The University of South Bohemia in České Budějovice
Faculty of Science

T Regulatory Lymphocytes During MBTA Immunotherapy of Pancreatic Adenocarcinoma

Bachelor Thesis

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Annotation:

The aim of this bachelor thesis is to summarize the current knowledge in the field of T regulatory lymphocytes in cancer research as well as during intratumoral MBTA immunotherapy. In addition, methods to detect and target T regulatory lymphocytes are suggested in order to improve MBTA immunotherapy.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 08.05.2023

Isabel Simmer

Acknowledgments:

First I would like to thank RNDr. Jan Zenka CSc., for being my supervisor and for making it possible to write my thesis about such an interesting topic.
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A special thank goes to my family, for enabling me to study at a university as well as for giving me always new motivation to overcome any difficulties.

Contents

1 Aim of the work

- Summarize current knowledge regarding T regulatory lymphocytes and intratumoral MBTA therapy in different tumor models.
- Suggest detection and targeting methods in order to improve the efficiency of intratumoral MBTA therapy where current knowledge is missing.

2 Cancer

In the 19th and the beginning of 20th century "Cancer" was initially described as an invasion of metastases, which were shown through rough results of autopsies or surgeries [1]. The current definition according to the National Cancer Institute is similar and states that "Cancer is a disease in which some of the body‘s cells grow uncontrollably and spread to other parts of the body." [2].

In general, during cell division new and healthy cells are produced which are needed in the human body. However, if damaged and unhealthy cells develop faster than healthy cells, those will form neoplasms, known as tumors [3].

There are two characteristic types of tumors, malignant and benign tumors. On the one hand, malignant tumor cells are developing in an uncontrollable way. They occupy surrounding cells or tissues, and are able to spread into the whole body, which means this type of tumor is cancerous. They form so called metastasis, which extend through the lymphatic system as well as through the blood stream. Such metastasis can be found everywhere in the body; however, they are mostly present in lungs, bone, brain and liver. If tumors are diagnosed early enough, surgery combined with chemotherapy or radiotherapy is a possible treating method. However, if the tumor is already spreading other cells or tissues, a systemic treatment, like chemotherapy or immunotherapy, is required [3].

On the other hand, benign tumors remain located at their initial location and do not invade and damage other cells. Usually, they are larger than malignant tumors, develop slower and have different borders. After benign tumors are surgically removed, they normally do not return and do not cause any problems anymore. However, since their size increases, the surrounded tissues are compressed, which further leads to medical issues and pain. Typical examples for this type of tumor are lipomas in the skin and fibroids in the uterus. Sometimes benign tumor cells are changed into malignant tumor cells, such as colon polyps. Is this the case, a surgical removal is necessary [3].

Worldwide cancer is the main reason for morbidity and mortality despite any development of humans. In 2020, the number of new cancer cases in the whole world was 19.3 million and nearly 10 million patients died from cancer. The most cancer death of men and women, approximately 60%, occurred in Asia. Nevertheless, the majority of

the world's population lives in Asia. Almost ten percent of the population in the world resides in Europe, whereas 23% suffer from cancer and 20% cancer death are counted. All in all, the most diagnosed type of cancer of both sexes is the female breast cancer which accounts for 12% of the total cases. It is followed by lung, colorectal, prostate as well as stomach cancers. The major part, which are about 20%, of cancer deaths of women and men is caused by lung cancer, which is simultaneously the main reason for cancer deaths in men. Compared to this in women breast cancer is the most frequently diagnosed type of cancer and additionally causes the most female cancer deaths. It is expected that there will be an increase of cancer patients of about 47% in the future. Therefore, it is necessary to put a considerable amount of effort in creating measurements to prevent cancer and to develop sustainable and effective therapies [4].

2.1 Cause of Cancer

In principle, there exist environmental hazards and lifestyle-related risks that might lead to growth of cancer cells. Although certain lifestyle risk factors such as alcohol consumption or tobacco smoking decreases in Northern America and Western Europe, the number of patients with cancer still increases. Therefore the continuously changing environment where new carcinogenic factors are developed plays a huge role in cancer development [5, 6].

Tobacco smoking, alcohol consumption, diet, obesity, overweight as sedentariness belong to lifestyle-related risks [6]. Thereby, it is stated that smoking might cause a series of cancers such as lung, mouth, throat, stomach, liver, cervical, and pancreas cancer. Passive smoking is harmful as well and might cause leukaemia in children for instance [5]. The reason why smoking is so dangerous is because a huge number of mutagens like nitrosamines, polycyclic aromatic hydrocarbons as well as other tumor promoters are ingredients of tobacco smoke. These ingredients make smoking totally carcinogen [6]. In addition, the consumption of alcohol especially promotes the growing of mouth, liver, breast, oesophagus and larynx tumors [5]. In comparison to tobacco smoking, ethyl alcohol is principally co-carcinogen and therefore it is able to activate carcinogens which are either a promoter or a mutagen [6]. An unbalanced diet combined with the lack of sufficient number of physical activities cause obesity as well as chronic inflammations and

therefore promote the development of tumors, especially in breast, colorectal, pancreas and stomach. Nitrosamines which are mainly part of pickled meat are carcinogenic as well and force the development of gastric cancer [5].

The following cancer-causing agents belong to the environmental risk factors.

An increased exposure to electromagnetic fields, ionizing radiation as well as ultraviolet radiation enhances the development of cancer. Electromagnetic field increases the probability of breast cancer and leukaemia. However, even more dangerous are ionizing radiations which can spontaneously cause the development of cancer cells in any organ. Finally, ultraviolet radiation and therefore an intensive and chronic exposure to sunlight, might promote the development of skin cancer cells [5]. Additionally, certain types of oncogenic microorganisms and viruses are cancer causing agents. The most common DNA viruses are the hepatitis B and the human papilloma virus. These two viruses act differently. The hepatitis B virus causes chronic inflammation and thus generates reactive oxygen species whereas the human papilloma virus directly contributes via persuading viral genes [6]. In addition, there are other chemicals leading to genetic mutations. Examples are pollutants from industries and automobiles, pesticides, hair dye, chemicals in household and agriculture or toxic industrial waste [5].

Finally, also infections might lead to tumors such as infections from *Heliobacter pylori* may cause gastric cancer [5].

Besides these risk factors, there exist genetic and/or epigenetic transformations which can cause cancer development. Such changes on the one hand produce foreign antigens called neo-antigens and on the other hand force immortality. These processes are somatic gene mutations and thus lead to altered genes [7].

2.2 Cancer Treatment

There are several approaches to treat cancer and patients often get a combination of different methods, dependent on the type of cancer and how severe it already is. One method to treat cancer is removing the tumor by surgery. Cancer surgery is essential to cure cancer worldwide. Since it is possible to use this method in a preventative, diagnostic, supportive, curative, reconstructive or palliative way it is versatile applicable. As an example, tissues which are endangered to become cancer are preventative removed

as well as diagnostic surgery methods such as biopsies are necessary to detect tumors. To treat tumors especially breast cancer, surgeries are essential to remove the tumor. Moreover, surgical resections are important for palliative treatment. However, the disadvantage of this option is the ability of cancer cells to spread the whole body and so it is not possible to treat the patient completely. Therefore, additional treatments such as chemotherapies, radiological medical examinations or endoscopies are needed [8, 9].

Chemotherapy is a further widespread option to treat cancer, which is based on cytostatic drugs that attack the cell cycle. These drugs can be divided based on their target into alkaloids, alkylating agents, and antimetabolites. They attack the cytoskeleton and mitosis, the cellular DNA or RNA including their metabolism or pyrimidine and purine metabolic enzymes, respectively. The advantage of this treating method is that it ensures treating many different cancer types with success. However, using this method shows up severe side effects due to its non-specificity, that shows up signs of toxicity and chronic toxicity, respectively. Toxicity can be immediately observed on hair, skin, blood, etc. The reason for these immediate observations is that chemotherapeutic drugs target fast-dividing cells like tumor cells. However, there are other cells in the body which share this property, such as cells of the hair follicles, bone marrow, lymphatic cells, and red blood cells. Thus, chemotherapies also attack these cells, which leads to the negative observations. Moreover, this makes such therapies unsuitable for treatments over a long period of time. In addition, chronic toxicity shows up in infertility or drug resistance [8, 10, 11]. The selectivity of chemotherapeutic drugs can be improved by modified drugs called prodrugs. Prodrugs are small molecule drugs which are chemically adapted and biologically inactive. There are several requirements they have to fulfil, such as reducing toxicity and pain, improving oral and local absorption, decrease inappropriate taste etc. [11]. Furthermore, to reduce the arising side effects, chemoprotective agents, monoclonal and liposomal antibody therapies or hematopoietic stem cell transplantation are possible side-effect reducing methods [8, 10].

In addition, radiation therapies are a common treating method against cancer. Imaging systems such as image-guided radiation therapies lead to the big advantage of radiation. With these systems surrounded healthy tissues become less destroyed compared to chemotherapies. Radiation therapies are based on gamma radiation or proton beams

which destroy cancer cells. However, the effectiveness of such therapies is often decreased due to the heterogeneity of tumors which cause radiation resistance [8, 12].

Furthermore, there are so-called targeted cancer treatments. Compared to chemo- or radiation therapies the advantage of the targeting methods is that they only treat and destroy cancer cells and not neighboring healthy cells. Examples for this treatments are growth signal inhibitors which stop the development and division of tumor cells, apoptosis-inducing drugs and endogenous angiogenesis inhibitors, that inhibit the development of blood vessels for tumors [8].

Finally, immunotherapies are used to treat various types of cancers. This alternative treating method is based on forcing the own immune system to eradicate tumor cells. Further details of immunotherapies are discussed in chapter 6 [7, 13].

3 Immune System

The two main tasks of the immune system are to defend the organism against infections and pathogens as well as to clean the body from dead or ill cells like tumor cells. Therefore, several mechanisms are needed to recognize and eliminate them. These mechanisms within the different elements of the immune system must cooperate with each other to fight against foreign pathogens or damaged and dead cells effectively [13, 14].

