CD28 and CTLA-4 have the same ligands, CD80 (B7.1) and CD86 (B7.2) [105]. However, CTLA-4 competes more effectively with CD28 for binding to the ligands on the APC, favoring the displacement of CD28 from its association with CD80/86. When CTLA-4 binds to these ligands on APCs, it triggers an inhibitory response that suppresses the immune response by blocking T lymphocyte activity, reducing the proliferation of T lymphocytes, inhibiting the function of T_{Reg} lymphocytes, decreasing cytokine secretion, and ultimately leading to immunosuppression [109]. In 1994, it was discovered that the expression pattern of CTLA-4 differs significantly from that of CD28. After TCR/CD3-mediated T cell activation, CTLA-4 expression increases for 2–3 days, starting approximately 24 hours after TCR triggering, while CD28 is expressed on naive T cells. These findings indicate that CTLA-4 plays a crucial role in regulating activated T cells, as its absence leads to uncontrolled T cell proliferation [110].

While CTLA-4 is expressed by activated $CD8^+$ effector T cells, its primary physiological function appears to involve distinct effects on the two key subsets of $CD4^+$ T cells: one effect is the suppression of helper T cell activity, while another effect is the enhancement of T_{Reg} cell immunosuppressive activity. T_{Reg} cells constitutively express CTLA-4 as it is a controlled gene of the Foxp3 forkhead transcription factor, which determines the T_{Reg} cell lineage. When T_{Reg} cell-specific CTLA-4 deletion or inhibition occurs, their ability to control autoimmune and antitumour immunity is significantly reduced. Consequently, the mode of action of CTLA-4 blockade likely involves both an increase in effector $CD4^+$ T cell activity and a decrease in T_{Reg} cell-dependent immunosuppression, both of which are crucial aspects [105, 110].

Initially, there were doubts about the effectiveness of blocking CTLA-4 due to the lack of tumor specificity in the expression of CTLA-4 ligands, except for certain types of myeloid and lymphoid tumors. Furthermore, the knockout of CTLA-4 in mice resulted in severe autoimmune and hyperimmune reactions, suggesting that blocking this receptor could lead to significant immune toxicity [105]. However, Allison and colleagues conducted preclinical studies that demonstrated a potential therapeutic window by partially blocking CTLA-4 using antibodies [111]. These studies showed promising antitumor responses without noticeable immune toxicities when using CTLA-4 antibodies as standalone treatments in mice with partially immunogenic tumors. On the other hand, poorly immunogenic tumors did not respond to anti-CTLA-4 treatment alone but exhibited positive responses when anti-CTLA-4 was combined with a GM-CSF-transduced cellular vaccine. These findings indicated that CTLA-4 blockade could enhance the endogenous antitumor immune response in animals with existing tumor implants, leading to tumor regression. Additionally, for poorly immunogenic tumors that do not stimulate significant endogenous immune responses, the combination of a vaccine and a CTLA-4 antibody could generate a robust immune response capable of slowing tumor growth and, in some cases, eradicating established tumors [105, 111].

The discovery of these preclinical findings provided the impetus for developing and testing two CTLA-4 antibodies that are fully humanized: ipilimumab and tremelimumab. These antibodies entered clinical trials in 2000 [105]. Ipilimumab is a human IgG1 mAb that effectively inhibits the function of CTLA-4. It was recommended for the treatment of melanoma, after gaining approval in 2011. Ipilimumab is also used in the treatment of advanced RCC, highly unstable MSI (MSI-H) or DNA mismatch repair (MMR) metastatic colorectal cancer, malignant pleural mesothelioma, non-small cell lung cancer (NSCLC), and hepatocellular carcinoma, when combined with nivolumab [112]. In 2020, the FDA announced that the combination of nivolumab and ipilimumab administered intravenously

can provide therapeutic benefits to adult patients with unresectable malignant pleural mesothelioma (MPM) and NSCLC (with tumor PD-L1 expression ≥1% and absence of EGFR/ALK aberrations) as a first-line treatment [113]. However, it is important to note that the blockage of CTLA-4 with ipilimumab, a negative regulator of T cell immune responses, can lead to immune-related adverse effects (irAEs) such as colitis and enterocolitis due to the disturbance of autoimmunity prevention [110].

Contrary to ipilimumab, tremelimumab has not shown promising results in research studies, making it less likely to be widely used in monotherapy [114]. Reported response rates of 15% and 30% were observed in hepatocellular carcinoma (HCC) patients treated with tremelimumab and nivolumab, respectively [115]. Nevertheless, ongoing research is being conducted to explore the combination of tremelimumab and durvalumab as a potential treatment option [116].

2.6.2 PD-1 and ligands

PD-1, another immune-checkpoint receptor, is gaining recognition as a promising focus, highlighting the range of molecularly defined strategies to stimulate the patient's own immune system and elicit effective antitumor immune responses [105].

Unlike CTLA-4, PD-1 has a primary function of restricting T cell activity in peripheral tissues during an inflammatory response to infection and in preventing autoimmunity. It plays a crucial role in programmed death signaling to regulate the immune response mediated by T cells. This results in a significant immune resistance mechanism within the TME [105, 110]. Engagement of PD-1 can limit the secretion of cytokines like IL-2, IFN-γ, and TNF-α, as well as inhibit cell proliferation by interfering with the CD28-costimulatory signaling pathway. PD1 is expressed more extensively than CTLA-4, as it can be induced on various activated non-T lymphocyte subgroups such as B cells, DCs and NK cells. This induction leads to the limitation of their lytic activity [117]. Therefore, while the concept of PD-1 blockade is commonly associated with boosting the function of effector T cells in tissues and the TME, it is also likely to enhance NK cell activity within tumors and tissues. Additionally, it may indirectly or directly enhance antibody production through its effects on $PD1^+$ B cells [118].

Similarly to CTLA-4, PD-1 is highly expressed on T_{Reg} cells, where it may promote their proliferation in the presence of a ligand. Since many tumors are heavily infiltrated with T_{Reg} cells that further suppress effector immune responses, blocking the PD-1 pathway may enhance antitumor immune responses by reducing the number and/or suppressive activity of T_{Reg} cells within the tumor [119].

The PD-1 receptor has two ligands – PD-L1, also called B7-H1 and CD274, and PD-L2, also known as B7-DC and CD273 [120]. These ligands, belonging to the B7 family, share 37% sequence similarity and have emerged from gene duplication, placing them within 100 kb of each other in the genome. PD-L1, which can be expressed by both tumor and immune cells, serves as a valuable biomarker for predicting the response to anti-PD-1/PD-L1 antibodies in certain cancer patients [121]. PD-L1 plays a role in inhibiting the cancer-immunity cycle by binding to negative regulators of T-cell activation like PD-1 and CD80. Consequently, the binding of PD-L1 prevents the migration and proliferation of T cells, thus limiting the ability to eliminate tumor cells [110].

Prolonged exposure to antigens, as seen in cases of chronic viral infection and cancer, can result in elevated levels of persistent PD-1 expression. This, in turn, leads to a state of exhaustion or anergy among specific T cells that recognize these antigens. This state has been observed in both mice and humans with various chronic viral infections, and it appears that blocking the PD-1 pathway can partially reverse this condition [122]. Additionally, apart from its primary role in limiting immune responses in tissues and tumors, the PD-1 pathway can also modulate the balance between T cell activation and tolerance at the initial stages of T cell responses to antigens within secondary lymphoid tissues, resembling CTLA-4's function at a similar stage. Overall, these findings suggest a complex range of mechanisms through which PD1-pathway blockade operates [105].

PD-1 is present on a significant portion of TILs in various types of cancers. The increased expression of PD-1 on $CD4^+$ TILs can be attributed to the high level of PD-1 expression on T_{Reg} cells, which often make up a large proportion of $CD4^+$ T cells within the tumor. The enhanced PD-1 expression on $CD8^+$ TILs could indicate either a state of reduced responsiveness or exhaustion, as suggested by the lower cytokine production of $PD\text{-}1^+$ TILs compared to $PD\text{-}1^-$ TILs in melanoma cases [123]. Similarly, PD-L1 and PD-L2 are frequently upregulated on the surface of tumor cells in various tumors. The expression patterns of PD-1 ligands play a critical role in determining the suitability of therapeutic blockade targeting this pathway, as PD-1 primarily functions to inhibit lymphocyte activity when engaged by its ligands [105]. Initially, high expression levels of PD-L1 were reported in melanoma, ovarian, and lung cancer samples [124, 125]. Subsequently, it was discovered that many other human cancers also upregulate PD-L1 expression. In renal cancer, the presence of PD-L1, either on tumor cells or TILs in primary tumors was associated with a worse prognosis (decreased overall survival) [126]. Although most studies have focused on PD-L1, PD-L2 has also been found to be upregulated in various tumors. PD-L2 shows significant upregulation in certain B cell lymphomas, including primary mediastinal B cell lymphoma, follicular cell B cell lymphoma, and Hodgkin's disease [127].

Based on scientific research, it is reasonable to assume that inhibiting the PD1/PD-L1/2 pathway would enhance the body's ability to fight against tumors. Evidence has shown that immunotherapies, which focus on the PD-1 pathway, have brought a significant change in the treatment options available for various types of cancers. These include Merkel cell carcinoma (MCC), melanoma, head and neck squamous cell carcinoma (HNSCC), and NSCLC. The FDA has given its approval to three monoclonal antibodies - Nivolumab, Pembrolizumab, and Cemiplimab - as PD-1 inhibitors [128].

Nivolumab is a fully human IgG4 mAb inhibitor that targets and blocks the interaction between the ligands and the PD-1 receptor, effectively suppressing PD-1 activity. This innovative therapeutic approach combats the ability of tumor cells to evade the immune system by compromising the PD-1/PD-L1 pathway, thereby diminishing the cellular immune response [129]. Nivolumab received FDA approval in 2014 and 2015 for the treatment of melanoma and RCC, respectively. Additionally, the FDA endorsed nivolumab in 2015 for its efficacy in treating squamous cell lung cancer (SCLC) and NSCLC [130]. Despite the occurrence of irAEs, such as renal, gastrointestinal, pulmonary, hepatic, cutaneous, rheumatological, and endocrine manifestations in patients with metastatic RCC, nivolumab has demonstrated a satisfactory safety profile [131].

Pembrolizumab (Keytruda, Merck) is a humanized IgG4 mAb that disrupts the PD-1/PD-L1 pathway. The FDA has approved pembrolizumab for treating various types of tumors due to its strong objective responses and favorable pharmacokinetic and safety profile [132]. In 2021, the FDA confirmed that a combination of pembrolizumab and chemotherapy drugs, with or without bevacizumab, can provide therapeutic benefits to patients with recurrent metastatic cervical cancer whose tumor cells express high levels of PD-L1 [133]. Several studies have shown that pembrolizumab can induce complete and powerful responses, not specific to any particular tumor type, as it targets the immune system rather than the tumor cells themselves [134]. Furthermore, the FDA has recently approved pembrolizumab as the first tissue-agnostic/site-agnostic drug for the treatment of patients with deficient MMR (dMMR) or metastatic MSI-H [134]. Although the FDA's approval of pembrolizumab introduces it as a potential therapeutic option for patients with rare, advanced cancers, further investigation is needed to gather clinical evidence regarding the drug's efficacy and safety profile in these patients [110].

Cemiplimab (Libtayo®, Regeneron Pharmaceuticals/Sanofi) is a fully humanized IgG4 mAb that effectively blocks the interaction between the PD-1 receptor and its ligands. It is prescribed for patients diagnosed with metastatic or locally advanced cutaneous squamous cell carcinoma (CSCC) who are not suitable candidates for curative resection or radiotherapy. In September 2018, it received approval from the FDA and in June 2019, it

was also approved by the European Medicines Agency (EMA) [135]. Cemiplimab holds the distinction of being the first FDA-approved drug specifically for the treatment of CSCC and has proven to be superior in terms of overall survival (OS) and progression-free survival (PFS) when compared to epidermal growth factor receptor (EGFR) inhibitors and chemotherapy, highlighting its immense potential in the treatment of CSCC patients [136].

As for anti PD-L1 agents in cancer therapy, the FDA has granted approval to three, specifically Atezolimumab, Durvalumab, and Avelumab. These inhibitors have been successfully utilized in the treatment of various solid tumors, such as NSCLC, HNSCC, melanoma, and MCC [128].

Atezolizumab (Tecentriq, Genentech) is an IgG1 mAb. It has been designed with a modified Fc domain to decrease antibody-mediated cellular cytotoxicity, thus preventing the depletion of T cells that express PD-L1 [137]. This antibody is indicated as a standalone treatment for patients with locally advanced or metastatic bladder cancer who have previously undergone platinum-based chemotherapy or have contraindications for these drugs [138]. It is also approved for the treatment of patients with locally advanced or metastatic NSCLC who have undergone prior chemotherapy or targeted therapy, depending on their EGFR or anaplastic lymphoma kinase (ALK) mutation status [139].

Avelumab (Bavencio, Merck KGaA, Pfizer) is a fully human antibody that has a dual effect. It not only hinders the connection between PD-L1 on tumor cells and PD-1 on T lymphocytes but also activates ADCC activity by binding to receptors on immune system effector cells. There has been extensive research conducted to understand the mechanism of action and effectiveness of avelumab in the treatment of neoplastic diseases, particularly due to its ability to enhance ADCC [140]. Avelumab has received approval for the treatment of advanced MCC [141]. The FDA has also granted approval for avelumab as a second-line drug in combination with platinum chemotherapy or after its use in locally advanced/metastatic urothelial cancer [142]. Additionally, avelumab was approved for RCC after demonstrating better PSF (13.8 months vs. 8.4 with sunitinib) when used alongside axitinib [143].

Durvalumab (Imfinzi, Medimmune/AstraZeneca) is an immunotherapy treatment approved by the FDA that works by binding to PD-L1 with high affinity and specificity, which prevents it from interacting with PD-1 and CD80 [144]. The FDA designated this treatment as a breakthrough therapy in February 2016, based on early clinical data from a Phase I trial. This trial showed promising results for patients with metastatic urothelial bladder cancer, specifically those whose tumor cells express PD-L1 and who have advanced disease after a standard platinum-containing chemotherapy regimen [145]. In May 2017,

the FDA also approved durvalumab for the treatment of patients with urothelial carcinoma, either metastatic or locally advanced, who have progressed during or after platinum-based chemotherapy. This includes patients who experienced disease progression within one year of treatment with a platinum-based regimen in the neoadjuvant or adjuvant setting followed by surgical resection [145]. Furthermore, in 2018, the FDA approved durvalumab for patients with unresectable stage III NSCLC, whose disease has not progressed after concomitant platinum-based chemotherapy and radiotherapy [146].

2.6.3 ICI-based immunotherapy biomarkers

While immunotherapy has yielded long-lasting clinical benefits, research has identified certain limitations in its effectiveness. Therefore, it is crucial to devote attention to studying biomarkers that can enhance prediction accuracy of clinical responses [147]. Extensive research has already been conducted to explore and develop biomarkers for predicting response to ICI treatment.

Several different types of biomarkers have been identified as potential factors that can enhance the response to immunotherapy in patients who are receiving ICIs.

2.6.3.1 Tumor mutational burden

TMB refers to the density of non-synonymous mutations found in the protein-coding area, or simply the number of mutations within the tumor. It is typically quantified as the total number of mutations per megabase of substitutions, insertions, or deletions in the coding region of the gene analyzed in the tumor sample, and generally is detected as mutations per million bases (Mut/Mb) [148]. Until now, whole-exome sequencing (WES) has been the preferred method to measure TMB, considered as the gold standard. However, due to the costly and time-consuming nature of WES, accurate TMB assessment can now be achieved through next-generation sequencing (NGS) panels [149].

Numerous recent reports have indicated that an increased TMB is linked to a positive response to immunotherapy with ICIs. Particularly in patients with NSCLC, TMB equal to or greater than 10 mut/Mb has been associated with improved OS when Nivolumab and Ipilimumab are combined, regardless of PD-L1 expression [150]. A TMB value of ≥ 10 mut/Mb has also demonstrated a correlation with enhanced survival in various tumor types, including SCLC [151]. Pembrolizumab has also proven to be effective in multiple cancer types with TMB ≥ 10 mut/Mb, predominantly solid cancers [152].

2.6.3.2 Microsatellite Instability /DNA Mismatch Repair

The MMR system plays a crucial role in identifying and correcting errors that occur during DNA replication, such as mismatches between bases and misincorporation. If this system is impaired, it can result in excessive mutations, leading to cancer [153]. In cancer immunotherapy, MSI status has been recognized as a potential biomarker for prediction. There is a close association between MMR functionality and MSI. When MMR functions properly. i.e., is in a proficient state (pMMR), it can repair MSI to maintain stability. On the other hand, when the expression of MMR-related proteins is disrupted, leading to dMMR, it impairs cellular repair mechanisms, enabling the accumulation of replication-induced DNA mutations. This ultimately contributes to the development of MSI [154]. MSI can be broadly categorized as MSI-H or lowly unstable (MSI-L) [153].

dMMR is common in various tumor types, particularly gastrointestinal cancers such as colorectal, stomach, small intestine, and prostate malignancies [155]. Detecting dMMR requires utilizing immunohistochemistry (IHC) to identify MMR proteins like MSH2/6, MLH1, and PMS2, as well as polymerase chain reaction (PCR) to identify small genomic alterations [156]. Research has demonstrated that dMMR/MSI-H tumors exhibit significantly higher somatic mutation rates compared to pMMR tumors. These tumors are also known to produce numerous shift-code peptides acting as neoantigens, thus enhancing the immune response[147] . Combining dMMR/MSI-H status with TMB and PD-1/PD-L1 expression has been suggested to predict the effectiveness of ICI-based immunotherapy. Notably, the CHECKMATE 142 study reported that Nivolumab, as a treatment option, could lead to durable responses in patients with dMMR/MSI-H metastatic colorectal cancer [157]. Moreover, pembrolizumab has shown promising and long-lasting outcomes in multiple tumor types, specifically dMMR/MSI-H tumors, in several clinical trials, including KEYNOTE-012, 016, 028, and 158 [147].

2.6.3.3 IFN-γ

Changes occurring in the signaling pathways associated with tumors also impact the effectiveness of ICIs. IFN-γ is a cytokine that stimulates the immune response and plays a crucial role in activating immune cells. It can also trigger cellular events leading to the death of tumor cells by binding to receptors on their surface. Additionally, IFN-γ can enhance the expression of PD-L1 in tumors and increase the expression of MHC [147].

A study conducted by Karachaliou *et al.* examined seven patients with NSCLC who were treated with pembrolizumab, and their findings suggested that high levels of IFN-γ expression may be linked to improved PFS and OS in NSCLC patients [158]. Higgs *et al.*

similarly demonstrated that patients with heightened IFN-γ-associated signaling had a longer median OS (18.1-22.7 months vs. 6.5-7.7 months) and a significantly higher objective response rate in advanced NSCLC [159].

Mutations in genes associated with the IFN-γ pathway have also been shown to contribute to unfavorable outcomes and resistance in patients receiving ICI therapy. The primary pathway involving IFN-γ signaling is the JAK/STAT pathway, which regulates immune responses and the development of tumors [110]. Mutations in JAK1/2 result in reduced production of IFN-γ. Shin *et al.* indicated that JAK1/2 mutations were associated with resistance to anti-PD-1 therapy in patients with colorectal cancer. These findings suggest that mutations in JAK can impair the effectiveness of ICIs [160].

2.6.3.4 PD-L1 expression

PD-L1 is extensively studied as a biomarker in clinical trials, particularly in relation to the effectiveness of ICIs. IHC is used to measure the expression of PD-L1 in tumors, and this method was one of the first biomarkers developed to predict the benefits of ICIs [161]. Numerous clinical trials have presented evidence showcasing the viability of PD-L1, and PD-L1 expression has garnered approval from the FDA as a biomarker for adjuvant or second-line therapy [147].

Results from the KEYNOTE-024 trial indicated that a fixed dose of Pembrolizumab was associated with improved OS and PFS, as well as a lower incidence of treatment-related adverse events in NSCLC patients with PD-L1 tumor proportion score ≥50% compared to chemotherapy [162]. Furthermore, the results from the KEYNOTE-048 study revealed that Pembrolizumab improved OS in patients with recurrent or metastatic HNSCC as the expression of PD-L1 increased [163]. These findings demonstrate that PD-L1 expression serves as an indicator of response for ICIs.

2.6.3.5 Tumor-infiltrating lymphocytes

TILs represent a powerful adaptive immune mechanism that holds potential in fighting against tumors. Studies have shown that TILs are associated with prognosis and response to immunotherapy in various types of cancer. These specific TILs originate from the tumor tissue and possess the ability to recognize and destroy autologous tumors through MHC-restricted tumor lysis activity [164].

When it comes to tumor immune infiltration, the link between immune inflammation and immunotherapy with ICIs is evident. Immune inflammation is characterized by the presence

of $CD8^+$ and $CD4^+$ T lymphocytes within the tumor tissue, along with the expression of IC molecules. This suggests that ICI treatment can trigger an immune response against the tumor [165]. Analyzing the tumor samples prior to treatment revealed a higher abundance of $CD8^+$ T cells at the margins of patients who responded well to the treatment, and monitoring the patients during treatment demonstrated an increased infiltration of $CD8^+$ T cells into the tumor tissue [166]. Additionally, data showed that patients with a higher density of $CD8^+$ TILs experienced longer PFS and OS compared to those with a lower density [167].

2.6.3.6 Liquid biopsy biomarkers

Liquid biopsy offers a less invasive approach compared to surgical sampling, reducing patient suffering. It also provides additional benefits not found in tissue biopsy. One advantage is its ability to overcome the inherent heterogeneity of tissue biopsy by allowing for multiple samplings. This enables real-time monitoring of tumor changes and yields more comprehensive results. The most promising biomarkers commonly used in liquid biopsy are circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomes [168].

ctDNA is primarily released by deceased cancer cells but can also be directly secreted by CTCs. It contains information about the entire tumor genome and its data variability allows for dynamic monitoring of tumor progression throughout treatment [169]. Various studies have demonstrated that high ctDNA mutations are associated with OS and prognosis in patients with different types of cancer treated with ICIs. Lee *et al.* found that melanoma patients with consistently elevated levels of ctDNA during anti-PD-1 therapy had less favorable responses, resulting in shorter PFS and OS [170].

Recent findings have also shown a correlation between the detection of CTCs and tumor metastasis. In patients with advanced head and neck cancers, overexpression of PD-L1 in CTCs suggests that the combined detection of PD-L1 and CTCs may serve as a potential biomarker for predicting the efficacy of ICIs [171].

Exosomes, vesicles found outside of cells, contain various tumor-associated proteins, metabolites, RNA, DNA, and lipids. These components provide crucial information for biopsy and can be used as significant biomarkers [147]. According to Zhang *et al.*, exosome levels were found to be higher in liver metastases of gastric cancer patients. Furthermore, gastric cancer patients exhibited higher serum exosome levels compared to healthy individuals, and the quantity of exosomes in serum was positively associated with the stage of gastric cancer [172]. Additionally, it has been demonstrated that the presence of PD-L1 mRNA in plasma exosomes is linked to the effectiveness of ICIs. The presence of PD-L1

mRNA in exosomes might result in the suppression of effector lymphocytes involved in antitumor immunity, thereby reducing the efficacy of ICIs [173].

2.6.4 Immune-related adverse events induced by ICIs

Although ICIs play a beneficial role in activating T cells that target tumor antigens, they can also activate T cells to react to normal self-antigens, causing side effects similar to autoimmune diseases. These side effects, known as irAEs, are graded based on severity and can range from mild (grades 1-2) to severe (grades 3-4), sometimes resulting in death [174]. Since ICs have an important biological function in the body, they are expressed in normal tissues as well. Consequently, blocking them with ICIs can trigger a broad immune response, which may impact various organs within the body [175].

The frequency and affected organs of irAEs vary depending on the specific ICI treatment and the type of cancer being treated. For example, melanoma patients treated with a combination of ipilimumab and nivolumab experience irAEs in over 95% of cases, with severe irAEs occurring in approximately 60% of patients [176]. In contrast, irAEs are less common with PD-1/PD-L1 inhibitors, affecting around 65% of patients at any grade and 15% with severe irAEs [177].

Toxicities seen in more than 10% of patients treated with anti-CTLA-4 agents include loss of appetite, stomach pain, diarrhea, tiredness, nausea, itching, rashes, and vomiting. Similarly, arthralgia, diarrhea, fatigue, nausea, itching, and rashes have been observed in more than 10% of patients treated with anti-PD-1/PD-L1 agents [56]. It is essential to note that although severe irAEs are rare in patients undergoing ICI therapy, they can pose a life-threatening risk if not anticipated and managed properly. Although irAEs resemble autoimmune diseases, they usually have a more sudden onset and greater severity [174]. The most critical life-threatening toxicities associated with anti-CTLA-4 treatment and PD-1/PD-L1 agents are dysimmune colitis and interstitial pneumonitis, respectively. However, other severe toxicities linked to ICI treatment have also been reported, such as autoimmune anemia, infusion reactions, type-1 diabetes with ketoacidosis, Guillain–Barré syndrome, Stevens–Johnson syndrome, and thrombocytopenia with bleeding complications [56, 175].

Currently, guidelines for managing irAEs are predominantly based on expert opinion due to limited supporting evidence, and the primary objective of these guidelines is to promptly resolve irAEs. There is a need for more collaborative efforts in order to assess and establish standardized management approaches for various irAEs [174].

3. Hyperthermia as a cancer treatment

Although immunotherapy, specifically ICI-based immunotherapy, has shown promising results in some types of tumors, its efficacy largely relies on the immunogenicity of the tumor. Given the immune condition of the TME, tumors can be categorized as either "cold" or "hot". "Cold" tumors often exhibit low response rates to anti-PD-1/PD-L1therapies due to limited tumor mutation, minimal T-cell infiltration, decreased PD-L1 expression, and an abundance of immunosuppressive cells [178]. Treatment with anti-angiogenesis agents, radiotherapy, or chemotherapy enhances the effectiveness of ICIs by transforming the immune status of TME. This transformation involves unveiling tumor-specific antigens, normalizing the endothelial function, attracting immune cells, among other mechanisms [179, 180]. Hyperthermia can also influence the immune status of TME by exerting cytotoxic effects through elevated temperatures on the immune system.

Hyperthermia is a method of eradicating cancer cells or impeding their growth by elevating tissue temperature using an external heat source for a specific duration [180]. It has emerged as a promising approach in cancer treatment, addressing the limitations of traditional therapies. Hyperthermia holds immense potential due to its minimal side effects compared to conventional treatments and its cost-effectiveness, especially when combined with other therapies [180]. Over the course of four decades, hyperthermia has yielded impressive outcomes as a radiosensitizer or chemosensitizer and is currently being successfully employed in conjunction with radiotherapy or chemotherapy for various tumor types, such as recurrent breast cancer, bladder cancer, cervical carcinoma, head & neck cancer, soft tissue sarcoma, and melanoma [181]. Depending on the targeted organ, hyperthermia can be classified into local techniques (microwaves, radio waves, or ultrasound), regional approaches (hyperthermic intraperitoneal chemotherapy), and whole-body hyperthermia. Moreover, hyperthermia can be categorized based on temperature ranges, encompassing fever-range hyperthermia (39–40°C), mild temperature hyperthermia (heat shock temperature, 41–43°C), and thermal ablation (cytotoxic temperature, >43°C), usually used to kill carcinoma directly. Furthermore, the field of hyperthermia includes newly developed treatments such as magnetic nanoparticle hyperthermia, cryo-thermal therapy, and photothermal therapy [180].

3.1 Effects of hyperthermia in cancer cells

Hyperthermia influences the immune status of the TME by releasing danger signals through heat shock proteins (HSPs), and subsequently activating the immune system. These immunomodulatory effects not only render hyperthermia effective in fighting against

cancer, but also establish it as a reliable treatment for generating a TME characterized by increased expression of PD-L1 and accumulation of tumor infiltrating lymphocytes. There is a number of immune-related factors that are inducible by hyperthermia, with effects specifically on the immunogenicity and immunoreactivity of the TME, highlighting the beneficial adjunctive effect of hyperthermia on ICIs [180].

3.1.1 DNA damage

The immune system often responds to neoantigens resulting from accumulated DNA damage, known as the TMB. Hyperthermia can directly trigger the DNA damage response by inciting single-stranded breaks, double-stranded breaks, the formation of phosphorylated C-terminal serines foci on histone H2AX, and the phosphorylation of the ataxia-telangiectasia mutated protein. Additionally, it slows down DNA replication and repair by suppressing DNA polymerase and topoisomerase activity, and indirectly activates the DNA damage response. Through the promotion of reactive oxygen species (ROS) production, cell cycle arrest, cell cycle checkpoint arrest, cell death, and the deceleration of DNA replication, hyperthermia also induces the expression of tumor suppressor alternative reading frame. Furthermore, it significantly increases DNA damage in tumor stem cells that are typically resistant to conventional treatment methods. This approach becomes particularly effective in generating tumor neoantigens when hyperthermia is employed as an adjunct therapy alongside chemotherapy or irradiation, as it leads to irreversible cellular DNA damage [182, 183].

3.1.2 Immunogenic cell death

Immunogenic cell death (ICD), an innovative concept that has surfaced in the past decade, relies on the simultaneous production of ROS, Type I and the triggering of endoplasmic reticulum (ER) stress Type II. These mechanisms act as facilitators and inducers of phagocytosis towards recruited immune cells. ICD serves as a significant indicator of a favorable immunogenic TME, providing diverse functional infiltration of immunological cells and cytokines [180, 184].

Apoptotic, necrotic, or even live cancer cells represent a significant natural method of TAA production. Depending on the degree of temperature change, hyperthermia induces diverse forms of TAA. Typically, when temperatures fall within the fever range (37–40°C), cancer cells exhibit a protective response, allowing for the presentation of their constituents. In contrast, temperatures ranging from 41–43°C predominantly trigger apoptosis-induced

cell death, maintaining a delicate balance between pro-apoptotic and anti-apoptotic mechanisms. As the temperature continues to rise above this range, the pro-apoptotic factors gain dominance. Beyond 43°C (indicative of thermal ablation), tumor cells undergo destruction primarily through necrosis [180].

Thermal ablation-induced necrosis is a form of cell death that can elicit an immunogenic inflammatory reaction. Unlike thermal ablation, hyperthermia in the fever range merely affects cell membrane fluidity and stability, alters cell structure, and influences sodium-calcium levels within cells. During this temperature regime, simultaneous occurrences of the heat shock response and ER stress can arise. Heat shock response-driven HSPs can potentially mitigate activation or alleviate ER stress by activating a negative feedback mechanism known as the unfolded protein response (UPR), thereby preventing excessive activation. Moreover, HSPs can shield tumor cells against both caspase-dependent and caspase-independent apoptosis prompted by oxidative stress. Interestingly, this temperature range rarely induces eIF2α phosphorylation—a hallmark of immunogenic cell death [185, 186]. When the temperature ascends from the fever range to the thermal ablation range, tumor cells primarily undergo apoptosis, striking a balance between cellular self-destruction and self-preservation mechanisms. This process entails the induction of CCAAT/enhancer-binding protein homologous protein (CHOP), alterations in calcium levels, and the activation of ER proteases, specifically the calpain-calpastatin proteolytic system and caspase-mediated apoptosis. Furthermore, this process coincides with the upregulation of eIF2α phosphorylation [186, 187]. While both mild (43°C) and severe (45°C) hyperthermic exposures can induce cell death by activating apoptotic pathways, mild hyperthermia (43°C) orchestrates the apoptotic response in a more controlled manner to sustain apoptotic cell death [187].

According to conventional belief, apoptosis is considered non-immunogenic and does not prompt inflammation. Nevertheless, recent research indicates that certain types of therapies that trigger apoptosis in tumor cells may also result in the release of DAMPs and the induction of ICD. For cell death to be classified as ICD, it is crucial to have factors such as the exposure of calreticulin (CRT), the release of high mobility group box 1 (HMGB1), and the secretion of adenosine triphosphate (ATP) [188]. Intriguingly, subjecting cancer cells to heat-shock conditioning not only increased the translocation of CRT to the plasma membrane but also led to the release of HMGB1 protein. Additionally, both CRT and HMGB1 relocation were associated with an enhanced ability for antigen cross-presentation and the maturation of antigen present cells in response to mild hyperthermia [189]. Although the connection between hyperthermia-induced apoptosis and ICD generation requires

further clarification, it is evident that apoptosis triggered by hyperthermia plays a role in the generation of ICD [180].

3.1.3 Heat Shock Proteins

Hyperthermia can also regulate the release of HSPs. HSPs represent a group of highly conserved chaperone proteins produced in a variety of tumors as a response to stress. This group includes HSP70, HSP60, HSP90, and small HSPs. Interestingly, following hyperthermia, the overexpression of HSPs can also lead to an improved immune response [180]. There are three distinct forms of HSPs: intracellular, membrane-bound, and extracellular. Intracellular HSPs play a crucial role in maintaining the structure and function of their associated proteins, aiding in protein folding during cellular challenges [190]. In contrast to the intracellular counterparts, studies have revealed that small HSPs are found within or close to the cytoplasmic membrane, alongside cytoskeletal proteins. This sub-membranous localization of HSPs seems to be associated with cellular responses to heat-induced membrane damage. Additionally, extracellular HSPs released from tumor cells are considered potent adjuvants, aiding in the presentation of tumor antigens and the induction of antitumor immune responses [191].

3.1.4 Enhancement of immune responses

Given an elevated TMB and ICD, hyperthermia activates or boosts immunity, leading to a subsequent immune response throughout various stages of the cancer-immunity cycle. The activation of immunity by hyperthermia seems to be specific, persistent, and memorable. This not only positions hyperthermia as a viable treatment option to combat cancer but also establishes its reliability in augmenting the effectiveness of ICIs [180].

The influence of mild thermal stress on DCs' function in regulating infections and tumor progression has long been established. In the context of antigen presentation, hyperthermia primarily modulates the phagocytosis checkpoints by amplifying immunogenic "eat me" signals and suppressing tolerogenic "eat me" signals, as well as "do not eat me" signals [192]. Particularly, receptor-mediated endocytosis via pattern recognition receptors (PRR) is fundamental in enhancing the phagocytosis of APCs by leveraging immunogenic "eat me" signals originating from DAMPs. Similarly, hyperthermia decreases the expression of CD47 on the cell surface, thereby repressing the "do not eat me" signal [193]. Furthermore, hyperthermia can halt the transmission of tolerogenic "eat me" signals by facilitating the transformation of immature APCs or APCs displaying immunosuppressive phenotypes (M2

macrophages, N2 neutrophils, MDSCs) into a relatively mature state. This transformation encompasses the infiltration of activated monocytes into the TME, induction of immature DCs to differentiate into mature DCs, enhancement of macrophage polarization toward the pro-inflammatory M1 type, and stimulation of the release of inflammatory factors [191, 194].

Besides APCs, hyperthermia has also demonstrated the potential to restore defective $CD4^+$ T cell immune response by reorienting $CD4^+$ T cells towards Th1 and converting T_{Reg} cells into Th17 cells. This alteration creates a favorable TME capable of effectively responding to ICI treatment [195, 196].

Thermotherapy also has an effect in creating a favorable pro-inflammatory TME. Specifically, mild hyperthermia enhances the levels of L-selectin, P-selectin, and ICAM-1 within the blood vessel wall and stimulates the release of various pro-inflammatory cytokines and chemokines (such as IL−1β, IL-6, IL-8, IL-10, and CCL22) [180, 197]. These inflammatory molecules act at different stages to promote lymphocyte infiltration into the TME and facilitate the immune response against solid tumors [180].

Moreover, thermotherapy also promotes $CD8^+$ T cells' quantity and quality, by stimulating the differentiation of $CD8^+$ naïve T cells into antigen specific $CD8^+$ T cells, enhancing T cell cytotoxicity, and encouraging of the development of memory stem T cells [180].

3.1.5 Expression of coinhibitory molecules

Despite the well-established impact of hyperthermia on the immunogenicity and immunoreactivity of tumors, it is important to acknowledge that hyperthermia can also induce an increase in the expression of coinhibitory molecules. Studies have demonstrated that by subjecting tumor cells to temperatures ranging from 37 to 49°C, the expression of PD-L1 and IDO on their surface is systematically upregulated. Notably, this upregulation of PD-L1 expression is observed not only in the tumor margins but also in distant tumors after hyperthermia. It is yet to be fully understood whether the upregulation of coinhibitory molecules represents a cellular protective response aimed at preventing excessive immune activation or a byproduct of the heat shock response triggered by cellular damage [198, 199].

It is crucial to highlight that the amplified presence of coinhibitory molecules on tumor cells can hamper the function of $CD8^+$ T cells. However, the effects of this upregulated IC molecule can be counteracted by ICIs, leading to a synergistic outcome when combined with hyperthermia for tumor remission (refer to the subsequent section). Therefore, based on the aforementioned evidence demonstrating that hyperthermia can exert both an

activating and enhancing effect on the immune response while also upregulating PD-L1 in various types of tumors, scientists propose that hyperthermia treatment establishes a TME resembling that of a type I tumor, characterized by the presence of TILs, and augments PD-L1 expression to synergize with ICIs for more effective cancer therapy [180].

3.2 Combination of hyperthermia with ICIs

The concept of hyperthermia has been explored and studied for centuries, yet it is rarely utilized as a mainstream or adjuvant treatment for cancer. Clinical trials investigating the combination of hyperthermia with cytokines and DCs have yielded conflicting results, mainly due to variations in tumor tissue selection, antigen load both *in vitro* and *in vivo*, and the effectiveness of DC recruitment. Despite these inconsistencies, promising preclinical research has demonstrated optimistic outcomes when combining hyperthermia with ICIs. However, it is important to note that hyperthermia is predominantly utilized in nanoparticle-mediated hyperthermia and radiofrequency ablation scenarios [180].

Nanoparticle-mediated hyperthermia, a localized and non-invasive treatment with manageable irradiation, has emerged as an innovative approach in targeted cancer therapy. This technique encompasses photothermal therapy and magnetic hyperthermia. Studies have indicated that the combination of ICIs (CTLA-4, PD-L1, IDO) with nanoparticle-mediated hyperthermia can facilitate antigen capture, enhance the effect of ICD, suppress T_{Reg} cell function, promote M1 macrophage differentiation, multiply the recruitment of tumor-infiltrating lymphocytes, and establish enduring memory for inhibiting tumor growth in primary and distant sites [198-201]. The collaborative role of heat and ICIs is further supported by findings demonstrating enhanced tumor antigen-specific T cell responses and an increased effector T cell to T_{Reg} cell ratio in distant tumors when employing a combination of radiofrequency ablation and anti-PD-1 mAb administration [202].

In upcoming times, the combination of hyperthermia and ICIs could serve as an approach to amplify the local immune response and circumvent widespread immunotoxicity. The goal of merging hyperthermia and ICIs goes beyond converting the patient's immunosuppressed condition into an activated one; it also has the potential to stimulate the body's innate immunity. This holds advantages in terms of utilizing ICIs in low doses to mitigate the occurrence of irAE events. It is therefore necessary to persist in the exploration of hyperthermia in combination with ICIs and encourage more extensive and comprehensive investigations [203, 204].

3.3 imILT

Laser-based hyperthermia is a focal hyperthermia technique, also referred to as laser thermotherapy or laser ablation. It uses laser light as the heat source. Interstitial laser thermotherapy is its minimally invasive variant for the treatment of tumors deeper within the body. The primary objective of interstitial laser thermotherapy in oncological treatments is to eliminate the tumor without causing any harm to the surrounding structures and tissue. Direct cell death and coagulation are two factors that contribute to tissue destruction [205].

During laser-induced thermotherapy, light damages tissue by absorbing photons and transferring their energy through heat conduction into the tissue. Due to this heat conduction, laser thermotherapy creates a lesion that is larger than the area where light is absorbed. The modalities and parameters required to control tumor heating are determined by these two phenomena - direct light absorption and heat conduction, that are reliant on the specific characteristics of the tissue that is being treated [205].

Classic laser ablation is employed for the treatment of solid tumor masses found in various organs, with the goal of raising the temperature of the entire tumor volume to a minimum of 60°C. This process facilitates the coagulation of tissue in the targeted area, leading to a rapid cessation of cellular activity. To accomplish this, an optical fiber is positioned in the central region of interest, through which light is administered over a duration of 1 to 10 minutes, depending on the volume to be ablated and the specific device employed [205].

Immune stimulating interstitial laser thermotherapy (imILT) is a localized ablation technique that operates at non-coagulating temperatures in close proximity to the tumor border. This method involves establishing a temperature gradient within the tumor, resulting in a heating to 46°C at the tumor border or a few millimeters outside it. This temperature is maintained for an extended period of approximately 30 minutes in order to induce ICD at the tumor border. The manifestation of this effect is only observable 48-72 hours after treatment, stimulating an immune response [205]. Some preclinical models have previously demonstrated that the imILT protocol has the capability to induce a systemic immune response against tumors, commonly referred to as the "abscopal effect” [206-208]. However, the number of clinical applications reported thus far remains limited.

Numerous extensive preclinical investigations were conducted in order to demonstrate the immune stimulating properties of imILT. One particular study aimed to compare the immunologic memory elicited by imILT, in comparison with resection [205, 206]. The research involved the utilization of 280 rats, which were divided into four distinct groups: (1) rats with liver-implanted tumors that received treatment with imILT, (2) rats with liver-

implanted tumors that underwent surgical resection, (3) rats without tumors that were subjected to imILT, resulting in the ablation of normal liver tissue (sham imILT), and (4) rats without tumors that underwent resection of a portion of a healthy liver (sham resection). Rats in groups 1 and 2 received the implantation of adenocarcinoma and subsequent treatment after a period of 6-8 days. Following this initial procedure, a second tumor of the same nature was introduced in a different lobe of the rats, either 2, 5, or 10 weeks later. The animals were then observed for a maximum of 48 days after the second implantation, unless they exhibited signs of inactivity or distress prior to this endpoint. The evaluation of the tumor's vitality at the time of sacrifice was conducted in conjunction with other markers indicative of immune system activity. Notably, group 1, which underwent treatment with imILT, displayed a distinct response compared to the three other groups. In groups 2, 3, and 4, the secondly implanted tumor exhibited such significant growth that none of the rats survived for the full 48-day observation period. Conversely, rats in group 1 exhibited complete eradication of the second tumor by day 48. The combination of these findings, alongside the results obtained from immunology markers derived from blood tests, provides evidence that imILT elicits a robust immune response and generates immunological memory against the cancer being treated.