3.1 Different Parts and Workflow of Immune System

The immune system is split up into two different main parts known as innate and adaptive immune system. Basically, the innate immune system is the first barrier in host defense and thus starts with response reactions against the pathogen or damaged cell. Since the innate system consists of lots of different cell types, a fast initial response is possible. After a few days the adaptive immune system is activated by the innate immune system which leads to an increase of antigen-specific lymphocytes. However, only after the second contact to an antigen the adaptive immune system response is faster compared to the innate system, which leads to quicker elimination reactions. The adaptive system is composed of less types of cells. However, those cells are specific for different antigens individually. After recognizing the antigen, the number of immune cells increases, to achieve a successful response against the foreign pathogen [13, 14]. This process is called clonal expansion and very characteristic for the adaptive system. However, clonal expansion also occurs in the innate immune system when antigens must be fought. Besides expanding the number of lymphocytes to ensure protection against a certain antigen, clonal expansion is developing a numerous sum of cells via selecting and differentiating responding immune cells [15]. Therefore, while the innate immune system functions as immediate host defense, the adaptive system expresses itself for a longer time period [14].

Their cooperation is very crucial for the human health, because of several reasons. Besides several tasks such as presenting antigens or removing pathogens and dead or damaged cells, compounds of the innate immune system are additionally responsible for activating the antigen-specific B and T lymphocytes. Furthermore, adaptive immune

cells intensify their reactions through recruiting mechanisms of the innate system to control the foreign microorganisms [13, 14].

Each of those two main parts contains a humoral and a cellular part. The humoral part of the innate immunity is the complement system, and the cellular part is composed of macrophages, neutrophils, dendritic cells etc. In comparison, the humoral part of the adaptive immune system are antibodies and both, T and B lymphocytes are the cellular part. In addition, several mechanisms such as stimulation of antigen-presenting cells, identification of antigens, and activation of several cells such as T lymphocytes are included [13]. Moreover, the ability of T lymphocytes to recognize both, foreign antigens and self-antigens as a molecular complex is crucial to keep self-tolerance mechanisms [14]. The connection of the two parts are the antigen-presenting cells, short APCs, such as dendritic cells and macrophages, which present antigens to the T lymphocytes [13].

Since the immune system is able to destroy a huge number of dead cells as well as of different pathogens, toxins and other allergenic compounds, it is essential to protect the own tissues from immune cells, which is defined as self-tolerance. However, if the self-tolerance mechanism fails, consequently autoimmune diseases appear. Both parts of the immune system, innate and adaptive, carry out these mechanisms [14].

3.1.1 Innate Immunity

The innate immune system is the earliest defense system which works immediately after the human is born. It contains all properties of the immune defense mechanisms which are encoded in the host‘s germ-line genes in their mature functional form. The main function of the innate system is to ensure a first and fast defense against antigens [14, 16]. Part of this defense mechanisms are physical and chemical barriers. The main physical blocking organ is the skin, that is able to secret sweat and sebaceous which both have a low pH and thus have an antimicrobial function. Moreover, the skin is covered with different enzymes such as lysozymes as well as fatty acids that again hinder for example pathogens to enter the body [16].

In addition, the epithelial cilia functions as a physical barrier. It removes the mucus layer allowing it to be always clean after a contamination of ingested or inhaled molecules. Moreover, epithelial cell layers are part of these barriers and demonstrate close cell-cell

interactions. In general, the secreted mucus layer covers the epithelium in the gastrointestinal, respiratory as well as the genitourinary tracts [14].

The respiratory tract is additionally protected through hairs in the nose as well as through the cough reflex. To eliminate pathogens in the respiratory tract, phagocytic cells such as alveolar macrophages are present. The innate immune defense mechanisms of the gastrointestinal tract is composed of hydrolytic and proteolytic enzymes as well as a low pH in the stomach [16].

In addition, the innate immune system includes both, small molecules that are biologically active and soluble proteins such as interferons [14, 16]. After a pathogen is invaded, special cells such as monocytes, macrophages and polymorphonuclear leukocytes are eliminating the invader. Those cells are developed from myeloid precursors. Besides these cells dendritic cells and innate lymphoid cells belong to the innate system [16, 17].

The important function of the innate immunity is pattern recognition, which is based on the property of both innate cells and specific soluble mediators to recognize pathogen-associated molecular patterns, short PAMPs. PAMPs consist of several different microbial molecules with many different biochemical properties which send warning signals and thus are recognized as invading pathogens. The recognition of such PAMPs is enabled by pattern recognition receptors, abbreviated as PRRs. These receptors are located on the cell surface of immune cells, for example on dendritic cells and can be further divided into C-type lectin receptors, Toll-like receptors, NOD-like receptors, RIG-I-like receptors, and f-Met-Leu-Phe receptors. After foreign antigens are detected in the form of PAMPs, antigen-presenting cells present these antigens to the adaptive immune cells in lymphatic nodes. Besides PAMPs, other molecules are set free after injuries. These molecules are the so-called damage-associated molecular patterns, short DAMPs. These molecular patterns are molecules that contain a physiological role in the inside of the cell and are released by either dead cells or living cells which suffer from life-threatening stress. They are responsible for alarming the organism about any type of danger. Moreover, they activate inflammatory reactions and enhance regeneration processes [16, 18, 19].

Besides pattern recognition patterns, the complement system is a further soluble element

of the innate immune system. It is composed of around 25 proteins, that are mainly developed in the liver and help antibodies to get rid of bacteria. In addition, it is together with innate and adaptive immune cells responsible for inflammatory reactions like pain, swelling, redness etc. Each working step is done according to the complement cascade and finally the product is placed into the bacteria surrounding cell walls, which kills the pathogen or secondly, the bacteria is opsonized in order to improve the recognition by neutrophils and different phagocytes. It is called membrane attack complex, short MAC [16].

3.1.2 Adaptive Immunity

The adaptive immune system mainly develops antigen-specific cells as well as memory cells and exists only in vertebrates [16].
Contrary to the innate system, the adaptive immune system is highly specific to target antigens. The immune reactions are mainly dependent on antigen-specific receptors that are demonstrated on the surface of T and B lymphocytes. These receptors are encoded by genes of the germ-line gene elements, which are formed through somatic rearrangements to further develop genes for T cell receptors and immunoglobulins. This assembly of receptors is formed by hundreds of different gene elements and enables the development of several millions of antigen receptors that are individual for a huge number of different antigens [14].
Thereby antibodies are synthesized and secreted by B lymphocytes, which is part of humoral immunity. In contrast, T lymphocyte responses are defined as cellular immunity or cell-mediated responses, because of their production of diverse cytokines and T lymphocyte subsets. Compared to B cell receptors which are able to directly bind to antigens, T cell receptors are not able to directly bind antigens and thus cannot be directly activated. They recognize peptides which are secreted when antigens are bound to antigen-presenting cells like dendritic cells and macrophages or peptides which are in contact with the major histocompatibility complex, short MHC. To finally activate T lymphocytes a second signal is required, namely the ligation of complimentary membrane molecules with co-stimulatory molecules which are expressed by T lymphocytes [16].

The organs which belong to the adaptive immune system are the lymphatic organs and are the place where B and T lymphocytes are maturated, differentiated, and multiplied. Lymphatic organs can be subdivided into primary and secondary organs. Thymus and bone marrow belong to the primary organs because there the development of T lymphocytes and B lymphocytes, respectively occurs. In the thymus positive and negative selection of T lymphocytes takes place. During positive selection, the double-positive cells react with epithelial cells which convey MHC molecules. The MHC molecules consist of peptides that are connected in the peptide-binding channels. If this interaction is successful, the next step the cell must undergo is the negative selection. During negative selection, the cells which are very reactive against self-MHC molecules are sorted out and thus the organisms is prevented from severe autoimmune diseases. After their maturation and selection, T and B lymphocytes move through the blood stream to the secondary lymphatic organs such as lymph nodes and spleen. In these organs the proliferation and differentiation of the lymphocytes takes place [16].

In general, B and T lymphocytes constantly flow between the lymph, lymphoid organs, tissues, and blood. The binding of a B or T cell receptor with cell-surface adhesion molecules, short CAMs, that are placed on high endothelial venules enables an extravasation of B and T lymphocytes to tissues where activated immune cells are present [16]. The main property of the adaptive immune system is that it is able to develop long-living immune cells that continue in an inactive state but are immediately activated after the invasion of antigens that are already specific for them. Thus, the adaptive immunity is able to create immune memory, which leads to a more successful and faster host reaction after the specific pathogen entries a second time [14].

4 Cancer and Immune System

In general, the major aim of studying tumor immunology is to analyze and subsequently use findings for diagnostic or therapeutic approaches [16]. Since antigenic molecules are expressed by malignant cells on their cell surface, several mechanisms of the innate and adaptive immune system are activated when it comes to recognition of the antigens [16, 20–23]. Tumor antigens are induced by several biological transformations such as gene activation, mutation, or clonal amplification and are separated into immunogenic and nonimmunogenic tumor antigens. Immunogenic tumor antigens are able to cause immune reactions, whereas nonimmunogenic tumor antigens are commonly self-antigens and thus are tolerated by the host. To overcome this tolerance, some amino acids of peptides, which are placed on the surface of self-antigens, become more immunogenic, because of fast replication of tumor cells or reparation processes leading to possible mutations [16].

The process describing the recognition and eradication of tumor cells by the immune system has been named as immunosurveillance [16, 20–23].

However, the resistance against tumor cell development does not necessary mean that the tumor cells do not develop immunogenic antigens. The reason is the occasional failure due to systemically decreasing the activity of tumor specific immune responses, which are evolved naturally in order to hinder cancer development [16]. Nevertheless, primary tumors or multiclonal tumors actively possess decreased immunogenicity and therefore are able to escape from immune recognition and elimination by the immune system [16, 21–23]. Besides that, cancer cells actively suppress immunosurveillance by subversion of the immune system, known as immunosubversion. Thus, the interaction between immune system and cancer cells leads to different consequences, such as to complete elimination of cancer cells, which are less immunosuppressive as well as to an increased number of cancer cells that block anti-tumor response of the immune system [21–23].

These opposing properties of protecting the organism and promoting cancer development are represented by the process immunoselection or immunoediting [16, 21–23]. Immunoediting is composed of three parts, namely elimination of tumor cells through (i) immunosurveillance, (ii) equilibrium, which describes the inactivity of the immune

system after partial elimination of tumor cells and finally (iii) escape in which the number of tumor cells, which exceeded the anti-tumor mechanisms of the immune system, increases [16, 20, 21]. The different steps of cancer development as well as the comparison of cell-extrinsic and cell-intrinsic properties during cancer development are summarized in Figure 1 [21].