II. Study design and aims

1. Study design

The current work presents the findings of a flow cytometry study carried out in three distinct pilot studies, conducted with the primary objective of determining the optimal conditions and optimizing protocols for conducting a more extensive research project, wherein the primary aim is to assess the synergistic antitumor impact of combined laser-based thermotherapy and immunomodulators (anti-PD-1 and anti-CTLA-4) in a poorly immunogenic B16F10 melanoma mouse model. The project is sponsored by Clinical Laserthermia Systems AB (Lund, Sweden), and the research work is being developed in IPO-Porto - Instituto Português Oncologia do Porto Francisco Gentil, EPE and i3S – Instituto de Investigação e Inovação em Saúde da Universidade do Porto.

Prior to performing the final study, there was a need to perform four pilot studies, to optimize the conditions to take further to the final project, in an effort to minimize unexpected events, thus increasing the probability of success of the final study. Pilot studies 0, II and III will be described in detail in the following sections. Pilot study I was performed by other members enrolled in the project at the animal facility of i3S, and therefore will not be described in detail in the present dissertation. This study aimed at evaluating the tumor growth kinetics of different inocula of B16F10 melanoma cell line in female C57BL/6 mice, to determine the optimal cell inocula to take further to the different pilot studies and final study.

1.1 Pilot study 0 – Immune cell populations

Oncology constitutes a primary focal point within the realm of medical research, and animal models have been developed to comprehend its underlying mechanisms. The C57BL/6 mice model is conventionally employed to investigate cancer immunology. Nevertheless, we noticed a lack of consensual literature regarding the depiction of the distribution of the most prevalent immune populations in the C57BL/6 wild type (WT) mice strain. Specifically, we noticed heterogeneity of data published in the literature, depending on the strain, sex and age of the animals. This scarcity impedes the reliable interpretation and assessment of the values obtained in samples coming from manipulated animals.

Consequently, a pilot study was designed with the aim of delineating the distribution of the most common immune populations, specifically B lymphocytes, T lymphocytes, NK cells, DCs, neutrophils, and monocytes, within the peripheral blood of C57BL/6 WT

female mice. This understanding would allow for a precise and accurate interpretation of the resulting immune cell population frequencies observed in samples obtained from manipulated animals.

1.2 Pilot study II - Laserthermotherapy

ImILT consists of a precision temperature-controlled destruction of a tumor using a laser. The protocol ensures hyperthermia at the tumor border below coagulating temperature, resulting in ICD and with the potential to affect metastasis elsewhere. The aim of the second pilot study was to assess the effects of imILT, specifically the TRANBERG laser, on a melanoma mouse model. Pilot study II focused on optimizing the positioning of the fiber and temperature probe, as well as determining the ideal depth of the tumor for effective treatment. Additionally, the study aimed at investigating the impact of the imILT treatment on the frequency of immune cell populations in the tumor, spleen, and peripheral blood samples of the treated animals. Furthermore, Pilot study II also aimed at investigating the evidence of abscopal effect in the imILT treated animals.

1.3 Pilot study III – Immunomodulator concentration tests

The treatment of cancer patients with ICIs, specifically anti-PD-1 and anti-CTLA-4 agents, has been a significant breakthrough in the field of oncology in recent years and has contributed to a significant improvement in the outcome of treatment and prognosis of patients with different types of malignancies, with many drugs already approved by the regulatory agencies. Pilot study III aimed at determining the optimal dosage of antibodies (anti-PD-1 and anti-CTLA-4) without leading to full treatment, so that in the final study, it would be able to observe the combinatory effect of laserthermotherapy and immunomodulator therapy.

2. Aims of the dissertation

Since the present work consisted of conducting the pilot studies that preceded a larger and more comprehensive study, the main goal of this dissertation was to ascertain the ideal conditions and optimize the methodologies for the final study, specifically through the use of flow cytometry techniques, with the purpose of minimizing unexpected events and consequently increasing the likelihood of success of the final study.

Specifically, the tasks of the project were:

1. To assess, by flow cytometry, the distribution of immune system cell populations, namely B lymphocytes, T lymphocytes, NK cells, DCs, neutrophils, and monocytes, in the peripheral blood of C57BL/6 WT female mice.
2. To evaluate the effects of the TRANBERG laser therapy on the immunogenicity of melanoma tumors, as well as the spleen and peripheral blood of treated animals, by assessing the distribution of immune cell populations in those tissues by flow cytometry.
3. To assess the presence of any evidence of abscopal effect in imILT treated mice, by evaluating the immune cell distribution on the left tumor (not imILT-treated) by flow cytometry.
4. To evaluate, by flow cytometry, the effects of two anti-mouse PD-1 and anti-mouse CTLA-4 antibody dosages in the distribution of immune cell populations in tumors, spleens and peripheral blood of treated animals, and to determine the optimal dosage to take further to the final study.

III. Materials and methods

1. Antibodies for immunotherapy

Therapeutic anti-mouse PD-1 mAb (clone RMP1-14), rat IgG2a isotype control, anti-mouse CTLA-4 mAb (clone 9H10) and polyclonal Syrian hamster IgG1 isotype control were purchased from BioXcell (West Lebanon, USA). All antibodies were administered intraperitoneally to the mice.

2. Laserthermotherapy

Laserthermotherapy was carried out using the TRANBERG Thermal Therapy system. This is a patented technology by Clinical Laserthermia Systems AB designed to deliver laser energy to the target tissue. During a treatment session, heat and destruction can be controlled with high accuracy, while saving surrounding untargeted tissue. This system consists of a diode laser emitting laser light at 1064 nm and a temperature feedback unit connected to a PC.

The fiber is placed at a right angle into the center of the tumor and the feedback thermistor probe is placed perpendicular into the subcutaneous tissue, approximately 3 mm outside the tumor margin and at a depth of, at least, 2 mm from the skin surface. ImILT was performed for 30 minutes at a steady-state temperature of 46°C at the tumor margin (the thermistor location).

3. Animals

All animal related activities, including maintenance, treatment, and euthanasia, were carried out by my colleagues Catarina Rodrigues (Experimental Pathology and Therapeutics Group, IPO Porto), Cristiana Gaiteiro (Experimental Pathology and Therapeutics Group, IPO Porto) and Nuno Mendes (i3S, University of Porto) at the animal facility of i3S, University of Porto. C57BL/6 female mice used throughout the project were supplied by Charles River Laboratories (L'Arbresle, France).

For pilot studies II and III, C57BL/6 female mice were simultaneously inoculated on the right and left flanks. On the right side, a 150 000 B16-F10 melanoma cell inoculum was administered, which would subsequently be subjected to treatment. Conversely, on the left side, a 75 000 B16-F10 melanoma cell inoculum was administered, which would not be treated and instead serve to assess the presence of any abscopal effect. Exact

size of cell inoculum was determined in Pilot study I by other members who were actively involved in the project.

3.1 Pilot study 0

Peripheral blood of ten 12-week-old C57BL/6 WT female mice was collected into ethylenediaminetetraacetic acid (EDTA)-coated tubes to be analyzed by flow cytometry.

3.2 Pilot study II

Pilot study II comprised of three eight-week-old C57BL/6 female mice groups that were simultaneously inoculated with 150 000 and 75 000 cells on the right and left flanks, respectively. The number of cells for the inocula was determined considering the results obtained in Pilot study 1, performed by other members of the team (data not presented here). These groups included sham-treated group, imILT treatment group 1 and imILT treatment group 2. Each group comprised of five animals at the start of the study. However, due to unexpected heterogeneous and very rapid tumor growth, several animals in each group had to be euthanized, due to ethical and legal reasons, before the treatments began. Thus, sham-treated group and imILT-treatment group 2 ended up with two animals each, and imILT-treatment group 1 with three animals.

The sham-treated group was composed of two animals, with a right tumor depth of approximately 7 mm. In this particular group, the laser fiber was inserted into the right tumor without activating the laser unit, in order to investigate whether the fiber alone would elicit an inflammatory response. ImILT treatment group 1 consisted of three animals, in which the right tumor depth averaged 6.5 mm, that were treated with the TRANBERG laser 11 days post-inoculation. ImILT treatment group 2 consisted of two animals that presented a bigger right tumor depth (averaged 9.0 mm), that were treated 16 days post-inoculation. The purpose of the last group was to examine whether animals would experience any advantages from laser treatment at greater depths and volumes.

After sacrificing the mice, necropsy of each animal was performed and samples of the tumor (left and right), spleen and peripheral blood were collected for further evaluation by flow cytometry.

3.3 Pilot study III

For pilot study III, eight-week-old C57BL/6 female mice were simultaneously inoculated with 150 000 and 75 000 cells on the right and left flanks, respectively. On day 5 post-inoculation, animals were randomly assigned into eight different treatment groups, according to Table 1. Treatment with immunomodulators and their respective isotopic controls was performed on days 6, 9 and 13 post-inoculation.

After sacrificing the mice, necropsy of each animal was performed and samples of the tumor (right), spleen and peripheral blood were collected for further evaluation by flow cytometry.

Table1. Pilot study III treatment groups.

Group	Treatment	Number of animals
1	Rat IgG2a 2.5 mg/kg	3
2	Anti-mouse PD-1 2.5 mg/kg	4
3	Rat IgG2a 5.0 mg/kg	3
4	Anti-mouse PD-1 5.0 mg/kg	3
5	Hamster IgG1 2.5 mg/kg	3
6	Anti-mouse CTLA-4 2.5 mg/kg	4
7	Hamster IgG1 5.0 mg/kg	3
8	Anti-mouse CTLA-4 5.0 mg/kg	3

4. Flow cytometry

Flow cytometry data was acquired using a FACSCanto II flow cytometer (BD Biosciences) belonging to the Immunology Department of the Portuguese Cancer Institute of Porto (Instituto Português de Oncologia do Porto, IPO-Porto). Data analysis and interpretation was carried out using the specialized software Infinicyt 2.0 (Infinicyt™, Cytognos).

4.1 Pilot study 0

Following mice euthanasia, peripheral blood was collected into EDTA-coated tubes. The sample was processed, and data was obtained on the same day. Firstly, and prior to immunophenotypical staining, 100 µl of peripheral blood to be analyzed by flow cytometry was incubated with Mouse BD Fc Block™ (BD Biosciences) to minimize unspecific binding to Fc receptor, this way avoiding biased results. Subsequently, the expression of B and T lymphocytes, NK cells, DCs, neutrophils, and monocytes was assessed using a panel of eight target-specific antibodies conjugated with eight different fluorochromes (Table 2). After immunophenotypical staining, red blood cells were lysed using a commercial lysing solution (BD Pharm Lyse), and samples were washed once with flow cytometry staining buffer (FCSB) (eBiosciences), before being acquired in the FACSCanto II flow cytometer.

Table 2. Pilot study 0 antibody panel for immunophenotypical staining.

v450	**v500**	**FITC**	**PE**	**PerCP Cy5.5**	**PE-Cy7**	**APC**	**APC-H7**
Ly-6C (AL-21)	CD3e (500A2)	CD19 (1D3)	CX3CR1 (SA011F11)	CD45 (30-F11)	CD11c (HL3)	NK1.1 (PK136)	Ly-6G (1A8)
BD Biosciences	BD Biosciences	BD Biosciences	BioLegend	BD Biosciences	BD Biosciences	BD Biosciences	BioLegend

4.2 Pilot studies II and III

For both pilot studies II and III, tumor (right and left for pilot study II and right for pilot study III), spleen and peripheral blood specimens were collected from the animals. All samples were collected, processed and data was acquired in the same day.

TILs were isolated from tumors using GentleMACS and Mouse Tumor Dissociator Dissociation kit (Miltenyi Biotec), according to the manufacturer's instructions. Spleens were harvested and dissociated mechanically into single cell suspension in ice-cold phosphate buffered saline (PBS). Splenocytes were washed twice with PBS and re-suspended in FCSB (eBiosciences) before an aliquot of approximately one million splenocytes was taken to be stained with the cocktail of antibodies. Peripheral blood was collected into EDTA-coated tubes, and 100 µl were taken to be stained with the antibody cocktail.

The expression of T lymphocytes and macrophages/monocytes was assessed using a panel of eight target-specific antibodies conjugated with eight different fluorochromes (Table 3). For cytoplasmatic Foxp3 antigen staining, Foxp3 fixation/permeabilization 1x kit (eBiosciences) was used, according to the manufacturer's instructions. For peripheral blood, red blood cells were lysed with Lysing Buffer 1x (BD Pharm Lyse), while tumors and spleens were treated with Red Blood Cell Lysis solution (Citomed). After red blood cell lysis, samples were washed and resuspended in FCSB, before being acquired in the FACSCanto II flow cytometer.

Table 3. Pilot studies II and III antibody panel for immunophenotypical staining.

v450	v500	FITC	PE	PerCpCy5.5	PE-CY7	APC	APC-H7
LY-6C (AL-21)	CD3e (500A2))	F4/80 (CI: A3-1)	Foxp3 (FJK-16s)	CX3CR1 (SA011F11)	CD8 (53-6.7)	CD25 (PC61)	CD4 (GK1.5)
BD Pharmingen	BD Pharmingen	ThermoFisher	eBiosciences	BioLegend	BD Pharmingen	BD Pharmingen	BD Pharmingen

5. Statistical analysis

Statistical tests were carried out using Graphpad Prism 10 (GraphPad software, Inc., CA/USA, 2011). Data is presented as mean ± SD. For single comparisons, the significance of differences between means was determined by Student's t test. For multiple comparisons one-way or two-way ANOVA were carried out. A value of $p<0.05$ was considered to be statistically significant.

IV. Results

In every stage of the study, the following humanitarian endpoints were considered: any signals of distress, suffering or pain not controlled, body weight loss greater than 20-25% of the body mass, anorexia and moribund state, related or not to the experimental procedure. Euthanasia was executed by cervical dislocation under anesthesia with isoflurane.

For Pilot study II, 2 animals from the imILT-group 1 were euthanized before day 7 post-treatment: one showed signals of pain due to severe muscle exposure and the other due to an ulcer that did not heal. Before the sham treatment, one animal from the control group was euthanized, due to bodyweight loss > 20%. One animal from the imILT- group 2 was also euthanized before day 7 post-treatment due to tumor volume > 2000 mm^3.

For Pilot study III, none of the animals showed any signal of distress, pain nor weight loss.

1. Pilot study 0

1.1 Dot plots for Immunophenotypical Characterization of WT C57BL/6 female mice (Figure 5)

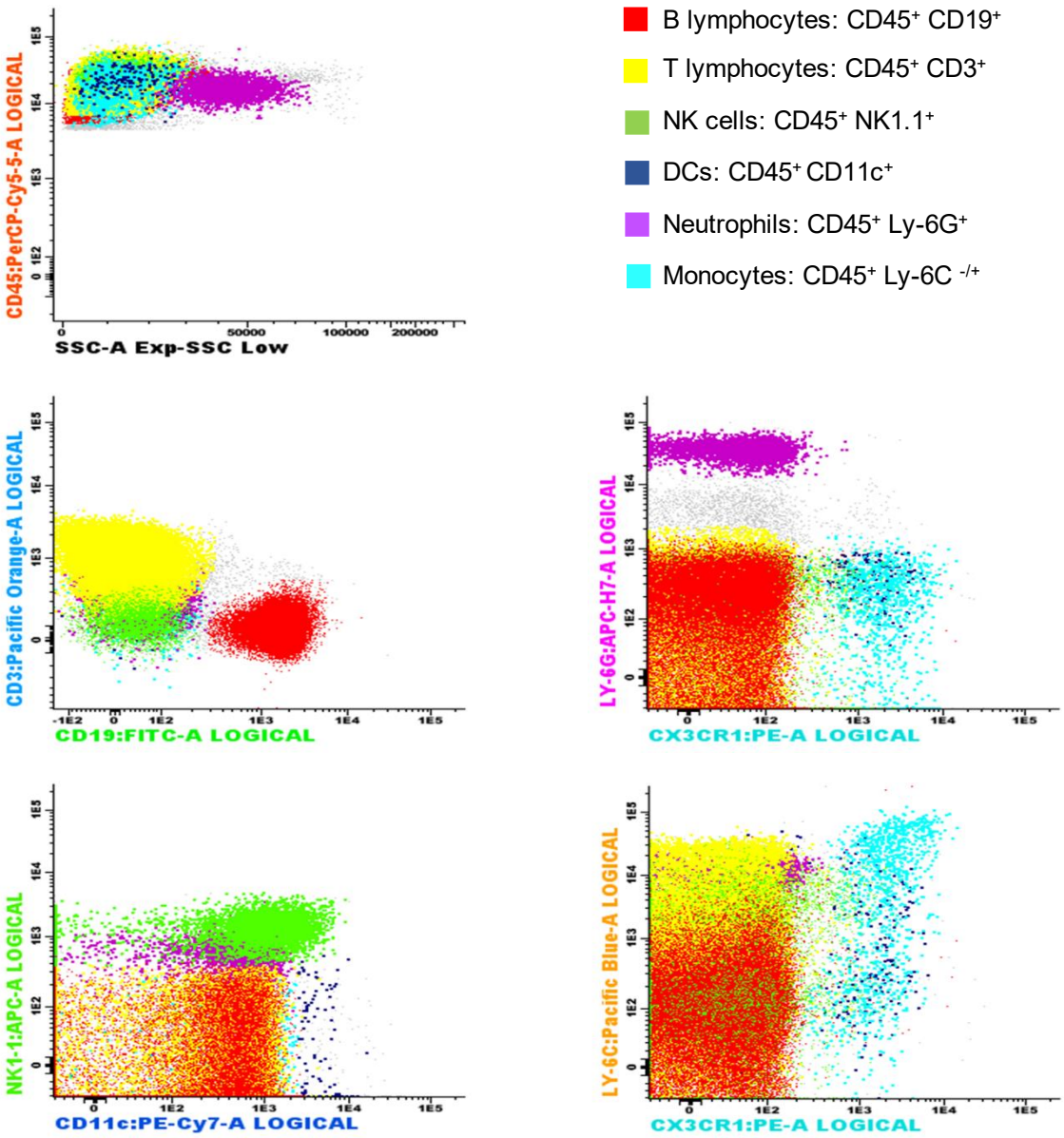

Figure 5. Representation of immunophenotypical staining of immune cell populations in the peripheral blood of a WT C57BL/6 female mouse

1.2 Cell population frequency

Table 4. Average frequency of the immune cell populations in the peripheral blood of C57BL/6 mice.

B lymphocytes	T lymphocytes	NK cells	Neutrophils	Monocytes	DCs
53.30 ± 1.75 %	24.48 ± 7.45%	4.11 ± 1.37 %	5.35 ± 2.09 %	2.02 ± 0.75 %	0.27 ± 0.12 %

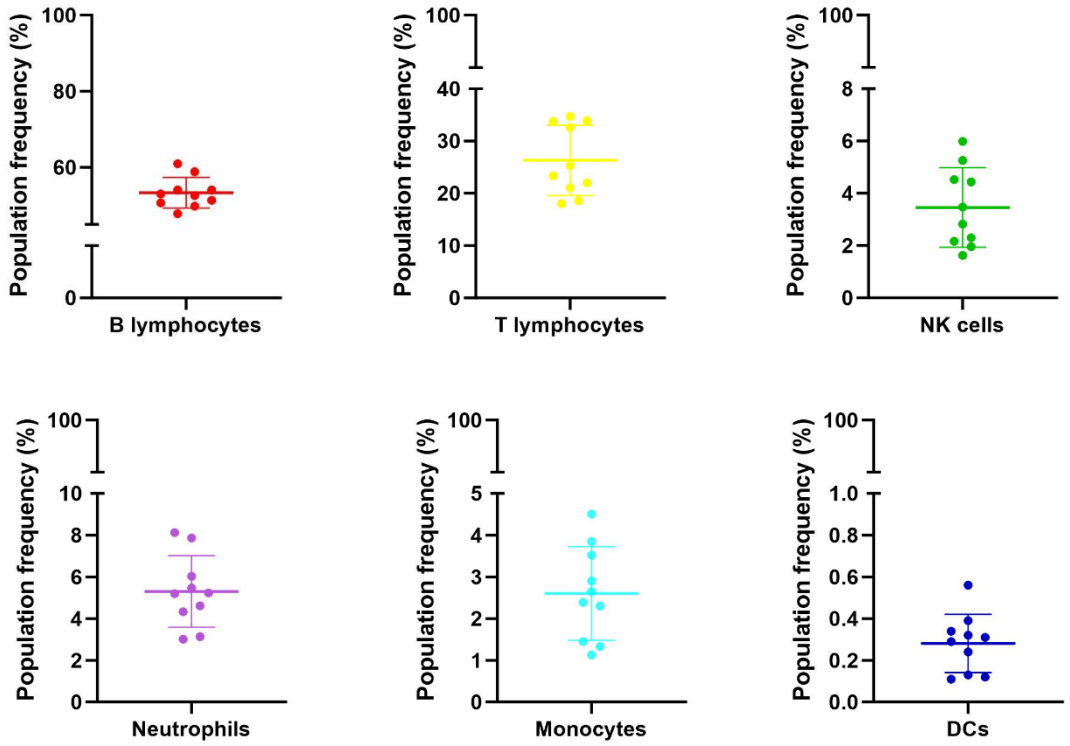

Figure 6. Representation of frequency of immune cell populations in the peripheral blood of C57BL/6 mice. Scatter plot represented by mean ± SD.

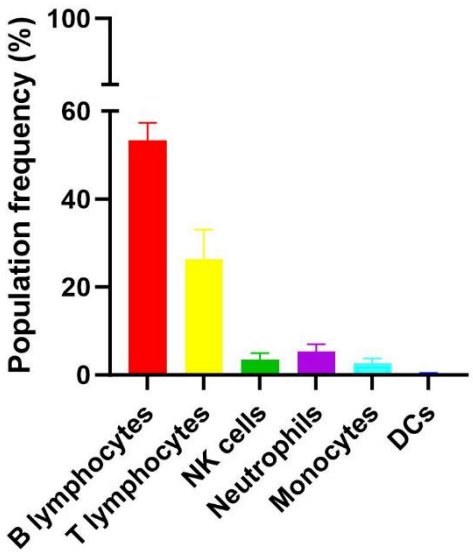

Figure 7. Overview representation of frequency of immune cell populations in the peripheral blood of C57BL/6 mice. Column bar graph represented by mean ± SD.

Out of the immune cell populations analyzed, lymphocytes represent the dominant population in the peripheral blood of C57BL/6 mice. Specifically, B lymphocytes are the most frequent population (53.30% ± 1.75%), followed by T lymphocytes (24.48% ± 7.45%). NK cells (4.11% ± 1.37%), neutrophils (5.35% ± 2.09%) and monocytes (2.02% ± 0.75%) constitute intermediate frequency cell populations in the peripheral blood of C57BL/6 mice, whereas DCs are the least frequent population (0,27% ± 0,12%) (Table 4, Figures 6. and 7.).

2. Pilot study II

2.1 Relative frequencies of lymphocytic populations

The distribution of T cell populations and subsets in right and left tumor, spleen and peripheral blood specimens of mice belonging to sham-treated group, imILT-treated group 1 and imILT-treated group 2 was assessed by flow cytometry.

Out of the T cell population ($CD3^+$ population), similar mean frequencies of $CD4^+$ and $CD8^+$ T cells were observed in the right tumor, spleen and peripheral blood of mice subjected to different treatments. In the left tumor of mice belonging to the imILT-treated group 2, significantly lower levels of $CD8^+$ T cells were observed, when compared to those of animals from imILT-treated group 1 (Figure 8., f.).

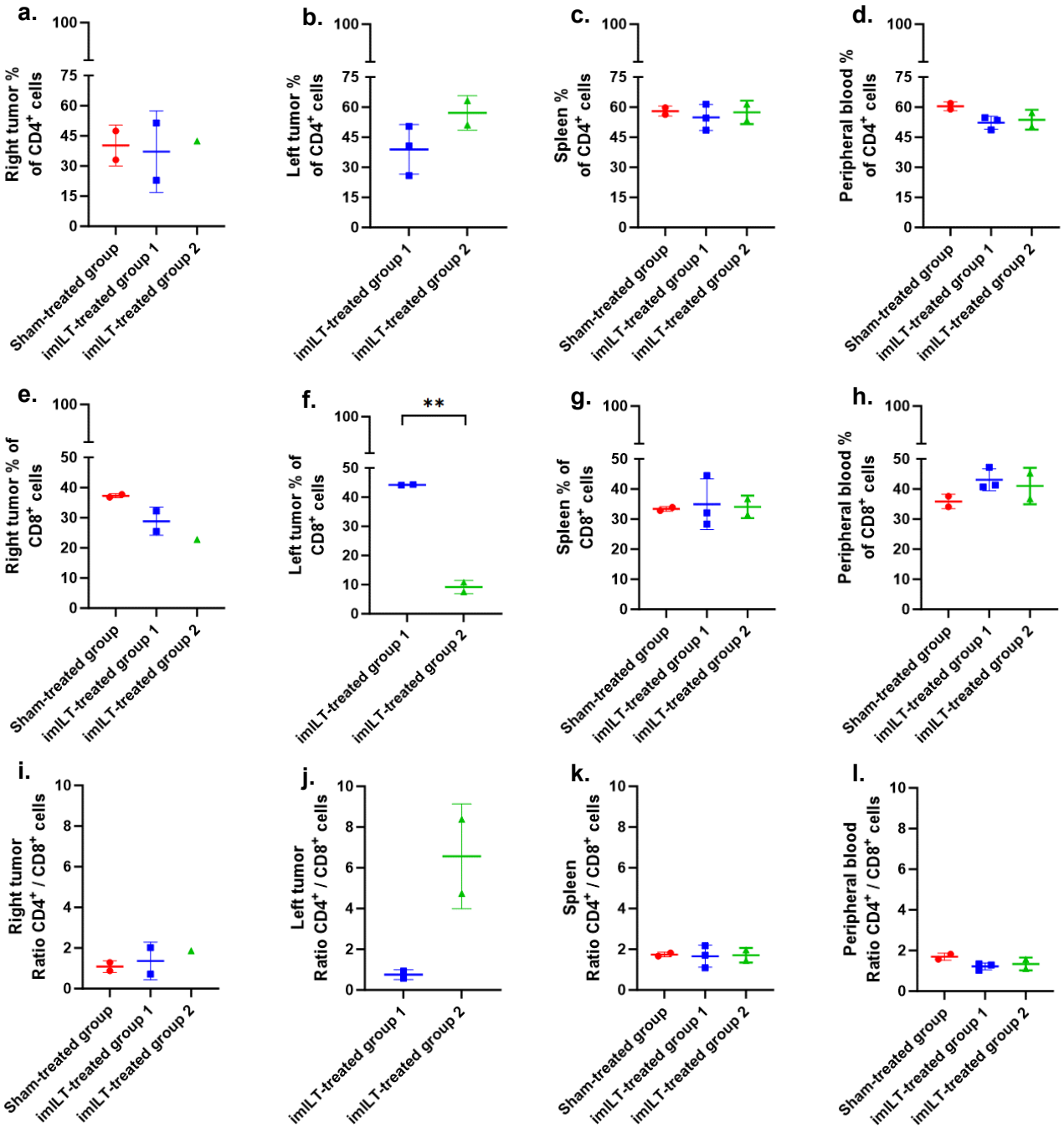

Figure 8. Flow cytometry analysis of T CD4⁺ cells, T CD8⁺ cells and T CD4⁺/CD8⁺ cell ratio (pilot study II). Percentage of T CD4⁺ cells (CD3⁺ CD4⁺ CD8⁻) (**a.- d.**), CD8+ cells (CD3⁺ CD8⁺ CD4⁻) (**e.- h.**) and CD4⁺/CD8⁺ T cell ratio (**i.- l.**) in the right tumor, left tumor, spleen, and peripheral blood of animals in the different treatment groups. Results are shown as a percentage of the total T cells (CD3⁺).
Scatter plots represented by mean ± SD. **p<0.01. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

As shown in Figure 9., T_{Reg} cells ($CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^+$ population or $CD3^+$ $CD8^+$ $CD4^-$ $CD25^+$ $Foxp3^+$ population) represent a small subset of $CD4^+$ or $CD8^+$ T cells in tumor, spleen, and peripheral blood specimens. Overall, similar levels of $CD4^+$ and $CD8^+$ T_{Reg} cells were observed in samples of mice across all treatment groups, except for a statistically significant increase in $CD4^+$ T_{Reg} cells in the right tumor and spleen of mice from imILT-treated group 2, when compared to mice belonging to the sham-treated group (Figure 9., a. and c.).

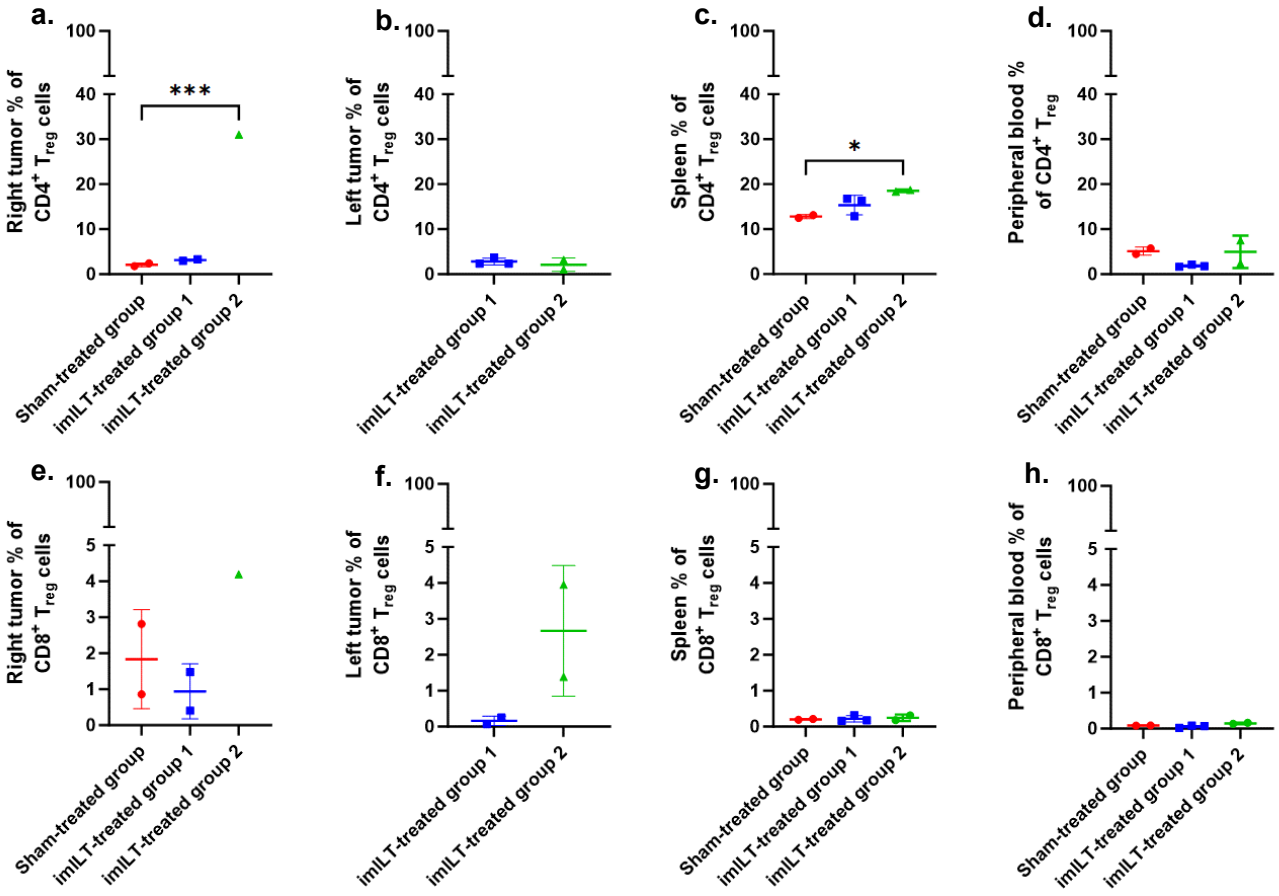

Figure 9. Flow cytometry analysis of $CD4^+$ T_{Reg} and $CD8^+$ T_{Reg} cells (pilot study II).
Percentage of $CD4^+$ T_{Reg} cells ($CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^+$) (**a.- d.**) and $CD8^+$ T_{Reg} cells ($CD3^+$ $CD8^+$ $CD4^-$ $CD25^+$ $Foxp3^+$) (**e.- h.**) in the right tumor, left tumor, spleen, and peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively.
Scatter plots represented by mean ± SD. *p<0.05; ***p<0.001. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

$CD4^+$ and $CD8^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^-$ $CX3CR1^+$ population or $CD3^+$ $CD8^+$ $CD4^-$ $CX3CR1^+$ population) were more prevalent in the right and left tumors of mice from all three treatment groups, while representing a residual population in the spleen and peripheral blood (Figure 10.). A statistically significant increase in $CD4^+$ memory T cells was observed in the left tumor of imILT-treated group 2 mice, when compared to imILT-treated group 1 mice (Figure 10., b.).

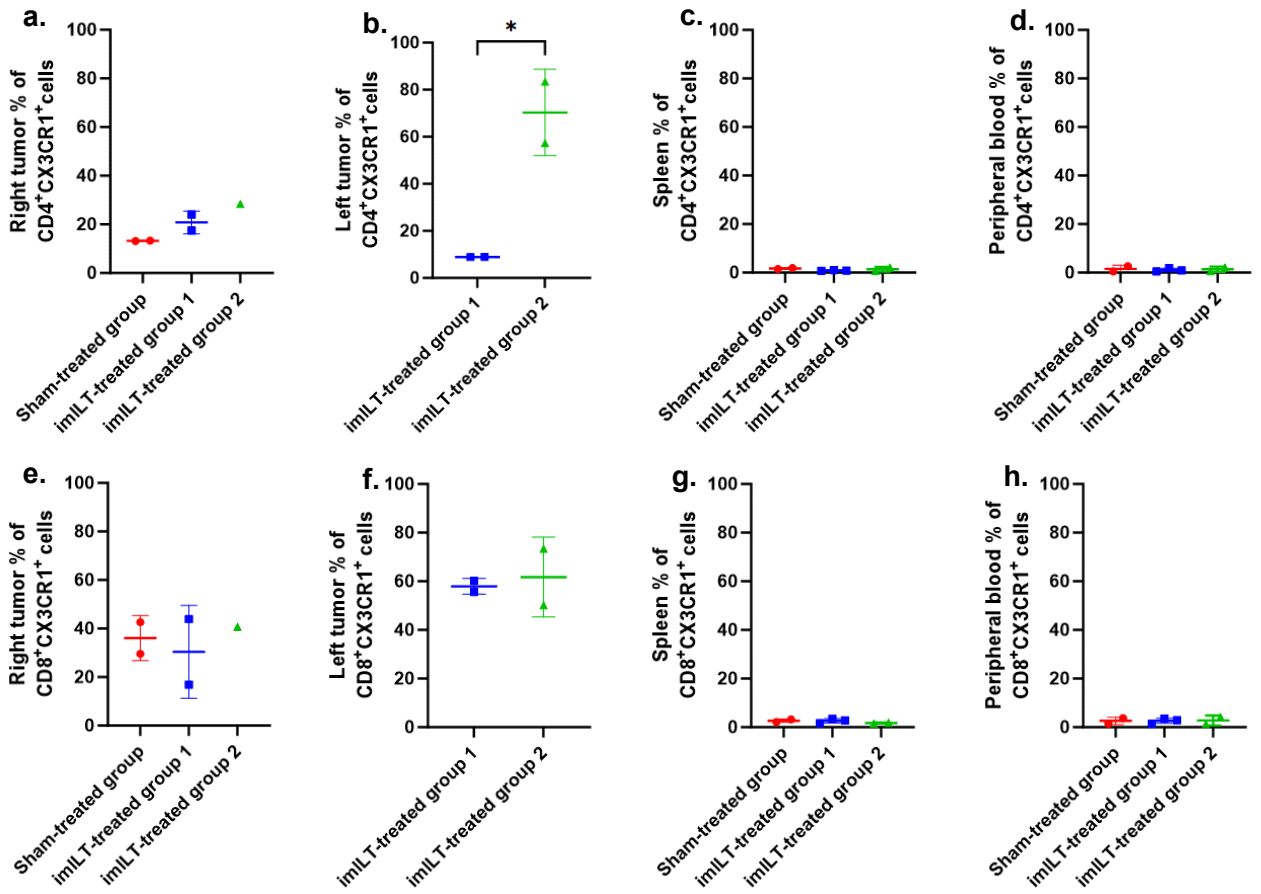

Figure 10. Flow cytometry analysis of $CD4^+$ memory T cells and $CD8^+$ memory T cells (pilot study II). Percentage of $CD4^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^-$ $CX3CR1^+$) (**a.**- **d.**) and $CD8^+$ memory T cells ($CD3^+$ $CD8^+$ $CD4^-$ $CX3CR1^+$) (**e.**- **h.**) in the right tumor, left tumor, spleen and peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively.
Scatter plots represented by mean ± SD. *$p<0.05$. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

$CD4^+$ $CD8^+$ T cells ($CD3^+$ $CD4^+$ $CD8^+$ population) were found as a small subset of the T cell population, representing less than 3% of the total of T cells in the tumors, spleens and peripheral blood of mice belonging to all treatment groups. $CD4^-$ $CD8^-$ T cells ($CD3^+$ $CD4^-$ $CD8^-$ population) were represented at higher levels, when compared to $CD4^+$ $CD8^+$ T cells, in the tumors. However, no significant differences were observed between mice across treatment groups (Figure 11.).

When gating specifically on the $CD4^+$ $CD8^+$ or $CD4^-$ $CD8^-$ T cells, it was found that a big percentage of those cells were $CD4^+$ $CD8^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^+$ $CX3CR1^+$ population) or $CD4^-$ $CD8^-$ memory T cells ($CD3^+$ $CD4^-$ $CD8^-$ $CX3CR1^+$ population) (Figure 12.). A significant increase in $CD4^+$ $CD8^+$ memory T cells was observed in the peripheral blood of imILT-treated group 2 mice, when compared to sham-treated group or imILT-treated group 1 mice (Figure 12., d.).

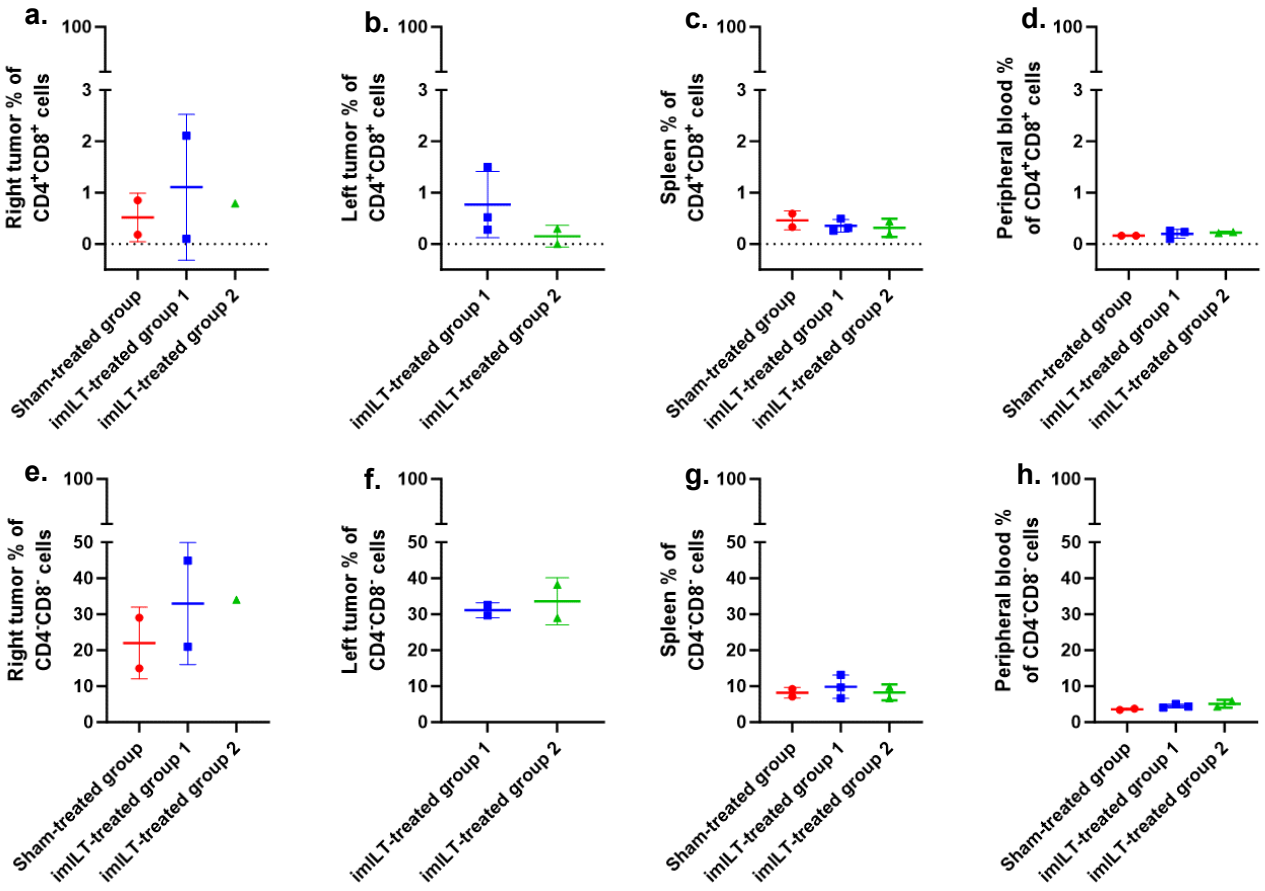

Figure 11. Flow cytometry analysis of $CD4^+$ $CD8^+$ T cells and $CD4^-$ $CD8^-$ T cells (pilot study II).
Percentage of $CD4^+$ $CD8^+$ T cells ($CD3^+$ $CD4^+$ $CD8^+$) (**a.**- **d.**) and $CD4^-$ $CD8^-$ T cells ($CD3^+$ $CD4^-$ $CD8^-$) (**e.**- **h.**) in the right tumor, left tumor, spleen and peripheral blood of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