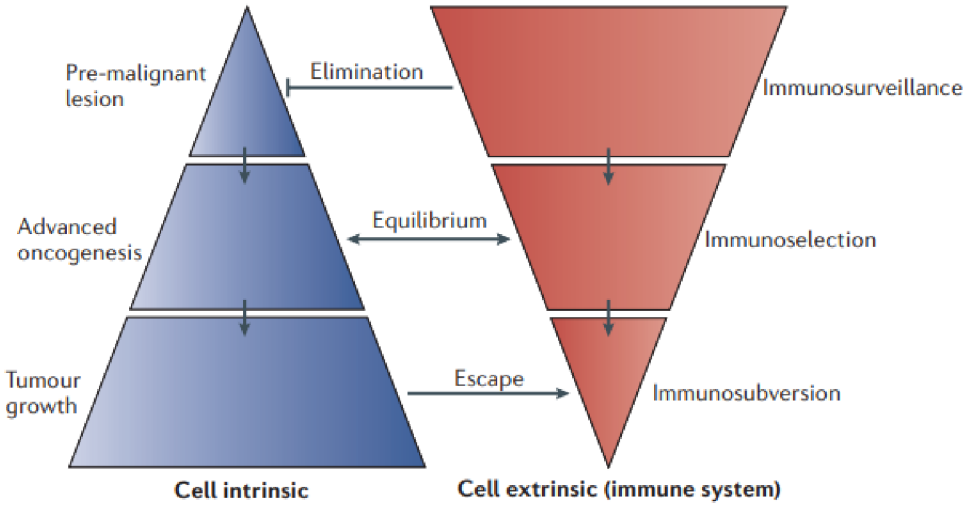

Figure 1: Comparison of cell-extrinsic and cell-intrinsic properties during tumor development [21].

A perfect example for an immunoediting process is the escape from an immune response mediated by T lymphocytes through decreased expression of HLA class I compounds. HLA is the abbreviation for human leukocyte antigens also known as the major histocompatibility complex in humans, short MHC. Figure 2 graphically shows how the MHC works. At first antigens, especially peptides are selectively bound by the MHC molecule and subsequently the bounded antigens are presented to a T lymphocyte having the corresponding T cell receptor. This takes place on the surface of the host cell. Therefore, tumor cells actively decrease the expression of MHC molecules in order to escape from their antigen recognition and subsequently from further immune reactions [16, 21].

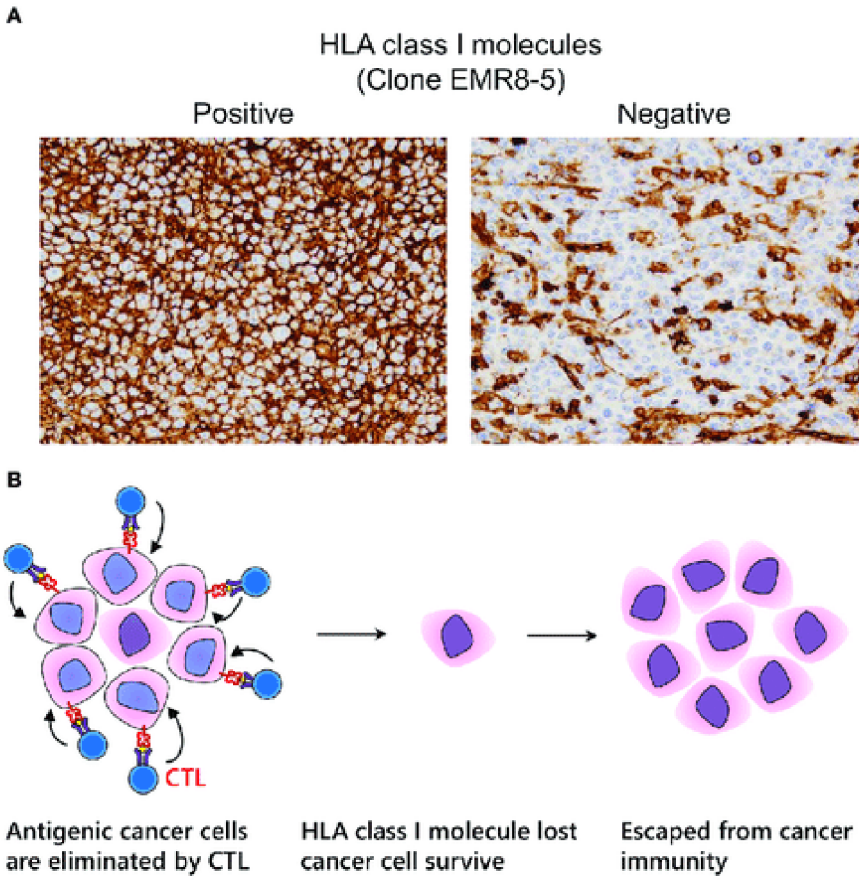

Figure 2: Loss of MHC molecules enables tumor the to escape from immune reactions [24].

This type of immunoselection is mainly observed in melanoma and epithelial-cell cancers. In contrast, in lung cancer an escape from immune reactions is only possible for tumor cells that totally lack in HLA class I development [16, 21–23].

5 Regulatory T Lymphocytes

Around 1970 a special cell population with suppressive properties against lymphocytes was observed. These cells were first named as T suppressor cells. However, at that time an isolation and clone of these T suppressor cells was not possible and thus scientists were not sure if this cell population is directly responsible for these suppressive actions. Approximately twenty years later the interest of suppressor cells rose again which led to the recognition of $CD4^+$ T lymphocytes, having the property to decrease the function of T lymphocytes. Subsequently, neonatal surgical removal of the thymus in mice showed higher development of autoimmunity diseases. To avoid these diseases, splenocytes of healthy donor mice with thymus were transferred into the mice with autoimmune disease. Due to this transfer of splenocytes the thymectomized mice did not develop the autoimmune diseases. This experiment showed that the splenocytes have suppressive properties and it was assumed that the splenocytes are connected to a specific group of $CD4^+$ lymphocytes, which was later named as suppressor or regulatory T lymphocytes, shortly Tregs [16, 23].

5.1 Types of Tregs

There are several types of regulatory T lymphocytes. One of them are natural regulatory T lymphocytes, short nTregs. The development of nTregs takes place in the thymus. After development they join the peripheral circulation, where they spread in spleens and minor reservoir lymph nodes. These autoreactive cells make up 5-10% of all T lymphocytes [16, 23].

In addition, there are induced or adaptive Tregs, abbreviated as iTregs. Induced Tregs are grown from naive T lymphocytes under the presence of the cytokines TGF-β (transforming growth factor β) and IL-10 (interleukin 10). They are responsible for adapting immune reactions against for example inflammatory bowel disease or other spontaneous inflammatory diseases. In addition, adaptive Tregs alter immune responses in infectious illnesses [16, 23, 25].

On the surface of both, iTregs and nTregs, the CD25 molecule is located, which is a component of the cytokine receptor IL-2 α chain. Besides that, they express Foxp3

(forkhead box p3 protein), which is a transcription factor that determines the lineage. Additionally, both types of Tregs express the protein CTLA-4 (cytotoxic T-lymphocyte associated protein 4) [16, 23].

Besides these groups of Foxp3$^+$ regulatory T lymphocytes, there are two more groups of Tregs also expressing the molecule Foxp3. The subgroups differ based on where they are developed. The first subgroup of Tregs develops in the thymus (tTregs), whereas the generation of pTregs takes place at the peripheral sites [25].

5.2 Different Types and Markers of Tregs in Mice and Human

In mice, between one and four percent of lymphocytes in secondary lymphoid organs are Tregs. Compared to human Tregs, regulatory T lymphocytes in mice are more homogeneous but the expression of the molecules CD4, CD25 and FoxP3 is typical for Tregs in both, mice and humans [16, 25–29]. In humans, regulatory T lymphocytes that express these markers are called FoxP3$^+$ CD4$^+$ T lymphocytes, which are not always homogeneous and immunosuppressive [16, 25, 29, 30]. Besides FoxP3$^+$ CD4$^+$ T lymphocytes there are Foxp3$^+$ CD8$^+$ Tregs, which can be found in tissue transplantations as well as in immune reactions that are caused by alloantigens [31]. In general, CD8$^+$ Tregs produce the cytokines IFN-γ and TNF-α in order to prevent or control different diseases such as infections or tumors. Both CD4$^+$ and CD8$^+$ regulatory lymphocytes share the marker CCR7 located on their cell surface [32].

There are three subgroups of FoxP3$^+$ CD4$^+$ T lymphocytes in humans, namely CD45RA$^+$ FoxP3^{lo} T lymphocytes, which are commonly referred to as resting Tregs and CD45RA$^-$ FoxP3^{hi}, which are activated Tregs. In addition, there are CD44RA$^-$ FoxP3^{lo} T lymphocytes which are effector T lymphocytes that are recently activated and express cytokines that are pro-inflammatory. Interestingly, in colorectal cancer infiltration of CD44RA$^-$ FoxP3^{lo} T lymphocytes in the tumor microenvironment (TME) leads to higher chance of patient to completely eradicate the cancer. However, enrichment of these CD45RA$^-$ FoxP3^{hi} T lymphocytes will be associated with worse progression in other cancer types [30].

Identifying heterogeneous intratumoral Tregs, the expression order on several surface molecules as well as the generation of inhibitory cytokines should be analyzed. A clas-

sification of the different subsets based on several markers is shown in Figure 3 [30].

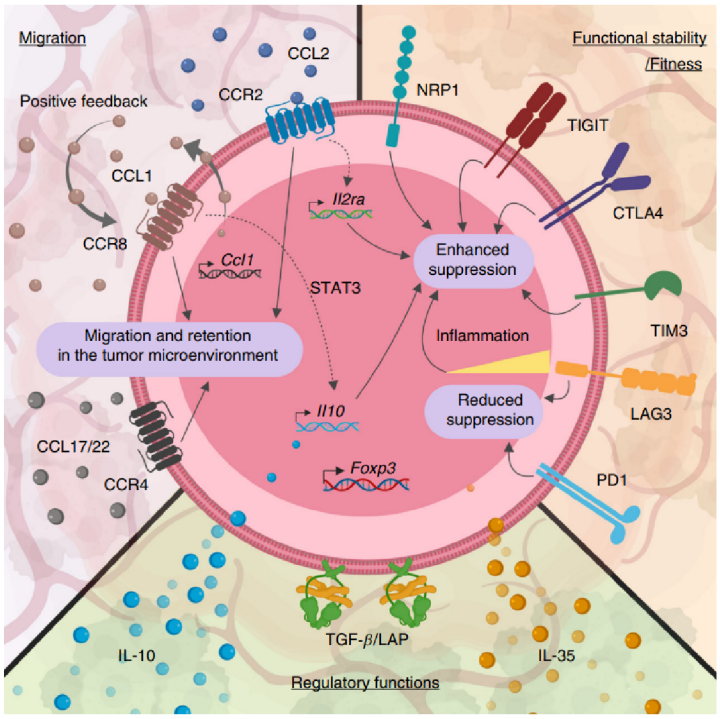

Figure 3: Identification of different Treg cell subgroups based on their markers [30].

There is an increased expression of activated Treg cells next to inflammations. Certain chemokine receptors, especially CCR8 increase the function as well as the stability of regulatory T lymphocytes. Moreover, these receptors enable chemotactic navigation in order to lead them to the microenvironment of the tumor. In addition, several inhibitory receptors, such as PD-1 and LAG3, are enhanced by Treg cells. As an example, the inhibitory receptor TIGIT enhances suppressive activity of regulatory T lymphocytes, whereas the inhibitory receptors PD-1 and LAG3 lead to less suppressive functions of regulatory T lymphocytes. Furthermore, different subgroups of regulatory T lymphocytes release inhibitory cytokines, like the transforming growth factor-β, abbreviated as TGF-β, interleukin-10 as well as interleukin-35 [30].

There are different cell types which are directly bound and regulated by FoxP3$^+$ Treg cells, such as helper T lymphocytes, cytotoxic T lymphocytes, B lymphocytes, dendritic cells, osteoblasts, macrophages, NK (natural killer) cells, NKT lymphocytes (natural killer T lymphocytes), and mast cells [23, 33].

Apart from these molecules tTregs, that are stable in humans and mice, are characterized by the marker Helios, which is a transcription factor. It is able to cause epigenetic

suppression of IL-2 formation and thus it functions as a regulator of Tregs. However, if is a lack of Helios development absent, IL-2 expression is increased, leading to an increased Treg development and subsequent enhanced suppressive activity [25, 34]. Additional common surface markers on regulatory T lymphocytes are the cytotoxic lymphocyte-associated antigen-4, short CTLA-4 as well as GITR which are glucocorticoid-induced TNF-receptors-related proteins [16, 23].

Table 1 and 2 represents a list of markers of regulatory T lymphocytes.

Table 1: Summarized markers of regulatory T lymphocytes [16, 23, 25–28, 30, 35–38].

Marker	**Human**	**Mouse**
CCR2	x	x
CCR4	x	
CCR7	x	x
CCR8	x	x
CD103	x	
CD127	x	x
$CD137^{pos}/CD154^{neg}$	x	
CD147	x	
CD25	x	x
CD3	x	
CD39	x	
CD4	x	x
CD45RA	x	
CD49d	x	
CD73	x	
CTLA-4	x	x
FoxP3	x	x
GITR	x	x
Helios	x	x
ICOS	x	x
IL-2	x	

Table 2: Summarized markers of regulatory T lymphocytes (continued) [16, 23, 25–28, 30, 35–38].

Marker	**Human**	**Mouse**
IL-35	x	x
IL-10	x	x
Ki67	x	
LAG3	x	x
LAP/GARP	x	x
Neuropilin-1	x	
NRP1	x	x
OX-40		x
PD-1	x	x
TGF-β	x	x
TIGIT	x	x
TIM3	x	
TNFR2	x	x

5.3 Function of Regulatory T Lymphocytes

Characteristic for regulatory T lymphocytes are suppressive properties, which lead to restrain host diseases as well as graft. Moreover, suppressive immune reactions against pathogens, allergens, or tumor cells are caused by regulatory T lymphocytes. These suppressive activities are not specific for antigens. Due to their suppressive properties Tregs are essential for maintaining immunological self-tolerance [16, 29, 39, 40].
In general, there are four different functions of Tregs leading to their suppressive actions. They are summarized in Figure 4 [41].

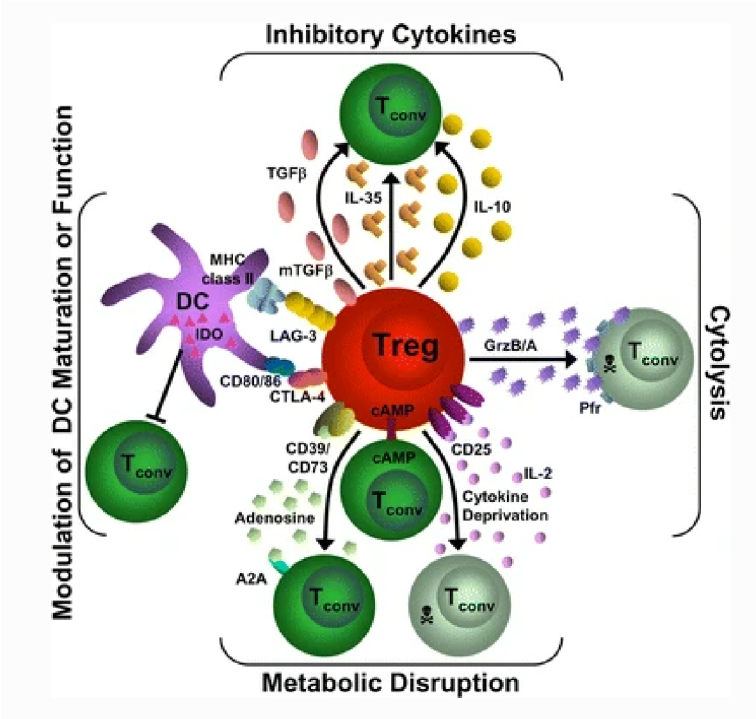

Figure 4: Different mechanisms leading to suppressive functions of regulatory T lymphocytes [41].

The first process is based on IL-10, IL-35 and TGF-β, which are cytokines with inhibitory properties. Regulatory T lymphocytes are expressing and using these cytokines for their suppressive actions [41].

Besides that, Tregs are able to induce cytolysis having either granzyme A (in humans) or granzyme B (in mice) in their environment [41].

In addition, metabolic disruption leads to the suppressive property of regulatory T lymphocytes. Conventional T cells (T_{conv}) are either suppressed by a loss of IL-2 causing cell death or adenosine is produced by the molecules CD39 and CD73. This further activates 2A, which is an adenosine receptor located on T_{conv} cells, leading to suppression of T_{conv} cells. The third possibility of metabolic disruption is the change of inhibitory cyclic antimicrobial peptides (cAMP) into normal T lymphocytes (T_{conv}) through gap linkages [41].

For the last mechanism Tregs need the cytokine interleukine-2 (IL-2) as well as several T cell receptors (TCRs). The main functions of IL-2 are forcing the development of T lymphocytes, increasing the destructive activity of natural killer cells, activating the development as well as differentiation of Tregs. The mechanisms, that are responsible

for the suppressive properties of regulatory T lymphocytes are visualized in Figure 5 [16, 42].

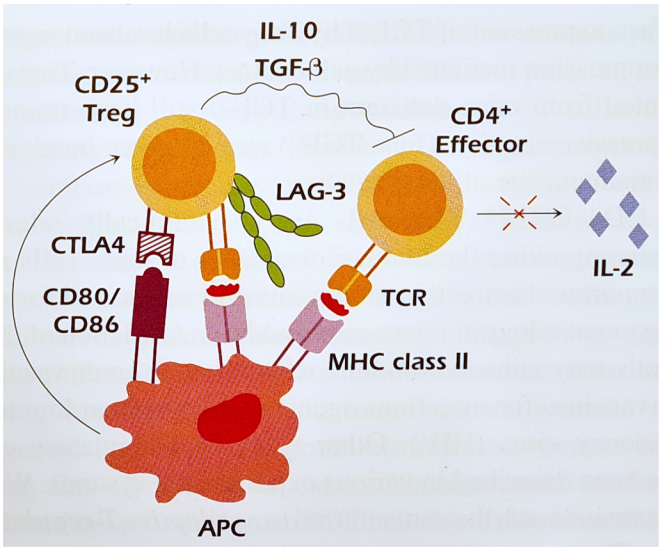

Figure 5: Adaption of dendritic cell development or function [16, 41].

An important molecule for the inhibition of helper T lymphocytes is the protein called CTLA-4, which is found on the surface of regulatory T lymphocytes. Besides that, the surface proteins CD80 and CD86, that are located on the surface of antigen-presenting cells, play a crucial role, because after collaboration of CTLA-4 and CD80/CD86 a co-stimulatory signal is produced which leads to suppressive reactions. Moreover, LAG3 (lymphocyte activation gene 3), which is an adhesion molecule, is found on the surface of Tregs as well. It attaches to MHC-II molecules, which reduce the number of CD80/CD86 molecules on APCs like dendritic cells. This leads to the release of IL-10 and TGF-β, and therefore the activity of effector T lymphocytes is hindered which is characterized by the lack in IL-2 production [16].

Thus, regulatory T lymphocytes inhibit immune reactions of external antigens and self-molecules. This is an important property, because a defect during the development or function of Tregs leads to autoimmune diseases. For example, the lack of Tregs leads to the IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome). Characteristic for the IPEX syndrome are autoimmune diseases in different endocrine organs. In general, an increase of Tregs is crucial for the treatment of autoimmune diseases or for the inhibition of allograft rejections. In contrast, reduction of regulatory T lymphocytes favors several immune reactions to vaccines against tumors

or viruses, such as HIV [16, 39].