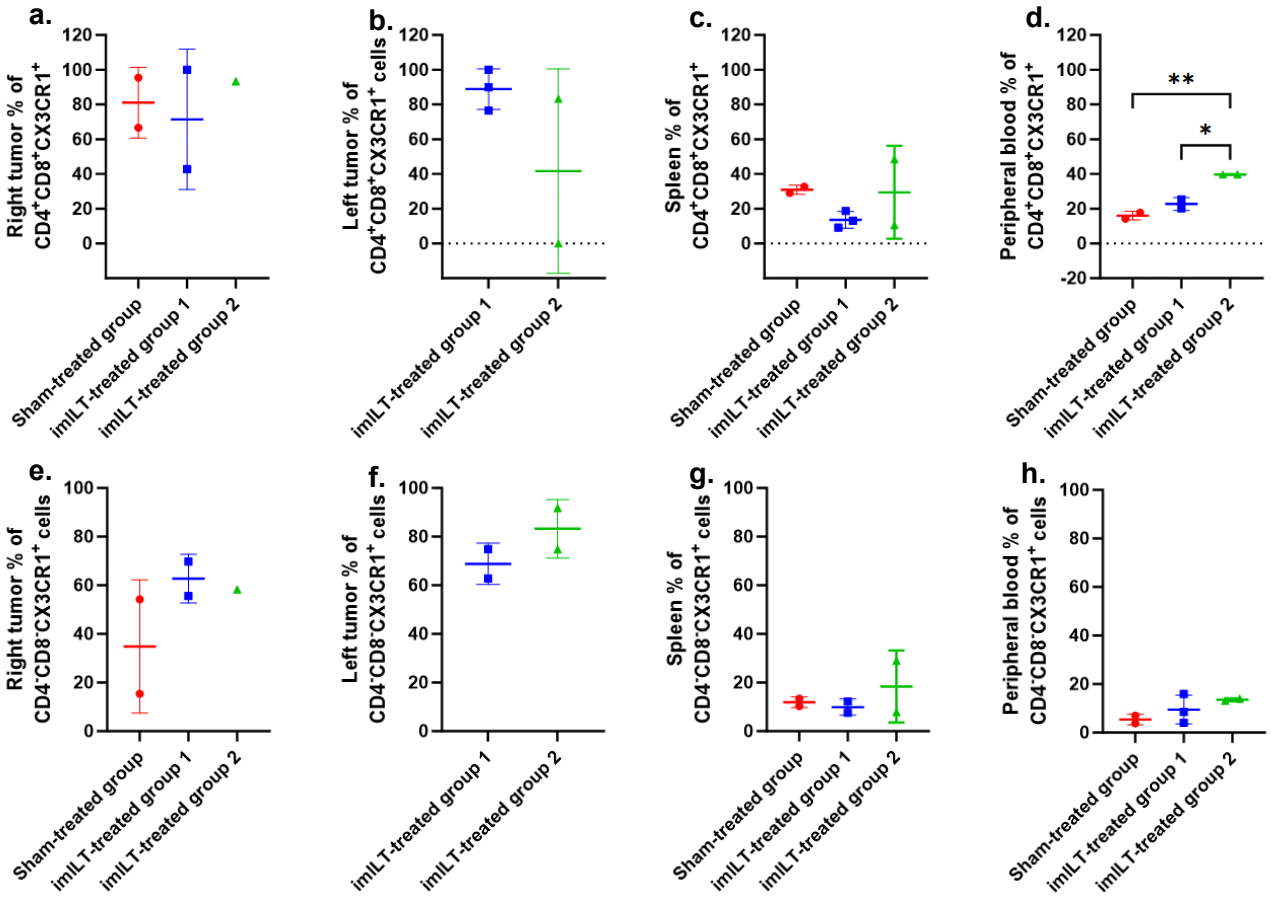

Figure 12. Flow cytometry analysis of $CD4^+$ $CD8^+$ memory T cells and $CD4^-$ $CD8^-$ memory T cells (pilot study II). Percentage of $CD4^+$ $CD8^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^+$ $CX3CR1^+$) (**a.**- **d.**) and $CD4^-$ $CD8^-$ memory T cells ($CD3^+$ $CD4^-$ $CD8^-$ $CX3CR1^+$) (**e.**- **h.**) in the right tumor, left tumor, spleen and peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ $CD8^+$ ($CD3^+$ $CD4^+$ $CD8^+$) or T $CD4^-$ $CD8^-$ ($CD3^+$ $CD4^-$ $CD8^-$) cells, respectively.
Scatter plots represented by mean ± SD. *p<0.05; **p<0.01. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

2.2 Relative frequencies of myeloid populations

The myeloid populations' distribution, namely M1 and M2 macrophages in the tumors and the spleens, and M1, M2 or intermediate monocytes in peripheral blood, was also evaluated by flow cytometry.

M1 ($F4/80^+$ $CX3CR1^-$ population) and M2 ($F4/80^+$ $CX3CR1^+$ population) macrophages were almost evenly distributed in the right tumors of mice from all treatment groups. However, some variability was found in mice belonging to the imILT-treated group 1. In the left tumor, there was a significant decrease in the frequency of M1 macrophages, and, accordingly, an increase in the frequency of M2 macrophages in imILT-treated group 2 mice, in comparison with imILT-treated group 1 mice. In the spleens, there was a higher frequency of M1 macrophages in imILT-treated groups 1 and 2, while in peripheral blood intermediate monocytes (LY-$6C^+$ $CX3CR1^{+/-}$ population) represented the majority of the monocyte population (Figure 13.).

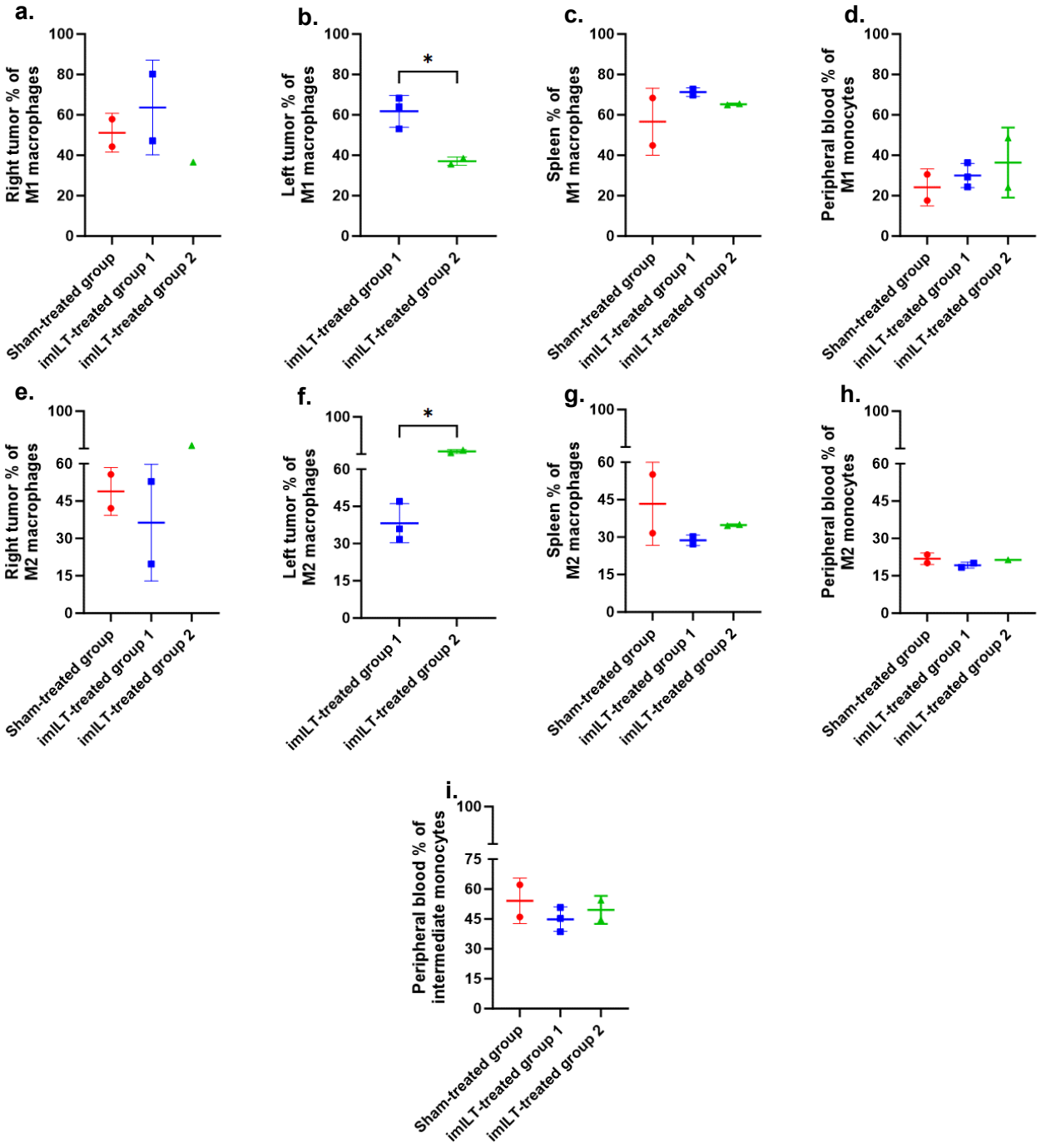

Figure 13. Flow cytometry analysis of M1 and M2 macrophages, and M1, M2 and intermediate monocytes (pilot study II).

Percentage of M1 macrophages (F4/80^{+} CX3CR1^{-}) (**a.**- **c.**) and M2 macrophages (F4/80^{+} CX3CR1^{+}) (**e.**- **g.**) in the right tumor, left tumor and spleen of animals in the different treatment groups, and percentage of M1 monocytes (Ly-6C^{+} CX3CR1^{-}) (**d.**), M2 monocytes (LY-6C^{-} CX3CR1^{+}) (**h.**) and intermediate monocytes (LY-6C^{+} CX3CR1$^{+/-}$) (**i.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total macrophages (F4/80^{+}) or monocytes.

Scatter plots represented by mean ± SD. *p<0.05. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

3. Pilot study III

3.1 Relative frequencies of lymphocytic populations

The expression of T cells and T cell subsets was assessed by immunophenotyping in tumor, spleen and peripheral blood specimens of mice belonging to the different Pilot III treatment groups: rat IgG2a at 2,5 mg/kg or 5,0 mg/kg, anti-mouse PD-1 at 2,5 mg/kg or 5,0 mg/kg, hamster IgG1 at 2,5 mg/kg or 5,0 mg/kg, and anti-mouse CTLA-4 at 2,5 mg/kg or 5,0 mg/kg.

In tumors, there was a significant increase in the frequency of $CD8^+$ T cells in the groups treated with the highest concentration of anti-mouse PD-1 antibody and anti-mouse CTLA-4 antibody, when compared to the respective isotypic controls at the same concentration, resulting in a decrease in the $CD4^+/CD8^+$ T cell ratio (Figure 14.)

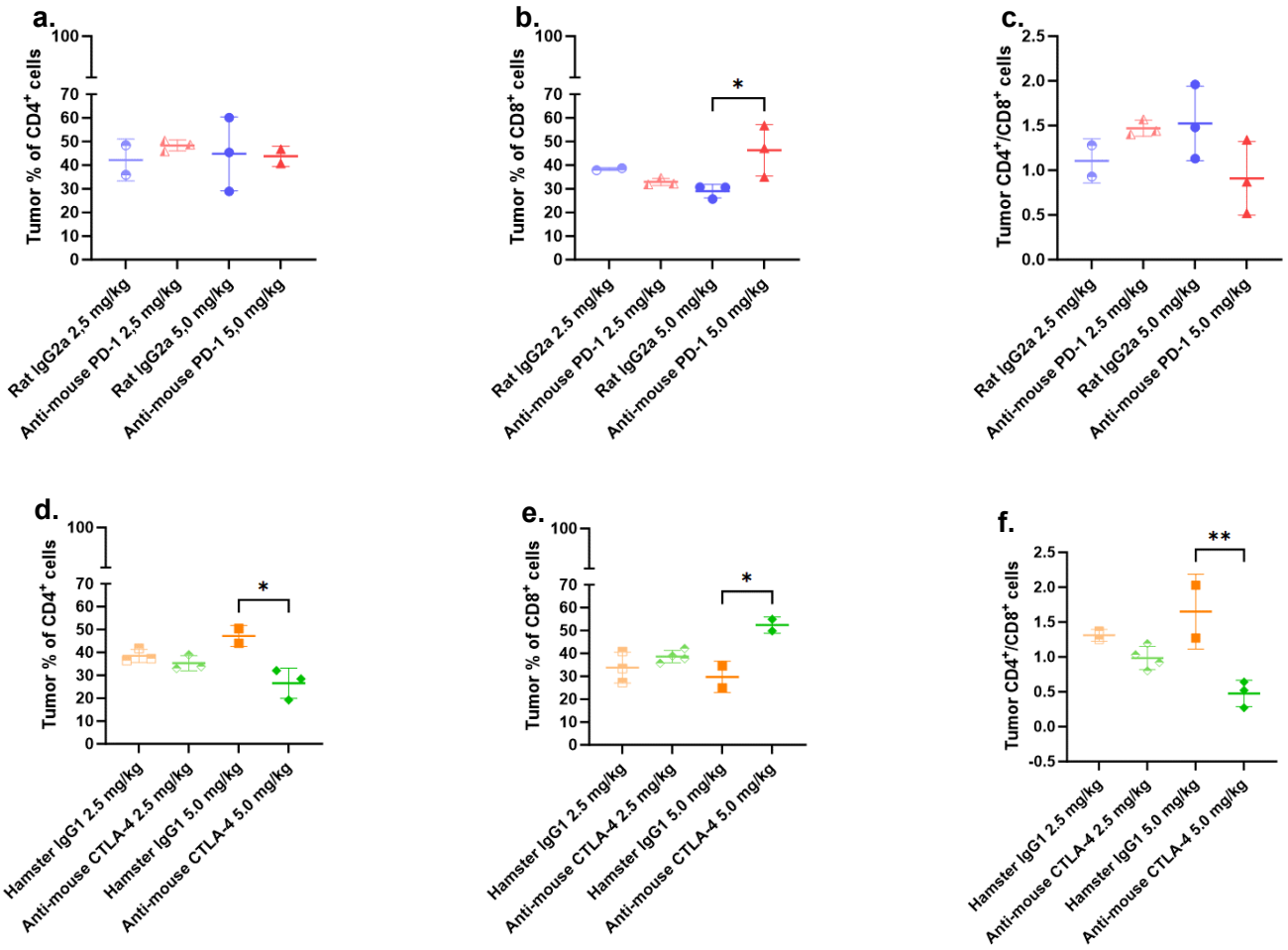

Figure 14. Flow cytometry analysis of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ T cell ratio in the tumor (pilot study III).
Percentage of $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD8^-$) (**a.** and **d.**), $CD8^+$ T cells ($CD3^+$ $CD8^+$ $CD4^-$) (**b.** and **e.**) and $CD4^+/CD8^+$ T cell ratio (**c.** and **f.**) in the tumor of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *p<0.05, **p<0.01. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

In the spleen, the $CD4^+$ T cell subset represented the majority of the total T cell population in mice across all eight treatment groups, resulting in a subsequent $CD4^+/CD8^+$ T cell ratio above one. However, the levels of both $CD4^+$ T cells and $CD8^+$ T cells were similar between treatment groups, with the exception of a significant increase in $CD8^+$ T cell population in the spleen of mice treated with 2,5 mg/kg of anti-mouse CTLA-4, when compared to the respective control (Figure 15.).

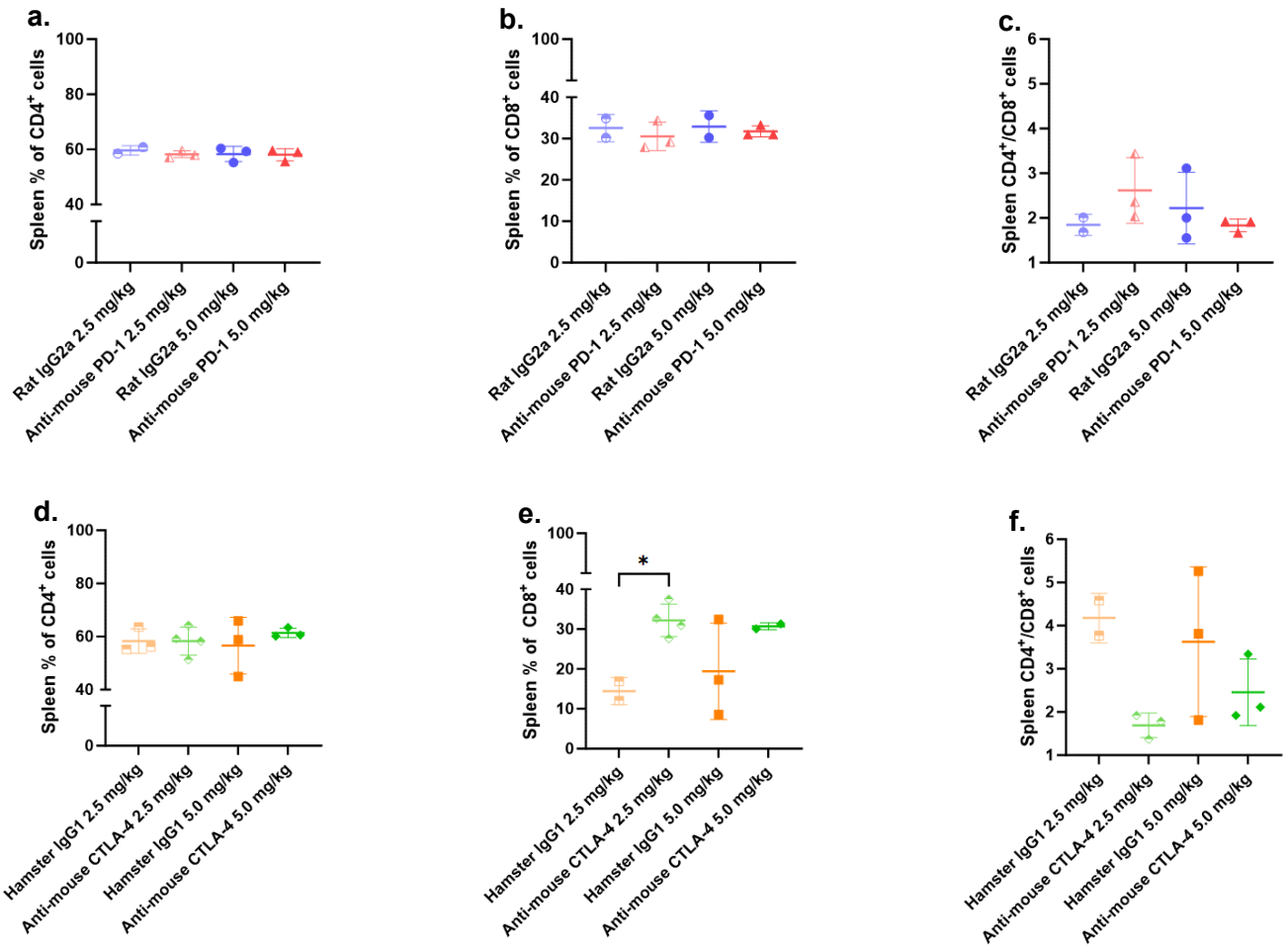

Figure 15. Flow cytometry analysis of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ T cell ratio in the spleen (pilot study III).
Percentage of $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD8^-$) (**a.** and **d.**), $CD8^+$ T cells ($CD3^+$ $CD8^+$ $CD4^-$) (**b.** and **e.**) and $CD4^+/CD8^+$ T cell ratio (**c.** and **f.**) in the spleen of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *p<0.05. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

In peripheral blood specimens, slightly higher levels of $CD4^+$ T cells were observed, in comparison to $CD8^+$ T cells, resulting in a $CD4^+/CD8^+$ T cell ratio between one and two. Nonetheless, no significant differences in $CD4^+$ and $CD8^+$ expression were observed between mice of different treatment groups (Figure 16.).