5.4 Importance in Cancer Development

Due to the ability of regulatory T lymphocytes to maintain immunological self-tolerance by stopping immune reactions, they may directly infiltrate into the microenvironment of the tumor and bind to tumor cells. There they suppress immune reactions against tumor cells, especially the action of tumor-reactive cytotoxic T lymphocytes required to eliminate the tumor cells. Subsequently, cancer tissues increase the change of naive T lymphocytes into regulatory T lymphocytes, which causes accumulation of Treg cells near the tumor and thus disrupt the development of effector reactions [21, 23, 29, 30, 33, 40]. Besides that, the stability and function of Tregs is improved by increased inflammation in the tumor microenvironment. This is done by an increased number of chemokines which facilitate the migration of regulatory T lymphocytes as well as by enhancing the production of inhibitory cytokines. In addition, blocking inhibitory receptors improve the function of Treg cells and thus are a barrier against cancer immunotherapies [30].
Due to these inhibitory properties of Treg cells, patients suffering for example from either melanoma or ovarian carcinoma have a reduced survival chance when Treg cells are present [21, 23]. Therefore, the reduction of regulatory T lymphocytes may promote the inhibition of endogenous immune mediated tumor and tumor antigen-specific immunity can be improved. Thus, tumor immunotherapies such as CTLA-4 blockade and vaccination is more successful when Treg cells are depleted [23, 33].
A murine model with pancreatic adenocarcinoma shows that the tumor enhances the development of regulatory T lymphocytes via various mechanisms such as stimulation of nTregs and the change of non-Tregs to Tregs. In addition, when analyzing the amount of $CD4^+CD25^+$ T lymphocytes, the number of Tregs in pancreatic adenocarcinoma in mice models increases when the tumor is growing. Similar to the mouse models, the number of Tregs is higher in humans with pancreatic cancer than in healthy ones. They are located in tumor-infiltrating lymphocytes and in the lymph nodes. In patients, having a low amount of regulatory T lymphocytes, the prognosis is more successful than in patients, having a high number of Tregs [23].
In contrast, the addition of $Foxp3^+$ regulatory T lymphocytes leads to an improved

prognosis of certain cancers such as colon and head tumors. This is due to various compositions of their subpopulations [29, 30, 33].

5.5 Therapies Against Tregs

In order to enhance immunity against tumor cells, regulatory T lymphocytes have to be reduced, which is done based on different targeting therapies [43].

The antibody ipilimumab is used to reduce the number of Tregs. It is an monoclonal antibody and especially targets the protein CTLA-4. However, this is disputed, because preclinical studies on mice showed that this antibody reduces Tregs via antibody-dependent cellular cytotoxicity, short ADCC. Whereas in humans, ipilimumab causes infiltration of $CD4^+$ and $CD8^+$ T lymphocytes into the tumor instead of decreasing regulatory T lymphocytes in the tumor microenvironment. Therefore, the precision of using CTLA-4 as a target has to be further studied [43].

Another preclinical targeting approach is using the anti-CD25 antibody that enables depletion of Tregs. In addition, surface proteins on Tregs such as immunoglobulin, G-protein-coupled receptor (GPCR), immune checkpoint receptor and TNFR can be possible targets as well [43].

Besides that, methods which hinder the function of regulatory T lymphocytes in an indirect way such as weaken immunosuppressive cytokines TGF-β, IL-10, IL-35, or hinder adenosine receptors, might be effective as well. In order to re-establish the function of T lymphocytes and subsequently make the tumor microenvironment proinflammatory, IDO is targeted. IDO is the abbreviation for indolamin-2,3-dioxygenase and is an enzyme that decreases tryptophan. Furthermore, tumor associated macrophages, short TAM, as well as myeloid-derived suppressor cells (MDSC) are possible target molecules as well in order to treat Tregs [43].

Due to the low specificity of targeting regulatory T lymphocytes, because several immune cells which are involved in treating tumor cells share the same receptors, there must be approaches which improve the efficiency of treating Tregs [43].

One interesting target is the transcription factor FoxP3. Because FoxP3 is an intracellular marker, it is not reachable for common antibodies and therefore, a method based on T cell receptors is studied. These TCR target certain peptides in Tregs that are

obtained from the transcription factor. Therefore, a so-called TCR mimic antibody that exactly binds the FoxP3 epitopes is created. After binding to the epitopes, Tregs are reduced through antibody-based cytotoxicity, shown in Figure 4A. Using AZD8701, which is a next-generation antisense oligonucleotide FoxP3 inhibitor, is another method to target the transcription factor FoxP3 leading to a significant decrease in FoxP3 development. This approach is visualized in Figure 6B. However, using both FoxP3 targeting approaches might lead to autoimmune diseases. Therefore, a screen based on clustered regularly interspaced short palindromic repeats screen (CRISPR), was performed in order to observe the phenotypes of Tregs. It turned out that ubiquitin-specific peptidase 22, short Usp22, and ring finger protein 20, abbreviated as Rnf20 regulate the expression of FoxP3. Experiments in which a Usp22 knock out (KO) mouse model, specific for Tregs was used, proofed that the expression of FoxP3 was decreased, the suppressive functions of Tregs were hindered and immunity against tumors was improved. Figure 6C illustrates the expression regulators of FoxP3 [43].

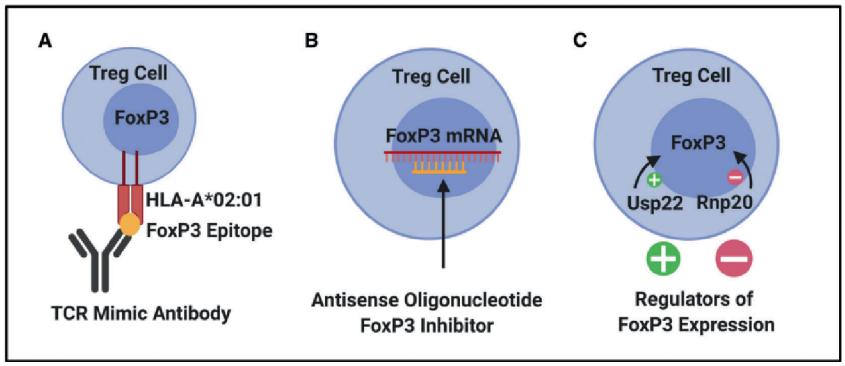

Figure 6: Illustrations of FoxP3 targeting approaches [43].

Besides that, the specificity of targeting Tregs is increased by using near-infrared photoimmunotherapy, short NIR-PIT. Therefore, a specific antibody is connected to a dye which is photoactivatable and sensitive to near-infrared light. After this antibody targets a certain receptor on Tregs, the NIR light is conducted to the tumor which makes direct targeting of Tregs possible. Another localizing therapy is injecting the anti-CD25 immunotoxin 2E4-PE38 intratumorally, which significantly decreases the amount of Tregs only in the tumor (not spleen). Because of the low immunotoxin concentration in the blood, distant cells such as $CD4^+$ and $CD8^+$ T lymphocytes in the spleen are not damaged. Both localizing methods are shown in Figure 7 [43].

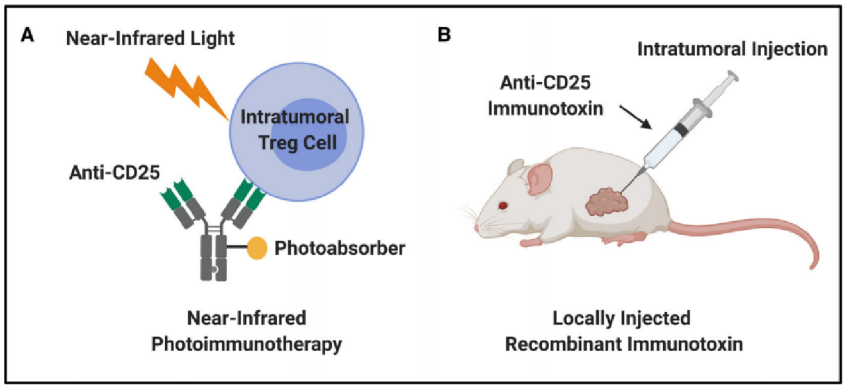

Figure 7: Graphic representation of localizing treatments [43].

Bispecific antibodies increase the specificity as well. An example for a bispecific antibody is the CTLA-4 x OX40 antibody. It hinders both CTLA-4 as well as the agonist OX40 at the same time which further leads to a direct reduction of intratumoral regulatory T lymphocytes and the stimulation of tumor-infiltrating effector T lymphocytes [43].
Additionally, an anti-CTLA-4 antibody is constructed in a way that Tregs located in the tumor microenvironment are reduced while Tregs in distant tissues are not affected. Therefore, the antibody is composed of an anti-CTLA-4 binding domain in the inside covered by a domain that targets tumors on the outside. After the tumor is reached by the antibody the outer part is cleaved by MT-SP1. MT-SP1 is the abbreviation for membrane type-serine protease 1 which is found in the tumor microenvironment. Subsequently, the inner part is revealed and the anti-CTLA-4 antibody is directly set free into the tumor microenvironment [43].
Furthermore, the kinetics of receptor expression differs based on the different immune cells. This can be used to improve the targeting specificity. As an example, Tregs express the protein CTLA-4, which is upregulated after TCR is stimulated. In contrast, effector T lymphocytes only express CTLA-4 after TCR is activated [43].
To sum up, the use of the monoclonal antibody ipilimumab which targets CTLA-4 on regulatory T lymphocytes is to date accepted by the Food and Drug Administration, short FDA. Additionally, several techniques are currently investigated in clinical trials, such as using an anti-CD25 antibody, the technique that is based on TCR to target FoxP3 peptides, the bispecific antibody CTLA-4 x OX40 and next-generation approaches [43].

5.6 Detection Techniques

Regulatory T lymphocytes can be detected from blood or tissue samples according to their markers mentioned before. A common method which is used for detecting Treg cells is flow cytometry analysis and sorting of cells based on their CD and other markers [44]. In general, flow cytometry enables a fast analysis of several parameters of different cells or cell populations. Therefore, lasers are needed as light sources in order to get fluorescent or scattered signals. The obtained signals are changed into electronic signals which are further read by the computer. There are several types of flow cytometry used in immunology such as immunophenotyping described above, which can be used to detect regulatory T lymphocytes. For this it is important to have a specific group of markers to get rid of possible disturbances from other cells. In order to make the transcription factor FoxP3 visible, intracellular staining with different fluorochrome-connected anti-FoxP3 antibodies is a common method. In addition, flow cytometry can be performed based on antigen specific responses as well as intracellular cytokine analysis, proliferation analysis and apoptosis analysis are additional methods [44, 45].

Besides flow cytometry analysis, another method to evaluate the amount and most importantly the localization of Tregs in tissue samples is labelling these cells immunohistochemically. Immunohistochemistry uses both, monoclonal and polyclonal antibodies in order to detect different antigens. With this the occurrence of antigens in different tissues is analyzed which makes it important for the diagnosis of several diseases especially of specific cancers [40, 46]. Another important advantage of immunohistochemistry staining of tissue slides is co-staining with hematoxylin and eosin (nuclei and extracellular matrix staining) enabling better analysis of tumor microenvironment [47].

Apart from these methods an important parameter for estimating regulatory T lymphocytes in tumor-bearing mice is the lymphocyte proportion of $CD4^+CD25^+$ to $CD4^+$ in the spleen and other secondary lymphatic organs [23].

Another detection technique which is used to identify regulatory T lymphocytes is single cell transcriptomic analysis, also known as single-cell RNA sequencing. Using this technique different immune phenotypes such as patterns of clonal expansion of different T lymphocyte families are investigated [48].

Due to epigenetic changes, such as different methylation structures it can be more clearly

distinguished between Tregs and effector T lymphocytes. In addition, Tregs can be identified by immunohistochemical approaches. However, for these methods a high number of tissues is needed. Besides that, the number of different cell types that can be analyzed at the same time as well as the amount of markers is limited [43].

6 Immunotherapy

The immunotherapy is an alternative treating method for several types of cancers which allows to use patients' immune system against cancer cells and therefore to treat cancer. It consists of different treating methods such as blocking immune checkpoint molecules, cancer vaccines, adoptive cell therapies etc. [7, 13].
As described above, the immune system is able to recognize and eliminate malignant cancer cells. In contrast, cancer cells are able to overcome this recognition and elimination of the immune system and proliferate [7]. The better understanding of the relationship of cancer and immune system enables us to target these regulation mechanisms and to increase the effectivity of anti-tumor mechanisms.

6.1 Different Types of Immunotherapy

To stimulate the processes that work against inhibitors and to favor the activity of the natural immunity of patients, different types of therapies can be applied [7]. Cancer immunotherapies can be divided into an active or passive therapy. The aim of active immunotherapies is to enhance the self-immunity to fight against tumor cells through non-specific immunomodulation, vaccination or targeting specific antigen receptors. In comparison, passive immunotherapies regulate the anti-tumor response of different agents like lymphocytes or cytokines [13].

6.1.1 Immune Checkpoint Inhibition

Cancer cells may be detected by the immune system as foreign antigens [7]. During immune reactions T lymphocytes are activated. For this activation the antigen must be presented to the matching T cell receptor (TCR) by the major histocompatibility complex (MHC) which is located on antigen-presenting cells (APCs). For a complete activation costimulatory CD28 and B7 molecules are needed. This step is controlled by inhibitory checkpoints which prevent autoimmune diseases [49]. However, tumors are able to express ligands which bind T cell receptors and thus suppress T lymphocytes resulting in resistance of tumor cells against immune anti-tumor responses [7].

Inhibition Using CTLA-4 Receptor One method includes suppression of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) which was discovered in the 1980s. With using this receptor, the activation of T lymphocytes is decreased, pre-existing T lymphocyte reactions against the cancer are activated and furthermore it is possible to elicit new immune responses [7, 49].

In general, the co-inhibitory molecule CTLA-4 functions as a regulator of the amount of T lymphocyte activation. After CTLA-4 reacts with the ligands CD80 and CD86, T lymphocyte activity is inhibited, and tumor development may be promoted. Therefore, this interaction has to be blocked to keep the T lymphocytes activated and further to destroy cancer cells [50]. The first part in Figure 8 shows that two immunological signals are needed for the activation of T lymphocytes. The first signal is activated after the recognition of an antigen by a TCR. In addition, CD28 is activated through the co-stimulatory molecule B7. The CTLA-4 receptor is expressed by the T lymphocyte and the B7 molecule by the antigen-presenting cell. After the B7 molecule interacts with the CTLA-4 receptor, it is not possible for the T lymphocyte to get completely activated [7].

In addition, in the second part of Figure 8 an antibody such as ipilimumab is used to inhibit the CTLA-4 pathway. Finally, this inhibition leads to the activation of T lymphocytes. However, there are some disadvantages of using the antibody ipilimumab. As an example, because of the unspecific increase of T lymphocytes in connection with the CTLA-4 receptor there might be severe immune-associated side effects in the patient. Moreover, it can take some month to observe an effective immune response [7, 49].

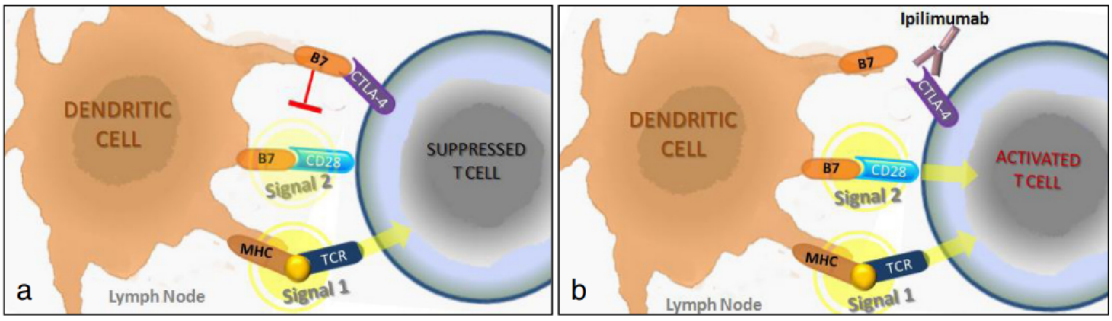

Figure 8: Inhibition using CTLA-4 receptors [7].

Inhibition Using PD-L1/PD-1 Receptor Another used inhibition receptor is programed death 1 (PD-1), which was discovered in 1992 [49].

After the activation of T lymphocytes, that occurs for example as a reaction to inflammation, they imitate expression of PD-1. However, tumor cells protect themselves by expressing PD-L1 that creates a bond with PD-1 on T lymphocytes to make the T lymphocytes inactive. Thus, this linkage may be blocked by monoclonal antibodies that bind to PD-1 or PD-L1 and therefore, lead to T lymphocyte-mediated tumor cell death in several tumor types [50].

There are two ligands named PD-L1 and PD-L2. Both, PD-L1 and PD-L2 are responsible for local inhibition of T lymphocytes within the tumor microenvironment. Generally, PD-L1 expression belongs to adaptive immune resistance mechanisms because the ligand is persuaded by IFNγ, which expression is through an active anti-tumor immune reaction. After PD-1 expression on the T lymphocyte, PD-1 binds to PD-L1 on tumor cells, resulting in T lymphocytes suppression, hindering immune reactions against the tumor. To prevent the binding of PD-1 and PD-L1, anti-PD-1 antibodies such as pembrolizumab, nivolumab and atezolizumab are needed [7, 49, 51]. When comparing the two receptors CTLA-4 and PD-1, PD-1 mainly affects the occurring immune reactions and CTLA-4 is mainly responsible for the regulation of new immune responses. Moreover, when PD-1 and PD-L1 are bound, the PI3 kinase signaling pathway is deactivated which suppresses the production of cytotoxic mediators that are needed for destroying healthy cells, but as soon as the inhibition is increased this suppression is immediately reversible. The main advantage of the interaction between PD-1 and PD-L1 are prolonged responses [7].

Figure 9 represents the inhibition using PD-1/PD-L1 receptors [7].

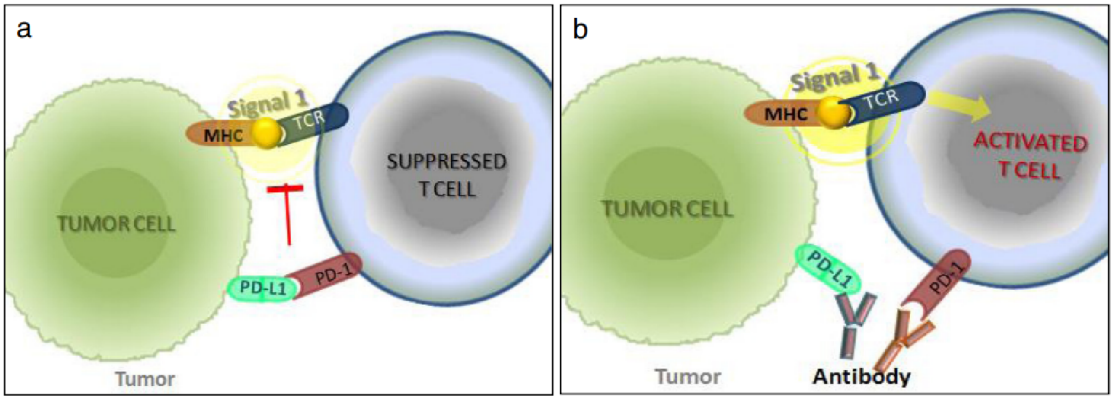

Figure 9: Inhibition using PD-L1/PD-1 receptors [7].

Anti-CD40 Antibody The membrane protein CD40 is part of the TNF-receptor superfamily and is basically found on antigen-presenting cells such as monocytes, dendritic cells, as well as B lymphocytes. In some cases, it is also expressed on non-immune cells such as B cell lymphomas and on epithelial malignancies. The bidirectional linkage between CD40 and CD40L is relevant for activating humoral immune reactions which are dependent on T lymphocytes as well as for cytotoxic T lymphocyte responses. These responses permit antigen-presenting cells to express the antigen and to stimulate responding precursors of $CD8^+$ cytotoxic T lymphocytes. In addition, cytotoxic myeloid cells are developed which are responsible to control cancer growth without T lymphocyte immunity. Therefore, agonistic CD40 monoclonal antibodies basically differ from other monoclonal antibodies like anti-PD-1 or anti-CTLA-4 because they do not inhibit negative immune checkpoints. The immunostimulatory properties of CD40 as well as the expression pattern on a variety of cancerousness demonstrate that CD40 is an interesting target for agonistic antibody treatments. In this process there are several mechanisms included, namely forming immune effectors like natural killer (NK) cells and complement-dependent cytotoxicity cells, direct signaling which causes apoptosis in cancerous cells as well as forcing antigen presentation. The therapy results in successful and long-lasting immune reactions against different types of cancer [52, 53].

Further Checkpoint Inhibition Therapies There are further immune checkpoint inhibition therapies in progress which contain the lymphocyte activation gene 3 protein (LAG3) as well as T cell immunoglobulin and mucin domain-containing 3 protein

(TIM3). The LAG3 protein reveals to T lymphocytes and therefore it is a selective indicator of regulatory T lymphocytes, which further means that it is involved in immune suppression by tumors. Based on these facts, it could be possible that the inhibition of LAG3 could improve antitumor immunity due to reversed exhaustion of T lymphocytes and it is either used as monotherapy, combined with anti-PD-1, or conventional therapies [7].