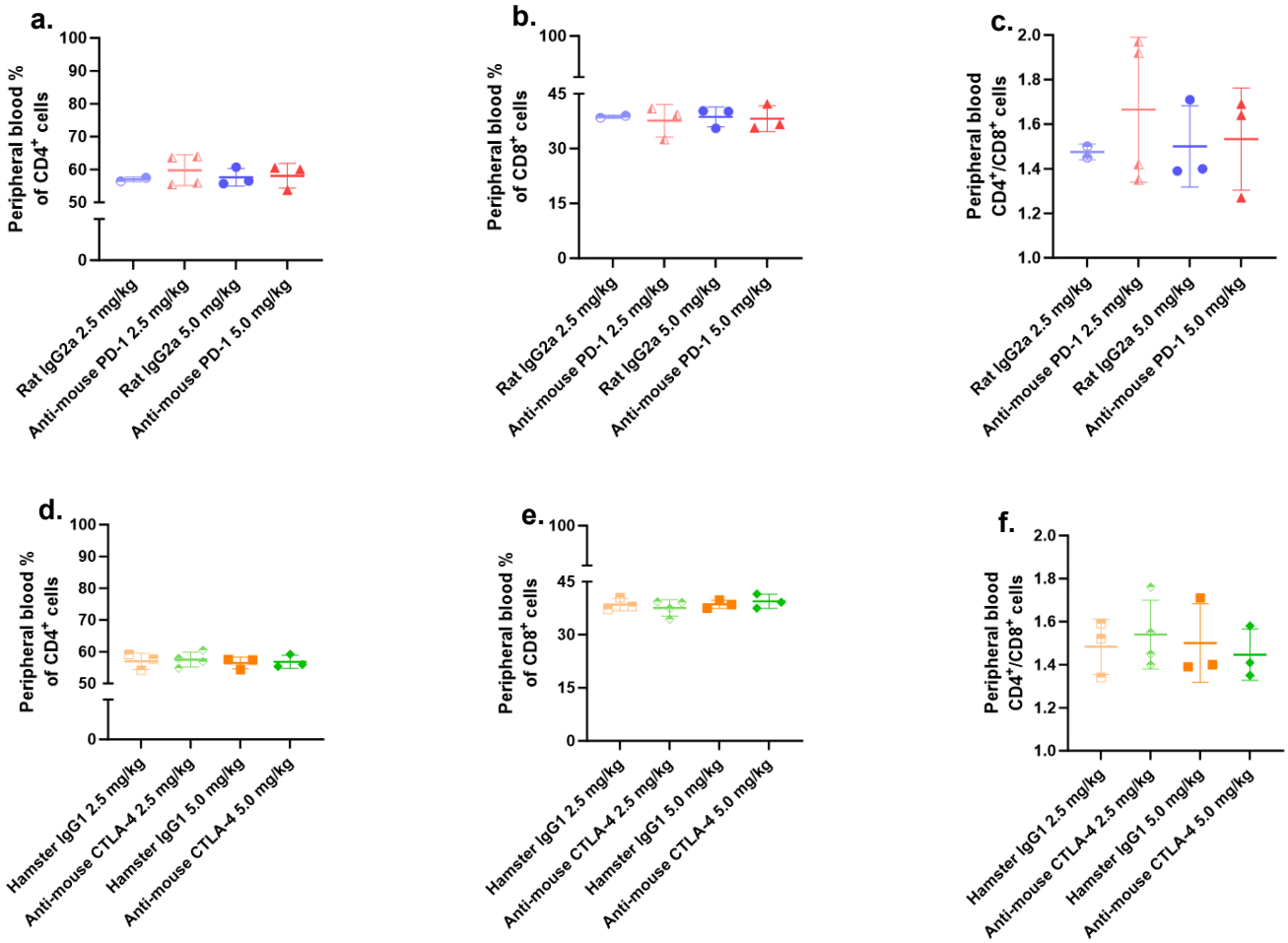

Figure 16. Flow cytometry analysis of $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ T cell ratio in the peripheral blood (pilot study III).
Percentage of $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD8^-$) (**a.** and **d.**), $CD8^+$ T cells ($CD3^+$ $CD8^+$ $CD4^-$) (**b.** and **e.**) and $CD4^+/CD8^+$ T cell ratio (**c.** and **f.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

Out of the $CD4^+$ T cell population in the tumor, up to 20% were $CD4^+$ T_{Reg} cells (Figure 17., a. and b.), whereas up to 40%, in some treatment groups, were $CD4^+$ memory T cells (Figure 18., a. and b.). A statistically significant decrease in the frequency of $CD4^+$ T_{Reg} cells was observed for mice treated with anti-mouse PD-1 antibody at 2,5 mg/kg, in comparison to the respective control. Besides, there was a tendency for a decrease in the expression of $CD4^+$ T_{Reg} cells in the tumor of mice belonging to anti-mouse CTLA-4 2,5 mg/kg or 5,0 mg/kg treatment groups, when compared to the isotypic controls administered in the same dosages. Regarding $CD4^+$ memory T cell frequency in the tumor, there was a statistically significant decrease for mice in the anti-mouse PD-1 5,0 mg/kg and anti-mouse CTLA-4 2,5 mg/kg treatment groups, when compared to their respective controls.

Concerning $CD8^+$ T cells, it was observed that only a small percentage of those are $CD8^+$ T_{Reg} cells (Figure 17., c. and d.), while $CD8^+$ memory T cells are represented at higher frequencies (Figure 18. c. and d.), for all treatment groups. No significant differences between treatment groups were observed for these two subsets of $CD8^+$ T cells, although there seemed to be a tendency for a decrease in the frequency of $CD8^+$ T_{Reg} cells in mice treated with anti-mouse PD-1 or anti-mouse CTLA-4 antibodies.

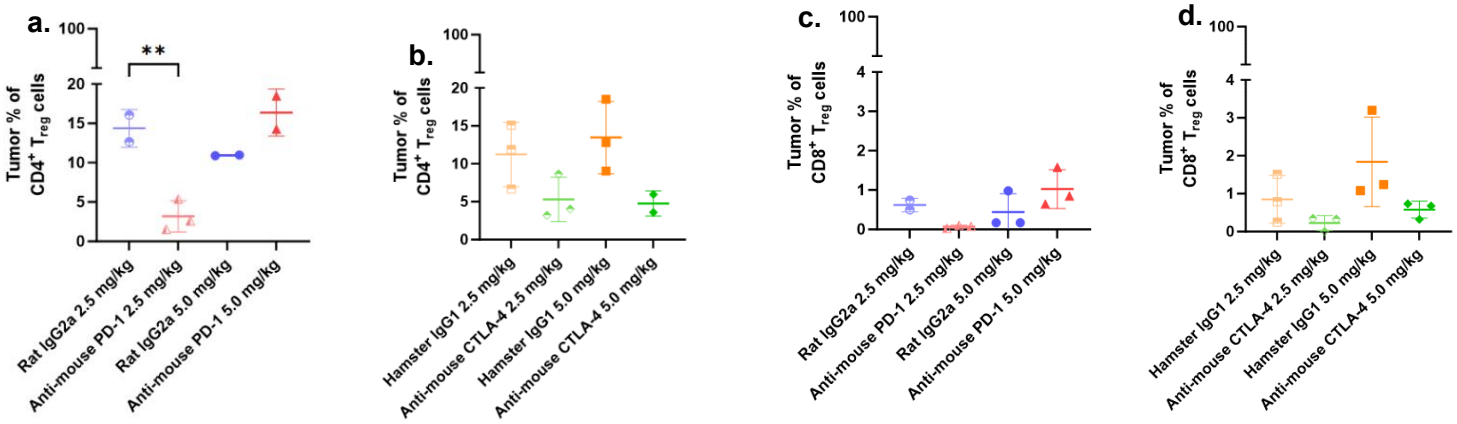

Figure 17. Flow cytometry analysis of $CD4^+$ T_{reg} cells and $CD8^+$ T_{reg} cells in the tumor (pilot study III). Percentage of $CD4^+$ T_{reg} cells ($CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^+$) (**a.** and **b.**) and percentage of $CD8^+$ T_{reg} cells ($CD3^+$ $CD8^+$ $CD4^-$ $CD25^+$ $Foxp3^+$) (**c.** and **d.**) in the tumor of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively. Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

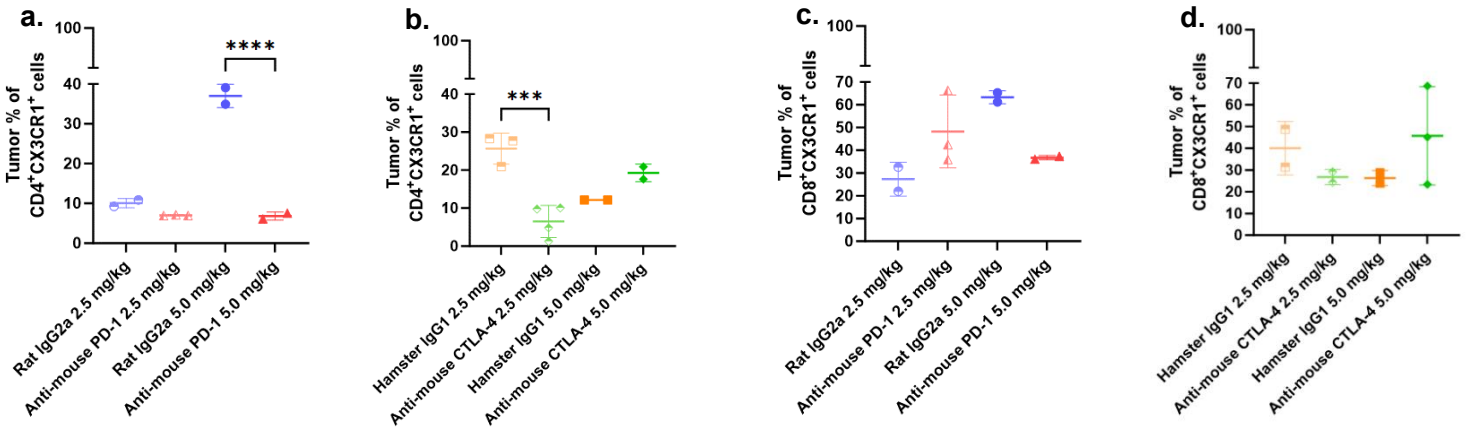

Figure 18. Flow cytometry analysis of CD4⁺ memory T cells and CD8⁺ memory T cells in the tumor (pilot study III).
Percentage of CD4⁺ memory T cells (CD3⁺ CD4⁺ CD8⁻ CX3CR1⁺) (**a.** and **b.**) and CD8⁺ memory T cells (CD3⁺ CD8⁺ CD4⁻ CX3CR1⁺) (**c.** and **d.**) in the tumor of animals in the different treatment groups. Results are shown as a percentage of total T CD4⁺ (CD3⁺ CD4⁺ CD8⁻) or T CD8⁺ (CD3⁺ CD8⁺ CD4⁻) cells, respectively.
Scatter plots represented by mean ± SD. ***p<0.001, ****p<0.0001. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

When analyzing the CD4⁺ T cell population in the spleen of the mice, it was found that less than 15% of those were CD4⁺ T_{Reg} cells (Figure 19., a. and b.), and less than 30% were CD4⁺ memory T cells (Figure 20, a. and b.). Overall, no significant differences in the frequency of CD4⁺ T_{Reg} cells and CD4⁺ memory T cells were found in the groups treated with anti-mouse PD-1 antibody or controls, but there was a statistically significant increase in CD4⁺ T_{Reg} cell population in the spleen of mice treated with 5,0 mg/kg of anti-mouse CTLA-4 antibody, and a decrease in CD4⁺ memory T cells in the same treatment group.

As for the CD8⁺ T_{Reg} cells in the spleen, they represented a residual percentage of the total CD8⁺ T cells (Figure 19., c. and d.), while the CD8⁺ memory T cells were represented at a higher percentage, but still less than 20% of the total CD8⁺ T cells in all treatment groups (Figure 20, c. and d.). The frequency of CD8⁺ T_{Reg} cells and CD8⁺ memory T cells remained similar across treatment groups in mice treated with anti-mouse PD-1 antibody and respective isotypes. Regarding anti-mouse CTLA-4 treatment groups, there was a significant decrease in the frequency of CD8⁺ T_{Reg} cells in the anti-mouse CTLA-4 2,5 mg/kg treatment group, comparing to the isotype control.

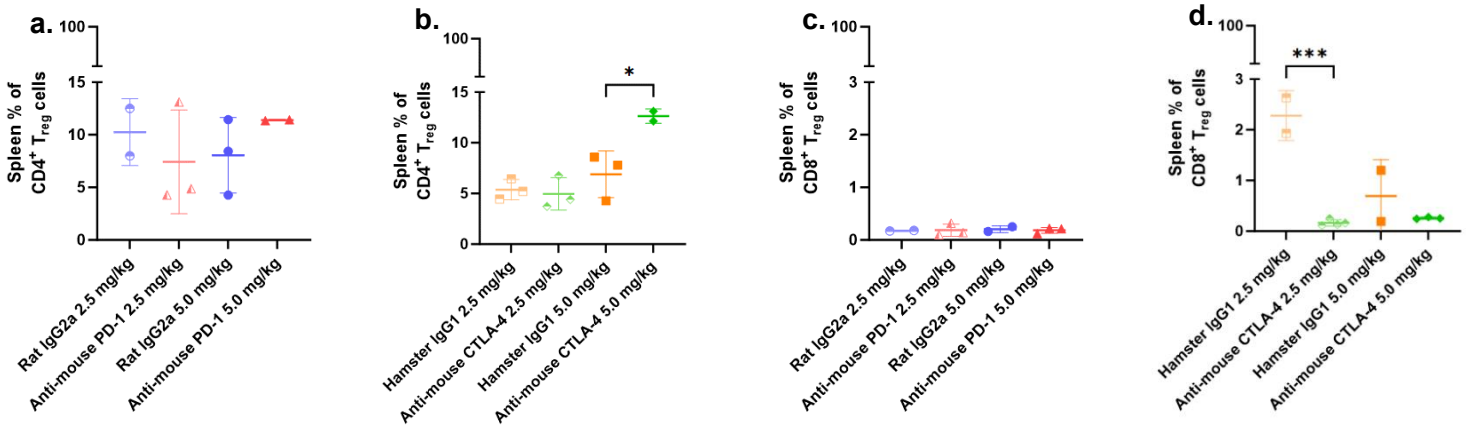

Figure 19. Flow cytometry analysis of $CD4^+$ T_{reg} cells and $CD8^+$ T_{reg} cells in the spleen (pilot study III).
Percentage of $CD4^+$ T_{reg} cells ($CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^+$) (**a.** and **b.**) and $CD8^+$ T_{reg} cells ($CD3^+$ $CD8^+$ $CD4^-$ $CD25^+$ $Foxp3^+$) (**c.** and **d.**) in the spleen of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively.
Scatter plots represented by mean ± SD. ***p<0.001. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

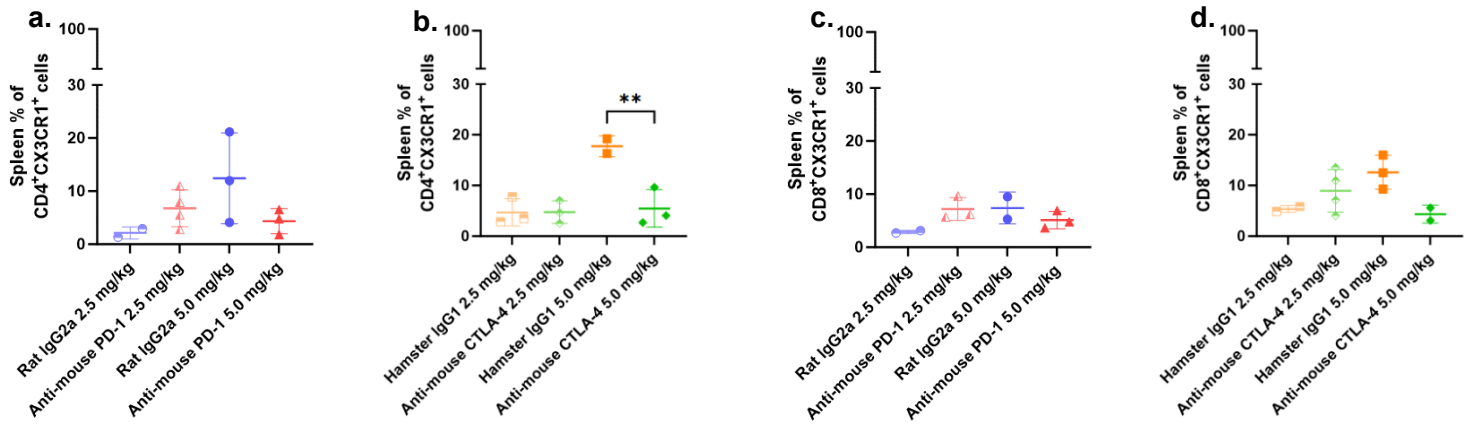

Figure 20. Flow cytometry analysis of $CD4^+$ memory T cells and $CD8^+$ memory T cells in the spleen (pilot study III).
Percentage of $CD4^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^-$ $CX3CR1^+$) (**a.** and **d.**) and $CD8^+$ memory T cells ($CD3^+$ $CD8^+$ $CD4^-$ $CX3CR1^+$) (**c.** and **d.**) in the spleen of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively.
Scatter plots represented by mean ± SD. **p<0.01. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

Regarding $CD4^+$ or $CD8^+$ T_{Reg} cells (Figure 21.) and $CD4^+$ or $CD8^+$ memory T cells (Figure 22.) they all were represented at small frequencies in the peripheral blood of mice belonging to all treatment groups, and no statistical differences were observed between groups. Besides, variability between mice belonging to the same groups was observed.

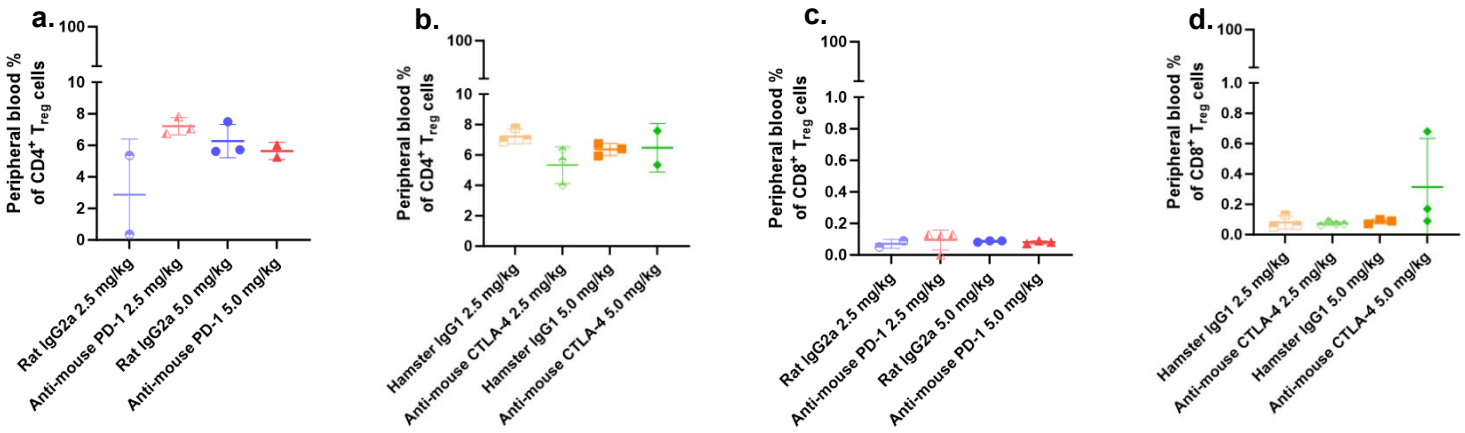

Figure 21. Flow cytometry analysis of $CD4^+$ T_{reg} cells and $CD8^+$ T_{reg} cells in the peripheral blood (pilot study III). Percentage of $CD4^+$ T_{reg} cells ($CD3^+$ $CD4^+$ $CD8^-$ $CD25^+$ $Foxp3^+$) (**a.** and **b.**) and percentage of $CD8^+$ T_{reg} cells ($CD3^+$ $CD8^+$ $CD4^-$ $CD25^+$ $Foxp3^+$) (**c.** and **d.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively.
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

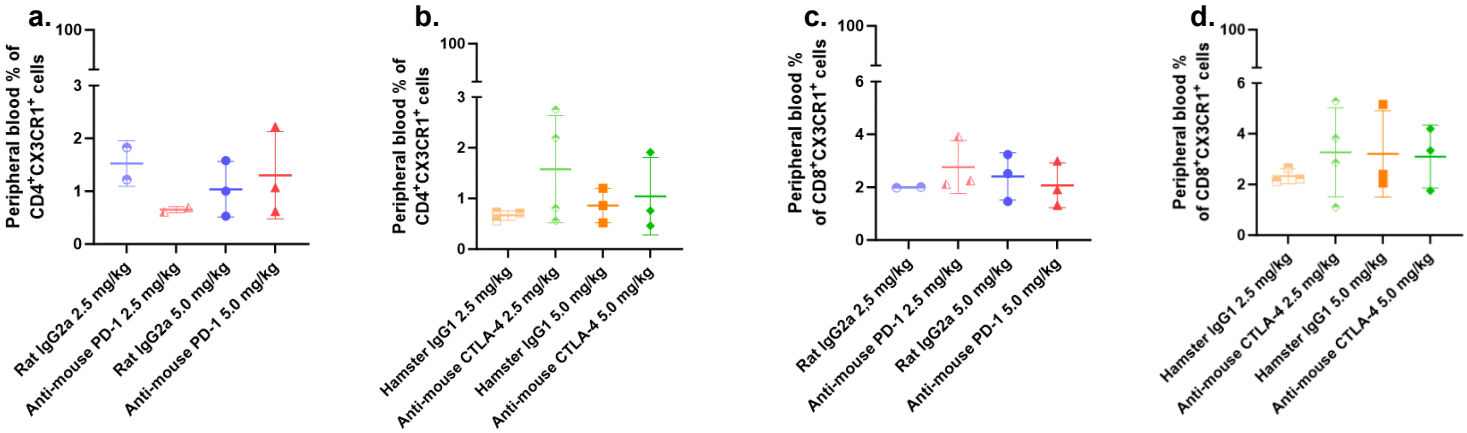

Figure 22. Flow cytometry analysis of $CD4^+$ memory T cells and $CD8^+$ memory T cells in the peripheral blood (pilot study III).
Percentage of $CD4^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^-$ $CX3CR1^+$) (**a.** and **b.**) and $CD8^+$ memory T cells ($CD3^+$ $CD8^+$ $CD4^-$ $CX3CR1^+$) (**c.** and **d.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ ($CD3^+$ $CD4^+$ $CD8^-$) or T $CD8^+$ ($CD3^+$ $CD8^+$ $CD4^-$) cells, respectively.
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.
groups.

$CD4^+$ $CD8^+$ T cells in the tumor of mice in the different treatment groups were represented at a maximum of 8% of the total T cells present in the tumor (Figure 23., a. and b.). A statistically significant decrease in the $CD4^+$ $CD8^+$ T cells was observed in the anti-mouse CTLA-4 2,5 mg/kg group, when compared to the isotype. Out of the total $CD4^+$ $CD8^+$ T cells, the majority of them seemed to be memory $CD4^+$ $CD8^+$ T cells, even though there was some variability in mice within the same group, specifically for the anti-mouse PD-1 2,5 mg/kg and anti-mouse CTLA-4 2,5 mg/kg treatment groups (Figure 24., a. and b.).

$CD4^-$ $CD8^-$ T cells are more prevalent in the tumor of mice when compared to $CD4^+$ $CD8^+$ T cells, and a significant decrease was observed in the anti-mouse PD-1 5,0 mg/kg treatment group (Figure 23., c. and d.). As for the memory $CD4^-$ $CD8^-$ T cells, high variability between mice from the same groups was observed, especially in the anti-mouse PD-1 treatment groups and control groups (Figure 24., c. and d.).

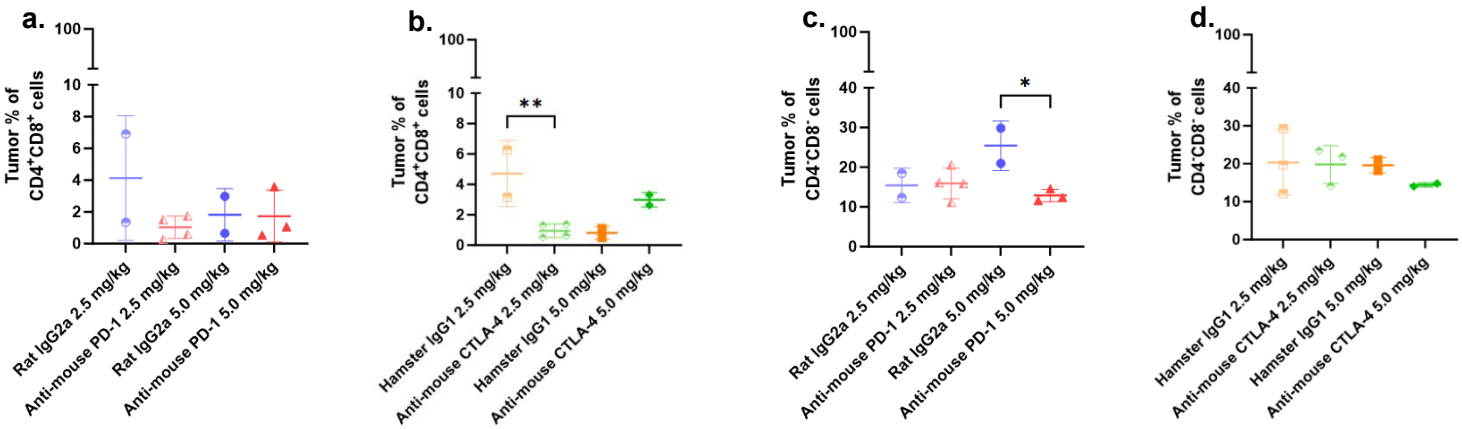

Figure 23. Flow cytometry analysis of $CD4^+$ $CD8^+$ T cells and $CD4^-$ $CD8^-$ T cells in the tumor (pilot study III). Percentage of $CD4^+$ $CD8^+$ T cells ($CD3^+$ $CD4^+$ $CD8^+$) (**a.** and **b.**) and $CD4^-$ $CD8^-$ T cells ($CD3^+$ $CD4^-$ $CD8^-$) (**c.** and **d.**) in the tumor of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *p<0.05, **p<0.01. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

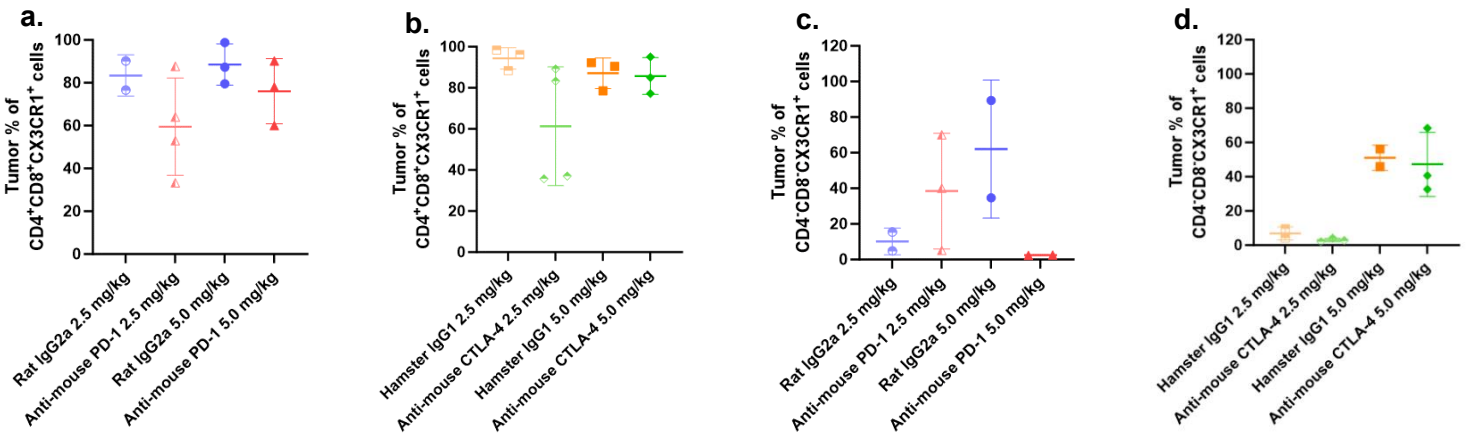

Figure 24. Flow cytometry analysis of $CD4^+$ $CD8^+$ memory T cells and $CD4^-$ $CD8^-$ memory T cells in the tumor (pilot study III).

Percentage of $CD4^+$ $CD8^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^+$ $CX3CR1^+$) (**a.** and **b.**) and $CD4^-$ $CD8^-$ memory T cells ($CD3^+$ $CD4^-$ $CD8^-$ $CX3CR1^+$) (**c.** and **d.**) in the tumor of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ $CD8^+$ ($CD3^+$ $CD4^+$ $CD8^+$) or T $CD4^-$ $CD8^-$ ($CD3^+$ $CD4^-$ $CD8^-$) cells, respectively.

Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

In the spleen, $CD4^+$ $CD8^+$ T cells were rare (less than 2%), while $CD4^-$ $CD8^-$ T cells were slightly more frequent (Figure 25.). A statistically significant increase was observed in the percentage of memory $CD4^+$ $CD8^+$ T cells in the anti-mouse PD-1 2,5 mg/kg treatment group, as well as a significant increase in the frequency of $CD4^-$ $CD8^-$ in the anti-mouse PD-1 2,5 mg/kg group, but a decrease in $CD4^-$ $CD8^-$ memory T cells in the anti-mouse CTLA-4 5,0 mg/kg treatment group (Figure 26.).

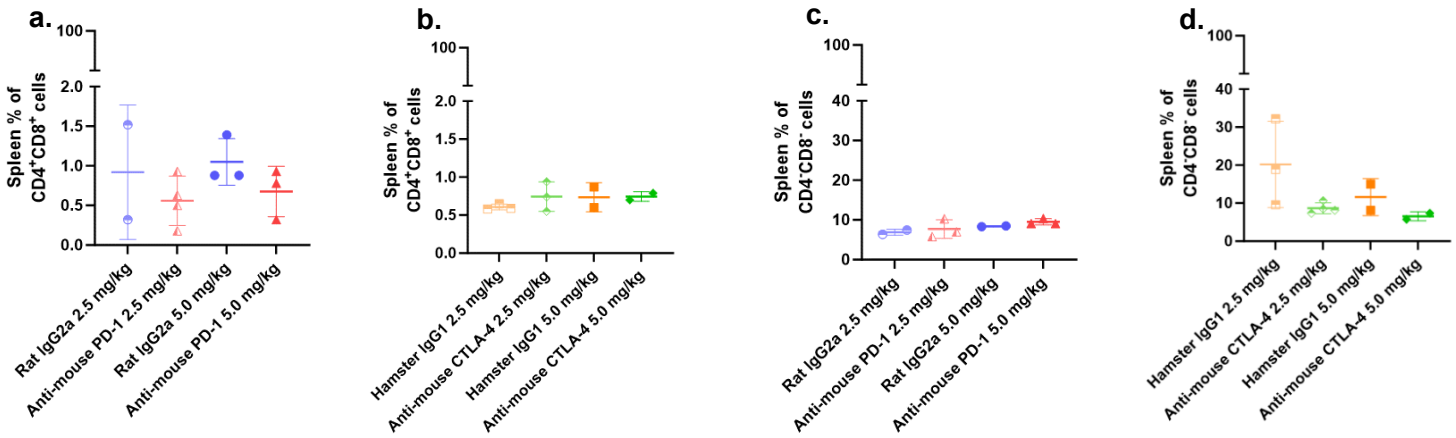

Figure 25. Flow cytometry analysis of $CD4^+$ $CD8^+$ T cells and $CD4^-$ $CD8^-$ T cells in the spleen (pilot study III). Percentage of $CD4^+$ $CD8^+$ T cells ($CD3^+$ $CD4^+$ $CD8^+$) (**a.** and **b.**) and $CD4^-$ $CD8^-$ T cells ($CD3^+$ $CD4^-$ $CD8^-$) (**c.** and **d.**) in the spleen of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

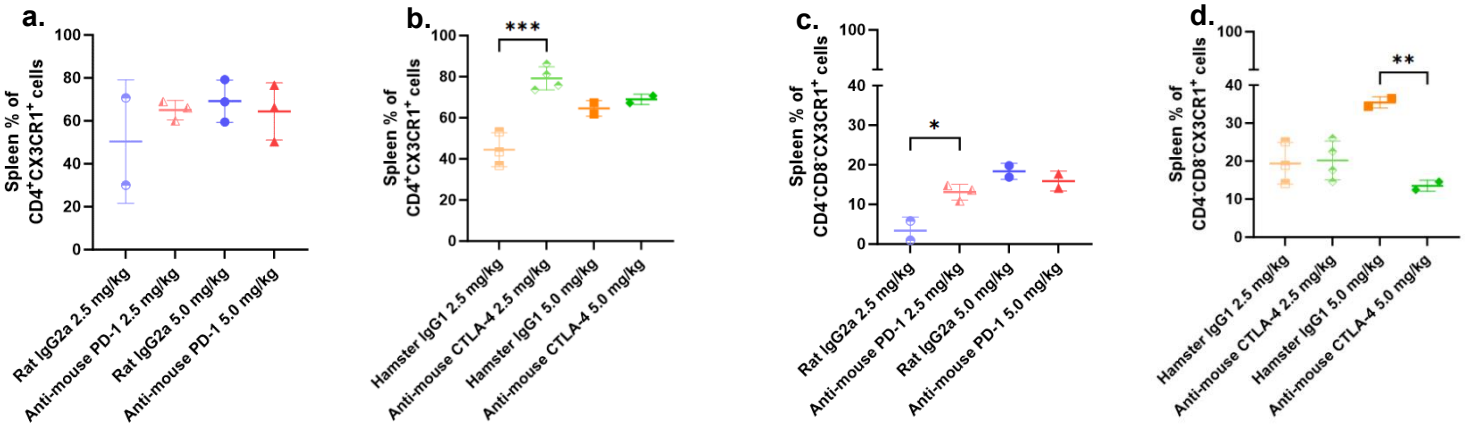

Figure 26. Flow cytometry analysis of $CD4^+$ $CD8^+$ memory T cells and $CD4^-$ $CD8^-$ memory T cells in the spleen (pilot study III).
Percentage of $CD4^+$ $CD8^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^+$ $CX3CR1^+$) (**a.** and **b.**) and $CD4^-$ $CD8^-$ memory T cells ($CD3^+$ $CD4^-$ $CD8^-$ $CX3CR1^+$) (**c.** and **d.**) in the spleen of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ $CD8^+$ ($CD3^+$ $CD4^+$ $CD8^+$) or T $CD4^-$ $CD8^-$ ($CD3^+$ $CD4^-$ $CD8^-$) cells, respectively.
Scatter plots represented by mean ± SD.*p<0.05, **p<0.01, ***p<0.001. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

In the peripheral blood specimens of mice belonging to all treatment groups, both $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^-$ T cells were present at low levels (Figure 27.). No statistical differences between groups were observed for the frequency of $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^-$ T cells, as well as $CD4^+$ $CD8^+$ memory T cells and $CD4^-$ $CD8^-$ memory T cells (Figure 28.).

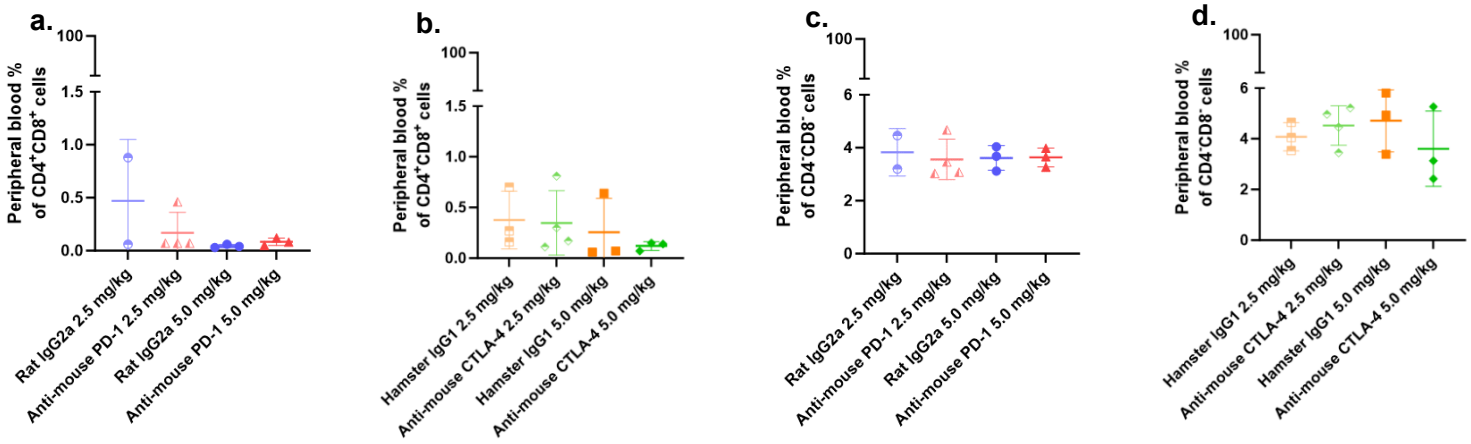

Figure 27. Flow cytometry analysis of $CD4^+$ $CD8^+$ T cells and $CD4^-$ $CD8^-$ T cells in peripheral blood (pilot study III).
Percentage of $CD4^+$ $CD8^+$ T cells ($CD3^+$ $CD4^+$ $CD8^+$) (**a.** and **b.**) and $CD4^-$ $CD8^-$ T cells ($CD3^+$ $CD4^-$ $CD8^-$) (**c.** and **d.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of the total T cells ($CD3^+$).
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

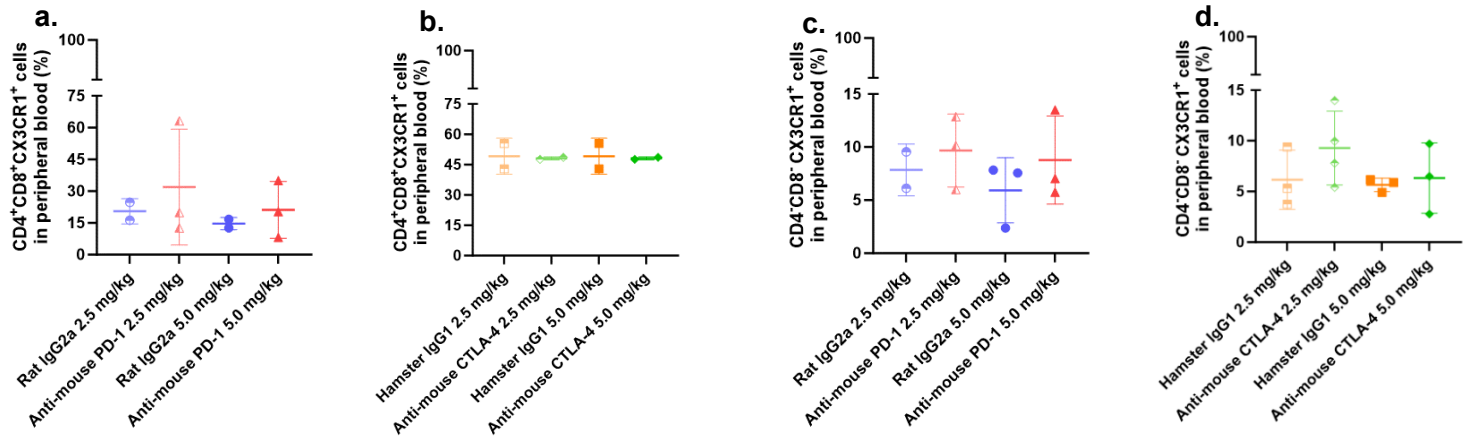

Figure 28. Flow cytometry analysis of $CD4^+$ $CD8^+$ memory T cells and $CD4^-$ $CD8^-$ memory T cells in peripheral blood (pilot study III).
Percentage of $CD4^+$ $CD8^+$ memory T cells ($CD3^+$ $CD4^+$ $CD8^+$ $CX3CR1^+$) (**a.** and **b.**) and $CD4^-$ $CD8^-$ memory T cells ($CD3^+$ $CD4^-$ $CD8^-$ $CX3CR1^+$) (**c.** and **d.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total T $CD4^+$ $CD8^+$ ($CD3^+$ $CD4^+$ $CD8^+$) or T $CD4^-$ $CD8^-$ ($CD3^+$ $CD4^-$ $CD8^-$) cells, respectively.
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

3.2 Relative frequencies of myeloid populations

The relative frequencies of myeloid populations, namely M1 and M2 macrophages, for tumor and spleen specimens, and M1, M2 and intermediate monocytes for peripheral blood specimens, were also evaluated in mice from Pilot study III treatment groups, by flow cytometry.

Regarding M1 and M2 macrophages in the tumor, M2 macrophages were more frequent than M1 macrophages in the anti-mouse PD-1 2,5 mg/kg group, and respective isotypic control at the same concentration, but the opposite was verified for the anti-mouse PD-1 5,0 mg/kg group and respective control, even though some variability within the anti-mouse PD-1 5,0 mg/kg group was observed. In the anti-mouse CTLA-4 treatment groups, a significant increase in M1 macrophages was observed for anti-CTLA-4 2,5 mg/kg treatment group, and a corresponding decrease in M2 macrophages was observed in the same treatment group (Figure 29.).

In the spleen specimens, higher frequencies of M2 macrophages were observed for the rat IgG2 control groups, and higher frequencies of M1 macrophages were observed for anti-mouse PD-1 treatment groups: a statistically significant increase in M1 macrophages frequency was detected in both anti-mouse PD-1 treatment groups when compared to the respective control groups. For the anti-mouse CTLA-4 and isotype control groups, the opposite was seen: higher levels of M1 macrophages were observed in the control groups, and higher levels of M2 macrophages were detected in anti-mouse CTLA-4 treatment groups (Figure 30.).

When analyzing the M1, M2 and intermediate monocytes in the peripheral blood of mice from the different treatment groups, no significant differences were observed in the distribution of these three monocyte subpopulations (Figure 31.).