The efficacy of checkpoint inhibition therapies is often improved when they are combined with other treatments such as chemotherapy. However, this treating method or checkpoint inhibitors alone have some disadvantages such as the cause of side effects in several organs including autoimmunity. Another problem is non-responsiveness of patients ranging from 20-50%, depending on the tumor type. Moreover, different tumors create different microenvironments and thus appropriate methods must be developed [50, 54].

6.1.2 Adoptive Cell Therapy

Adoptive cell therapy, short ACT, is an infusion with antiviral, anti-inflammatory or antitumor properties. Such therapy is based on the separation of lymphocytes from the patient such as, from tumor, peripheral blood or tumor-draining lymph nodes and subsequently activated ex vivo. Afterwards, the cells are transferred back to the patient, resulting in high amount of effector T lymphocytes with anti-tumor properties [7, 55].

In general, there are three different types of adoptive cell therapy which are based on tumor-infiltrating lymphocytes, short TILs, altered T cell receptors (TCRs) and CAR T lymphocytes, which are chimeric antigen receptor T lymphocytes [55, 56].

When using the therapy based on tumor-infiltrating lymphocytes, a combination of $CD4^+$ and $CD8^+$ T lymphocytes, developed from the surgically removed metastatic tumor, are collected and expanded ex vivo before transferring them back into the patient. Besides that, before reinfusion of regulatory T lymphocytes, they are grown in a mixture of different cytokines, which inhibit immunosuppressive functions of Tregs in the TME [7].

Before infusion of TILs, lymphodepleting is important for completely healing the patient. This is because lymphodepleting on the one hand rises the number of IL-7 and IL-15, which are homeostatic cytokines and on the other hand decreasing regulatory

T lymphocytes as well as myeloid-derived suppressor cells, which have immunosuppressive properties [7].

However, there are some disadvantages of TILs. Although the ACT is more efficient after lymphodepletion, this therapy might also have deadly side effects. In addition, developing the needed cells is cost and time intensive. Besides that, it is only possible to treat melanoma with the described therapy, because only the lymphocytes from melanomas are steadily sensitive against the own tumor and therefore, tumor-infiltrating lymphocytes are currently clinically active only in melanomas [7].

The remaining two ACT types contain T lymphocytes of the peripheral blood that are modified using gene transfer [55].

Figure 10 compares TCR and CAR T lymphocytes [55].

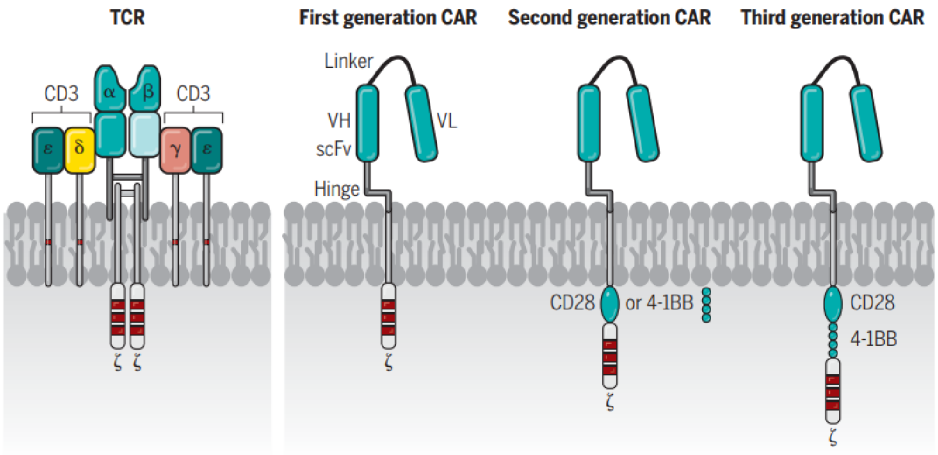

Figure 10: TCR compared to CAR T lymphocytes [55].

T cell receptors are composed of both, α-chain and β-chain, which are connected to the CD3 complex, located on the surface of the T lymphocyte. After the TCR realizes peptides attached to the MHC, that is located on the tumor cell surface or on APCs, T lymphocytes are activated [55]. This method is only applied, if the tumor possesses the related human leukocyte antigen allele and if for the T cell receptor it is possible to recognize the target antigen [7].

In comparison, chimeric antigen receptors include an immunoglobulin domain (variable) connected to a T cell receptor domain (constant) as well as further domains like OX40, CD137 as well as CD28, which have costimulatory properties. A main advantage of CARs compared to modified TCRs is that they are not dependent on the expression of MHC,

which enables T lymphocytes to recognize the target without any need of the MHC. This property is crucial especially when it comes to antitumor mechanisms, because the main part of tumor's immunoevasion is the lack of presenting antigens via the MHC [7, 55]. There are some disadvantages when using both TCRs and CARs, such as healthy tissues might express a similar target antigen which causes a secondary eradication of healthy tissues and development of autoimmunity as well as targets that are located outside the tumor cells are required [7, 55].

6.2 Intratumoral Application of Immunotherapy

The described checkpoint inhibition mechanisms usually target only a single molecule of the adaptive immune system, personalized vaccination can be quite expensive and for tumor cells it is possible to mutate rapidly. In addition, the described therapies might have lethal side effects, because the used agents might attack unwanted tissues as well. Another disadvantage of checkpoint inhibition therapies is that in order to get to the tumor microenvironment, different biological barriers have to be passed [7, 49–54]. Therefore, intratumoral applications may be used to overcome the disadvantages of systemic applications. Besides that, intratumoral immunotherapies are used because most of the tumors are immunologically cold and without any immune cell infiltration, checkpoint inhibitors are not working there. In addition, intratumoral application enables local stimulation of the immune system, resulting in better targeting of antitumor mechanisms within the tumor. Moreover, lower concentrations of drugs may be used during intratumoral administration compared to systemic administrations, leading to reduced toxicity [56].
Figure 11 summarizes the different types of intratumoral immunotherapy [56].

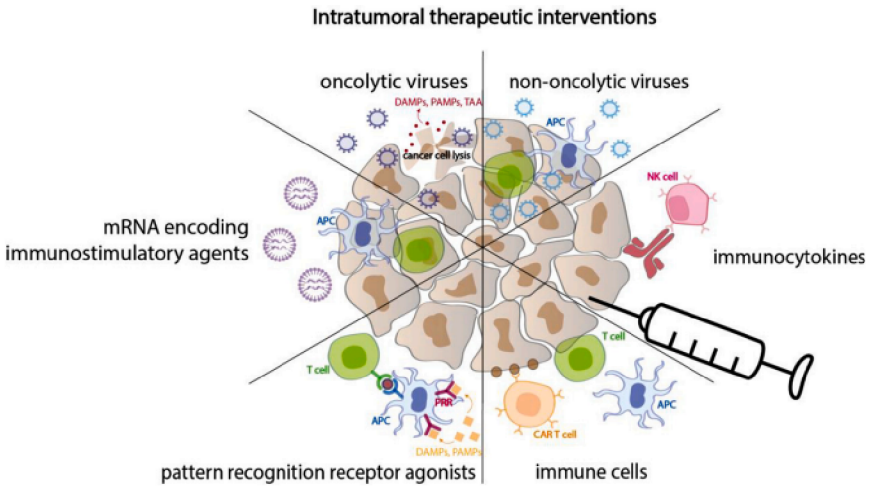

Figure 11: Summary of intratumoral applications [56].

6.2.1 Oncolytic and Non-oncolytic Viruses

Oncolytic virus therapies are based on human viruses, that are not virulent anymore, or on genetically modified viruses. These viruses are able to replicate only in tumor cells and in some cases even kill the tumor cells. The reason for the specificity is, that tumor cells commonly lack in protective mechanisms against viruses leading to decreased toxicity against healthy cells. During the death of tumor cells additional viral molecules are set free leading to immunogenicity induced by PAMPs. In addition, antigens that are specific to tumors activate reactions from the adaptive immune system. Examples for viruses that are commonly used are vaccinia viruses, adenoviruses, herpes simplex viruses etc. [56].

There are two methods of non-oncolytic virus therapies. The first one uses oncolytic viruses that are inactive and not able to replicate. A representative of this approach is the vaccinia virus Ankara, abbreviated as iMVA which is able to increase immunity against cancer. After dendritic cells are treated with iMVA, IFN-1 as well as chemokines and cytokines that are proinflammatory are secreted. In general, iMVA is able to modify the TME in a way to make it more immunostimulatory especially when effector T lymphocytes are activated. Antitumor immune reactions are improved when combining the described treatment with immune checkpoint inhibitors [56].

The second method uses non-oncolytic viruses such as the seasonal influenza vaccination, which works based on the ability of foreign antigens that initiate immune reactions in the

TME and leads to an increase of dendritic cells and $CD8^+$ T lymphocytes. The yellow fever vaccine is an additional example for non-oncolytic virus vaccinations. It contains living weakened viral strains that induce immunity against tumor cells mainly caused by $CD8^+$ T lymphocytes. In addition, the efficacy can be increased by performing pre-immunization, especially by producing immunity based on CD4 and CD8 lymphocytes [56].

6.2.2 Immunocytokines

Immunocytokines consist of an antibody fused with a cytokine, whereas the antibody enables to specifically target the microenvironment of the tumor leading to immune reactions against the tumor. In contrast, cytokines are used as immunomodulators and especially IL-2, IL-12 and TNF are responsible for the attraction and infiltration of B and T lymphocytes and other leukocytes to the TME. However, this approach might cause systemic toxicities or toxicities at neighbor cells also known as a cytokine storm. These bad side effects are minimized if an antibody is used, that is characteristic to tumor-associated receptors [56].

6.2.3 Immune Cells Transfer

A common approach, where immune cells are transferred intratumorally, is the dendritic cell therapy. Therefore, dendritic cells are used which express CCL21, that is a chemokine ligand, responsible for bounding between dendritic cells and T lymphocytes. For dendritic cell transfection an adenoviral vector is needed which expresses CCL21/SLC, that is a secondary lymphoid tissue chemokine. This dendritic cell therapy results in higher PD-L1 expression on tumor cells, increased penetration of $CD8^+$ T lymphocytes as well as efficient immune reactions. Thus, the reason for using dendritic cell treatments is to better regulate the tumor microenvironment in immunologically cold tumors in a way that a small number of dendritic cells are able to penetrate the tumor bed [56].