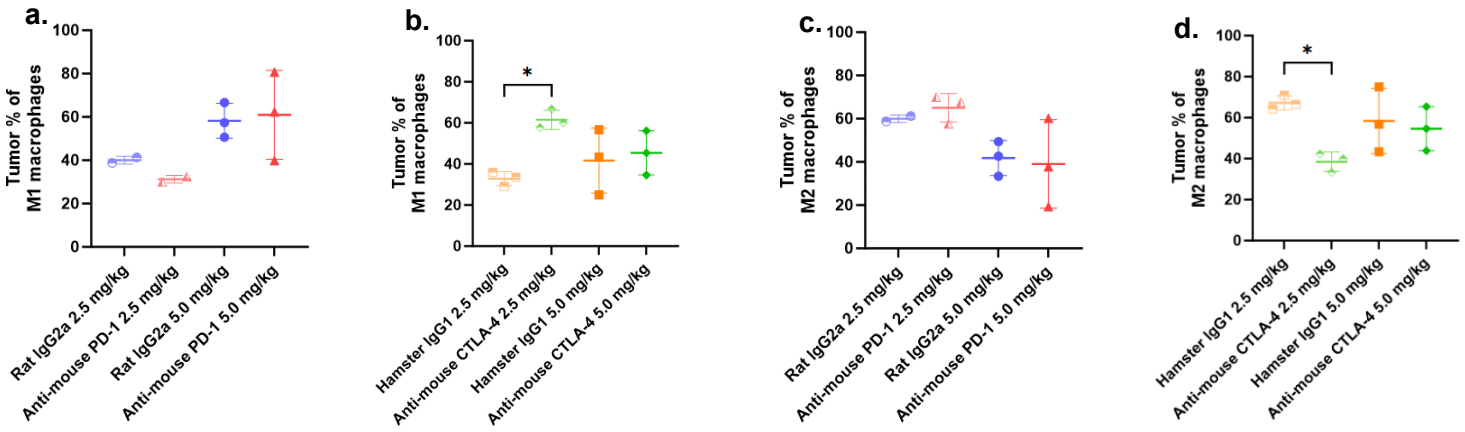

Figure 29. Flow cytometry analysis of M1 and M2 macrophages in the tumor (pilot study III).
Percentage of M1 macrophages (F4/80$^+$ CX3CR1$^-$) (**a.** and **b.**) and M2 macrophages (F4/80$^+$ CX3CR1$^+$) (**c.** and **d.**) in the tumor of animals in the different treatment groups. Results are shown as a percentage of total macrophages (F4/80$^+$).
Scatter plots represented by mean ± SD. *p<0.05. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

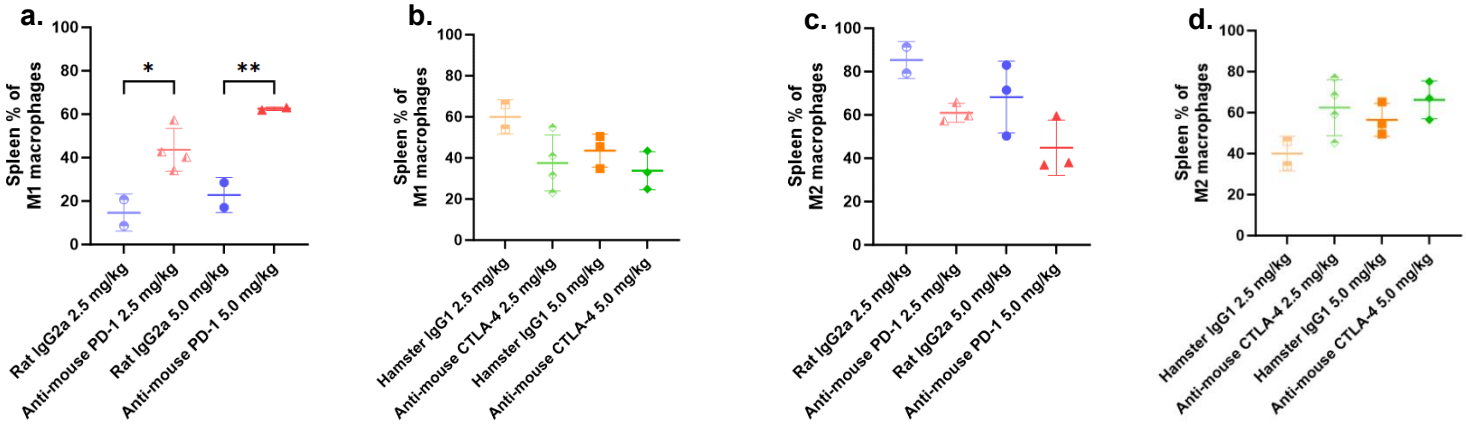

Figure 30. Flow cytometry analysis of M1 and M2 macrophages in the spleen (pilot study III).
Percentage of M1 macrophages (F4/80$^+$ CX3CR1$^-$) (**a.** and **b.**) and M2 macrophages (F4/80$^+$ CX3CR1$^+$) (**c.** and **d.**) in the spleen of animals in the different treatment groups. Results are shown as a percentage of total macrophages (F4/80$^+$).
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences between groups.

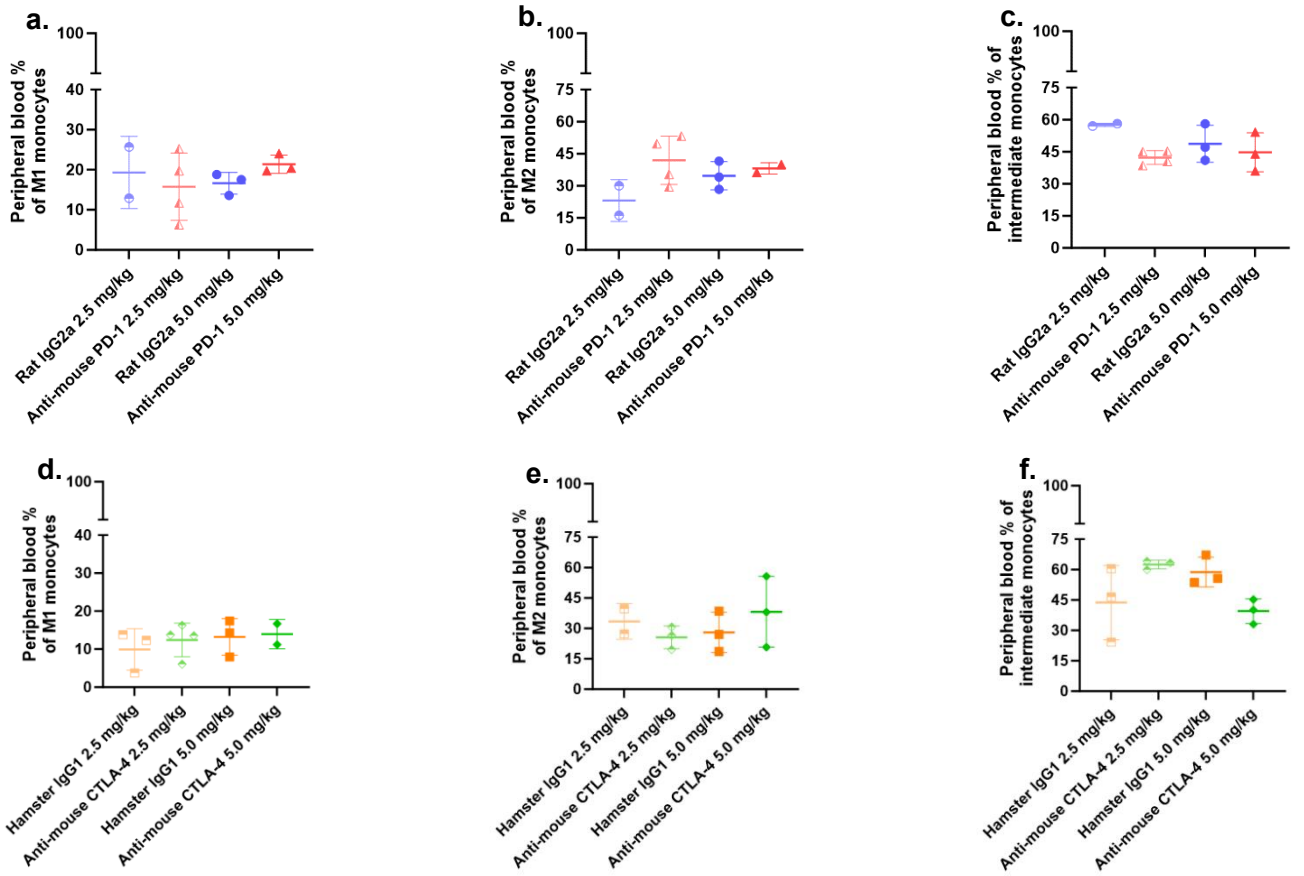

Figure 31. Flow cytometry analysis of M1, M2 and intermediate monocytes in peripheral blood (pilot study III). Percentage of M1 monocytes (Ly-6C^{+} CX3CR1^{-}) (**a.** and **d.**), M2 monocytes (Ly-6C^{-} CX3CR1^{+}) (**b.** and **e.**) and intermediate monocytes (Ly-6C^{+} CX3CR1+/$^{-}$) (**c.** and **f.**) in the peripheral blood of animals in the different treatment groups. Results are shown as a percentage of total monocytes.
Scatter plots represented by mean ± SD. *t-student* and *one-way* ANOVA tests were performed to detect statistical differences.

V. Discussion

Cancer diseases are a major contributor to global mortality, impacting millions of people annually. Consequently, the field of oncology assumes a central position in the domain of medical research [26]. Recent years have witnessed extensive investigation into the correlation between cancer and the immune system, with the inflammatory microenvironment of tumors and their capacity to evade immune responses now considered one of the cancer hallmarks [8]. The interaction between the immune system and tumor cells in the host constitutes a complex process that both impedes and facilitates tumor progression and development. This phenomenon is commonly referred to as cancer immunoediting, encompassing three main phases: elimination, equilibrium, and escape. In the elimination phase, both the innate and adaptive immune systems detect and eliminate immunogenic tumor cells. The immune pressure then leads to the emergence of poorly immunogenic tumor cells, resulting in a static equilibrium phase characterized by a balance between the growth and elimination of tumor cells, promoting a state of quiescence. Ultimately, tumor evolution favors the emergence and selection of immunoevasive traits in tumor cells, thereby driving tumor survival and growth [46].

The discovery of the dual impact of the immune system in relation to cancer has resulted in the advancement of immunotherapy as a mode of treatment for patients with cancer. At present, there exist six primary authorized methods of immunotherapy that can be administered to patients, specifically targeted mAbs, oncolytic virus therapy, cytokines, cancer vaccines, cell-based immunotherapy, and IC therapies [46, 55]. IC-based immunotherapy, which involves the utilization of ICIs against molecules such as CTLA-4, PD-1, and PD-L1, has emerged as a vital form of immunotherapy and has achieved considerable success in certain forms of both solid and hematologic malignancies. Nonetheless, despite the prolonged clinical advantages of ICI immunotherapy, there are certain constraints to its effectiveness, primarily attributable to the fact that ICI-based immunotherapy heavily relies on the immunogenicity of the tumor. Consequently, the clinical success of this approach in numerous cancer types remains greatly limited [178]. The treatment of cancer patients with more traditional anti-cancer strategies, such as chemotherapy or radiotherapy, could enhance the effectiveness of ICIs by transforming the immune status of the TME, thereby converting "cold" tumors into more responsive "hotter" tumors [179, 180]. This transformation can also be achieved using non-traditional, less invasive therapies, such as hyperthermia, which can impact the immune status of the TME by exerting cytotoxic effects on the immune system through elevated temperatures. This has led to the proposition that the combination of hyperthermia and ICIs could function as a method to amplify the local immune response

and overcome widespread immunotoxicity, thereby enhancing the clinical benefits of ICI-based immunotherapy [180].

The aforementioned hypothesis has prompted the team to design a study in which the impacts of combined immunotherapy using ICI-based treatment and imILT, a variant of laserthermotherapy would be evaluated in a poorly immunogenic mouse melanoma model. The research presented in this dissertation forms part of a more comprehensive research project, with the goal of assessing the mutually beneficial antitumor effect of combined laser-based thermotherapy and immunomodulators targeting CTLA-4 and PD-1, in a mouse model of B16-F10 melanoma, which is known for its limited immunogenicity. Here are presented the findings of three preliminary studies that were conducted with the primary objective of determining the most favorable conditions to advance to the larger project, as well as optimizing the methodologies to be employed. Despite the fact that the pilot studies, specifically II and III, also involved the analysis of tumor kinetics, a task carried out by other team members who are also enrolled in the project, this dissertation exclusively presents the results obtained through the implementation of flow cytometry analysis.

Pilot study 0 was exclusively dedicated to the evaluation, using flow cytometry, of the distribution of the most prevalent immune populations in the peripheral blood of 12 WT C57BL/6 mice. This was due to the lack of consensual information available in existing literature regarding the typical frequencies of these immune cell types in this particular strain of mice. The purpose of this study was to enable a precise and accurate interpretation of the frequencies of immune cell populations observed in samples obtained from manipulated animals. The study involved the application of immunophenotypical staining to the immune cells present in the peripheral blood of the animals, utilizing a panel of antibodies that would allow a comprehensive analysis of the most common immune cell populations, namely B and T lymphocytes, DCs, NK cells, monocytes, and neutrophils. The results of this study demonstrated that lymphocytic populations are the most dominant in the peripheral blood of C57BL/6 WT mice, whereas cell types typically associated with the innate immune system are present at lower proportions. This study provided insights into the typical distribution of immune cells and played a vital role in getting the team acquainted with mouse immune cell markers and gating strategies. Furthermore, it was of utmost importance to optimize and establish the ideal experimental conditions for the staining of immune cell populations in peripheral blood.

The general goal of pilot study II was to assess the impact of the imILT treatment, specifically utilizing the TRANBERG laser, on the melanoma mice model. The primary

focus was to optimize the placement of the fiber and temperature probe, as well as ascertain the optimal depth of the tumor for effective treatment. For this dissertation, specifically, the study aimed to investigate the influence of the imILT treatment on the frequency of immune cell populations in the tumor, spleen, and peripheral blood samples of the treated animals. Furthermore, it sought to evaluate the existence of any abscopal effect in the imILT treated animals by examining the distribution of immune cells in the second tumor, which had not undergone imILT treatment. To accomplish this, C57BL/6 mice were inoculated on the right (imILT-treated) and left flanks, with the left flank having an inocula half the size of the right flank, serving as a means to assess the presence of the abscopal effect. The mice were then divided into three groups: a control group (sham-treated), imILT-treated group 1, and imILT-treated group 2. The only difference between the last two groups was the depth of the tumor at the time of treatment. Flow cytometry analysis was performed by immunophenotypically staining the tumors, spleens, and peripheral blood of the mice in the different treatment groups, utilizing an antibody panel that allowed for the differentiation of various T cell subsets and macrophage/monocyte populations. The right-side tumors of the sham-treated group were compared to the treated tumors that did not fully necrose in animals from both imILT-treated group 1 and imILT-treated group 2. No significant differences were observed in the levels of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ T cell ratios as determined by this analysis. Similarly, there were no notable differences in the examined subpopulations in both the spleen and peripheral blood. Consequently, due to the limited number of animals that completed the study, which further exacerbated the disparity of the data, reliable results could not be obtained from the flow cytometry analysis. Consequently, no definitive conclusions could be drawn regarding the presence or absence of the abscopal effect. It is important to emphasize that based on the observations made by other members of the research team concerning tumor kinetics, none of the mice subjected to treatment exhibited the abscopal effect, and the tumors on the left side grew normally after laser treatment. Additionally, within imILT-treated group 1, two animals experienced complete tumor necrosis following laser treatment. Nevertheless, the treatment was ineffective in two mice: one displayed continued growth, while the other relapsed at the site of the treated tumor. With respect to imILT-treated group 2, both mice displayed a reduction in tumor volume after treatment, and one even exhibited complete necrosis at the tumor site. However, due to the cumulative tumor volume, the animals had to be euthanized earlier than anticipated, thereby preventing the derivation of substantial conclusions regarding the potential abscopal effect or relapse of the treated tumors. Overall, no definitive findings could be derived from pilot study II. Nevertheless, the study still carried significant value as it provided the research team with the opportunity to familiarize themselves with the

TRANGERG laser procedures, including the appropriate positioning of the fiber and temperature probe. Moreover, it allowed the realization that treatment of tumors at larger depths is not recommended, due to the fact that the volume's humanitarian endpoint would be reached sooner, thus decreasing the time window available to evaluate the effect of the therapy.

Pilot study III was conducted with the objective of ascertaining the optimal dosage of antibodies (anti-mouse PD-1 and anti-mouse CTLA-4) that would not lead to complete treatment. This was done so that in the final study, the combined impact of laserthermotherapy and immunomodulator therapy could be observed. Specifically, this dissertation aimed to achieve this goal by examining the distribution of immune cell populations in tumor, spleen, and peripheral blood specimens from animals that were treated with different dosages of anti-mouse PD-1 and anti-mouse CTLA-4 antibodies and compared to the respective isotypic controls at the same dosages. Once again, C57BL/6 mice were injected on both the right and left flanks, with the left flank receiving an inoculum half the size of the right flank. Following tumor inoculation, the mice were randomly assigned to eight different treatment groups: anti-mouse PD-1 at 2,5 or 5,0 mg/kg, and the corresponding isotypic control rat IgG2a at 2,5 or 5,0 mg/kg, as well as anti-mouse CTLA-4 at 2,5 or 5,0 mg/kg, and the corresponding control hamster IgG1 at 2,5 or 5,0 mg/kg. Flow cytometry analysis was conducted by staining the tumors, spleens, and peripheral blood of the mice from the various treatment groups using the same antibody panel as in Pilot study II. This allowed for the identification of different subsets of T lymphocytes and macrophages/monocytes. The most interesting finding in the flow cytometry data was an increase in $CD8^+$ T cells and a consequent decrease in the $CD4^+/CD8^+$ T cell ratio in the tumor for the mice belonging to the anti-mouse PD-1 5,0 mg/kg and anti-mouse CTLA-4 5,0 mg/kg groups (Figure 14., b. and e.), suggesting the recruitment of cytotoxic T cells to the TME. However, it was expected that the differences observed between the treatment antibodies and their isotype controls would be more pronounced. This lack of distinction can likely be attributed to the limited number of animals in each treatment group, which heightened the disparity of the data and hindered the drawing of reliable conclusions from the flow cytometry analysis. In relation to the tumor kinetics data collected by other team members, it is important to note that antibody treatment did not result in complete treatment in any group. Nevertheless, in certain groups, it appeared to prevent tumor growth when compared to the untreated control group (biological samples from this group were not subjected to flow cytometry analysis). This effect was particularly evident in the anti-mouse CTLA-4 5,0 mg/kg and anti-mouse PD-1 5,0 mg/kg groups, thereby demonstrating that tumor growth is dose-

dependent. Concerning survival time (results not shown), animals in the anti-mouse CTLA-4 5,0 mg/kg and anti-mouse PD-1 5,0 mg/kg groups had a superior median survival time compared to those in the anti-mouse CTLA-4 2,5 mg/kg and anti-mouse PD-1 2,5 mg/kg groups. However, once again, it was expected that the differences observed between the treatment antibodies and their isotype controls would be more pronounced.

Considering the results obtained from tumor kinetics and flow cytometry, particularly regarding the suboptimal discrepancy observed between the effects of isotype control antibodies and treatment antibodies, it can be inferred that the animals would benefit if a higher dosage (10 mg/kg) of anti-mouse PD-1 and anti-mouse CTLA-4 antibodies were administered, both in survival time and immunostimulation. Pilot study III results demonstrated that animals could benefit from this dose increasing, especially in combination with the laser treatment, increasing its efficiency. In fact, an additional pilot study (Pilot study IV) has been performed by the team, in which the combination of anti-mouse PD-1 antibody at 10 mg/kg with imILT was evaluated (results not presented in this dissertation). The findings from this study revealed that the combination of the treatment antibody with imILT led to an increase in the frequencies of NK cells and $CD8^+$ T cells within the tumor, indicating the recruitment of cytotoxic cells to the TME, as well as a decrease in the frequency of T_{Reg} cells.

Going into the final study, in order to ensure reliable and conclusive findings, it is crucial that each treatment group includes a greater number of animals to account for potential outliers. Additionally, it would be of great interest to conduct immunophenotyping on a broader range of immune populations using an expanded array of antibody panels. This approach would enable the detection of other significant immune-related molecules that actively impact the tumorigenesis process. For instance, the incorporation of CD152 and CD279 in the antibody panel would allow the identification of immune checkpoint molecules (CTLA-4 and PD-1, respectively). Moreover, the use of the CD11b marker would allow the identification of MDSCs, while the use of CD11c and I-A/I-E would aid in the identification of APCs. Furthermore, the activation markers CD69 and CD161a, along with pro-inflammatory cytokines like IFN-γ, would facilitate the identification of tumor antigen-specific T cells.

VI. Conclusion

The pilot studies performed prior to the final study were of great importance, given the fact that it allowed for the optimization of protocols and methodologies. Pilot study 0 allowed for a better understanding of the more common immune populations in the peripheral blood of WT C57BL/6 mice, as well as familiarizing oneself with the mouse immune cell markers, this way facilitating the optimization of the immunophenotypical panel to be used in flow cytometry studies.

Pilot study II enabled the team to get familiarized with the TRANBERG laser equipment, as well as to ascertain the optimal tumor depth for treatment. Moreover, it was also important to define gating strategies using a different antibody panel.

Pilot study III allowed for the realization that higher dosages of anti-mouse PD-1 and anti-mouse CTLA-4 antibodies than those employed in Pilot study III could bring benefits to the mice, both in terms of survival time as well as in immunostimulation.

Considering the pilot studies' findings, it is suggested to:

1. Perform an additional Pilot study V, with the goal of evaluating the effects of combining anti-mouse CTLA-4 antibody at 10 mg/kg with imILT in mice tumor kinetics and immunostimulation.
2. Perform an additional pilot study VI with the goal of studying the tumor kinetics when using a smaller cell inoculum on the right flank, and eventually performing the left flank inoculum at a later time, so that there is enough time to evaluate the effects of the therapy, before the sum of both tumors reaches the ethic limit and animals need to be euthanized.
3. Perform the final study, using a higher number of animals per group, using anti-mouse PD-1 antibody a 10 mg/kg, and the adequate dosage of anti-mouse CTLA-4 antibody, depending on the Pilot studies' V results, and the adequate cell inocula, depending on Pilot studies' VI results.
4. Include an extended array of antibody panels that would allow the identification of a broader range of immune cell populations and immune-related molecules with direct impact in tumor biology.

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