6.2.4 Pattern Recognition Receptor Agonists

In general, the main receptors that are responsible for the activation of the innate immune system are pattern recognition receptors, short PRRs. These PRRs consist of five

groups, which are Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), RIG-I-like receptors (RLRs), DNA sensors, and C-type lectin receptors (CLRs). Commonly, antigen-presenting cells, especially dendritic cells express these receptors. The receptors have the ability to recognize PAMPs, which are pathogen-associated molecular patterns, such as for example nucleic acids, lipopolysaccharides or even bacterial flagellins. In addition, DAMPs, which are known as damage-associated molecular patterns, are recognized by TLRs in order to keep homeostasis. DAMPs are able to activate APCs which subsequently leads to the activation of T lymphocytes. Examples for DAMPs are the compounds adenosine triphosphate and calreticulin [56, 57]. In the following paragraph toll-like receptor agonists are described in more detail. Due to their antitumor properties, they are used as vaccine adjuvants in immunotherapies against cancer [57–59].

Toll-like Receptor Agonists Toll-like receptor agonists are able to induce innate immune cells as well as to enhance the uptake and presentation of antigens which further leads to innate immune reactions. Subsequently, cytokines are induced which further activate the adaptive immune system. Thus, TLR agonists function as costimulatory factors. In general, human possess ten different groups of TLRs, which are located on immune cells, such as monocytes, dendritic cells, or macrophages as well as on tumor cells. TLRs are proteins, that are made of between 800 and 1000 amino acids, dependent on the localization of expression, which is either on the cell or endosomal plasma membrane. The localization of TLRs is crucial when it comes to immunotherapies. If TLRs are expressed on the plasma membrane, it is possible for TLR agonists to recognize them immediately leading to a fast activation of immune reactions. In contrast, if TLRs are located on the endosomal membrane of immune cells, immediate immune reactions are hardly possible. After the TLR and TLR agonist are bound together, different pathways are induced. As an example, the nuclear factor κB (NF-κB) is stimulated by TLR agonist through a pathway, which further controls the inflammation reaction in the tumor microenvironment. There are different TLR types which have different properties. For example, TLR3 and TLR5 show direct properties against tumor development such as apoptosis of cancer cells. In comparison, TLR4, -7, -8 and -9 demonstrate tumor promoting effects like forming chemoresistant phenotypes, proliferation of cancer cells or

forcing surveillance. Therefore, the choose of TLR agonists should be depended on TLR expression and on the effective results of TLR signaling after the reaction with a certain kind of tumor [57, 59, 60].

6.2.5 Immunostimulatory mRNA Agents

The administration of mRNA is a quite simple and safe method and is kind of a transcript-based immunotherapy. Since mRNA encodes proteins in a direct way, the probability for an insertion mutation is rather small compared to DNA. In addition, a small amount of mRNA is required since for a single mRNA molecule it is possible to copy a protein various times. Reasons for taking immunostimulating mRNA agents are producing a TME that is immunogenic as well as binding different cell receptors that are found in the TME. But the mRNA might be quickly destroyed by ribonucleases. Therefore, the mRNA has to be protected using proper packaging methods which lead to an improved transfer to the target cells as well [56].

7 MBTA Immunotherapy

Since several tumor types, such as pancreatic adenocarcinoma and pheochromocytoma, hardly have an immune cell infiltration (immunologically cold tumors), there is higher chance that existing systemic immunotherapies targeting only adaptive immune cells will not work in these tumors. Therefore, the team around Dr. Zenka have developed intratumoral immunotherapy on the basis of the combination of mannan-BAM, TLR ligands and anti-CD40 antibody (referred to as MBTA therapy) resulting in the stimulation of adaptive and innate immunity [61–63].

7.1 Principle of MBTA Immunotherapy

The principle of MBTA immunotherapy is based on infiltration and activation of innate and adaptive immune system within the tumor via intratumoral injection and subsequent recognition of tumor cells. This process forces phagocytosis of cancer cells and further enables recognizing tumor antigens. This strategy causes adaptive immune reactions which are tumor-specific [61].

For this type of immunotherapy several compounds such as mannan, TLR ligands as well as agonistic anti-CD40-monoclonal antibodies are needed. Mannan is a polysaccharide from *Saccharomyces cerevisiae* and functions as the ligand which activates the complement system, opsonization and subsequent phagocytosis of the tumor. To properly activate the opsonization of cancer cells, mannan has to be bound to tumor cells by a biocompatible anchor for cell membrane (abbreviated as BAM). The connection between mannan and BAM (short Mannan-BAM) enables the bounding between mannan and the cell membranes by the hydrophobic oleyl group of BAM. This leads to the recognition of mannan via pattern recognition receptors, concretely mannan-binding lectin. Furthermore, the complement is activated through the complement lectin pathway and phagocytosis, or frustrated phagocytosis of tumor cells occurs [61, 62].

The main principle is shown in Figure 12 [61].

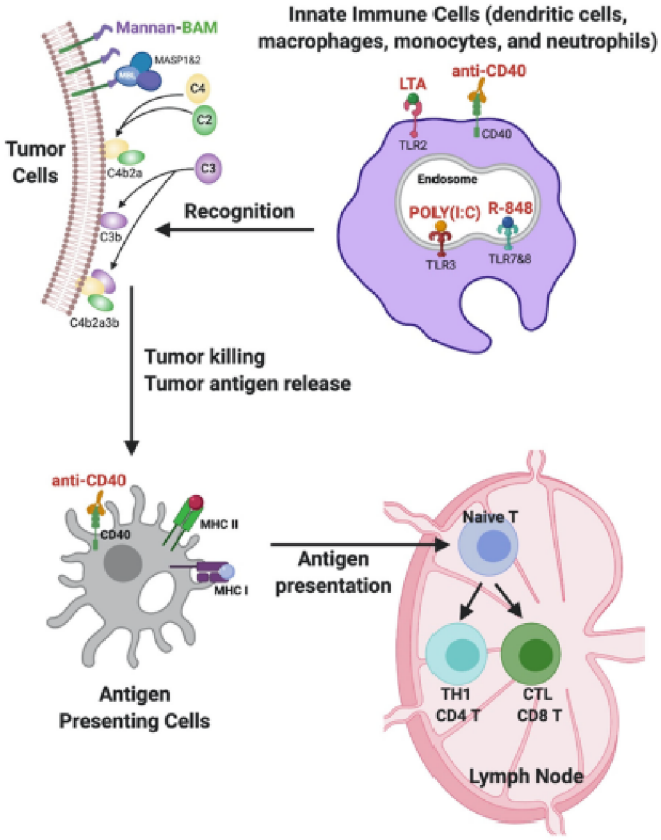

Figure 12: Principle of MBTA immunotherapy [61].

The inductive reaction of mannan-BAM on cells of the innate immune system is increased by various inflammatory processes which are forced by three toll-like receptor ligands, namely polyinosinic-polycytidylic acid (poly(I:C)), lipoteichoic acid (LTA) and resiquimod, abbreviated as R-848. The compound poly(I:C) is derived synthetically from viral dsRNA and starts the TLR3 signaling pathway, followed by activating APCs. This leads to adaption of tumor-phenotype associated macrophages to immunosupportive phenotypes. The second compound, lipoteichoic acid (LTA) gained from *Bacillus subtilis*, functions as an activator of TLR2 mediated inflammatory mechanisms that improves the TNFα production. The compound R-848 consists of imidazoquinolinamine and is synthetically derived from viral ssRNA. It is responsible for innate immune cell activation by starting TLR7-mediated signaling and Th1 cell immunity is induced. Finally forced anti-CD40 monoclonal antibodies are needed as an imitation of a CD40 ligand to activate antigen-presenting cells through the bond of CD40L. By ligation of CD40 with anti-CD40-mAb, dendritic cells, monocytes and B lymphocytes are activated and thus,

the adaptive immune system is induced [61, 62].

7.2 MBTA Therapy in Different Cancer Types

The MBTA immunotherapy was tested in different types of cancer, which were melanoma, pancreatic adenocarcinoma, pheochromocytoma, colon cancer and glioblastoma [61, 62, 64, 65].

Melanoma Melanoma is a malignant form of skin cancer, caused by mutations in cells that are responsible for pigment production, called melanocytes [66]. MBT immunotherapy, which is a therapy without anti-CD40, led to complete elimination of B16-F10 melanoma in 80% of mice which already was in an advanced growing stage, to anti-metastasis properties as well as to withstand re-transplantation of the tumor. Besides that, activation of the adaptive and innate immune system as well as their participation in this procedure could be observed [64].

Pancreatic Adenocarcinoma In general, pancreatic adenocarcinoma is the most abundant pancreatic tumor, which mostly has effect on the top of the pancreas [67]. The inhibition of tumor development in pancreatic adenocarcinoma mouse models was less effective after a MBT therapy than in melanoma mouse models. In addition, the results showed that there was no mouse treated with success. However, combining the MBT therapy with an agonistic anti-CD40 antibody, 80% were successfully treated [65]. Besides small tumors, larger pancreatic adenocarcinoma tumors were also studied. The experiments showed that there is a dependency of total elimination of tumor cells after MBTA immunotherapy on the size of the tumor at the beginning. Additionally, when analyzing $CD4^+$ and $CD8^+$ T lymphocytes, it was observed that $CD4^+$ T lymphocytes are not important when it comes to decreasing tumor development. However, these cells are crucial for longer survival, delayed tumor reactions as well as protection for retransplantations. Furthermore, it turned out that the memory which is caused by MBTA therapy is specific to antigens and thus specific to every type of tumor [65].
When studying bilateral pancreatic adenocarcinoma (Panc02), it was observed that only the development of the tumors was decreased by MBTA immunotherapy. Therefore,

MBTA therapy had to be improved which was done by treating the parallel tumor with different methods such as chemoablation, check-point inhibitors, radiotherapy, or the change of tumor desmoplasia. However, the most effective response was achieved after MBTA therapy was applied in both tumors simultaneously [68].

Pheochromocytoma Pheochromocytoma, short PHEO, is a neuroendocrine tumor, which is evolved from neural crest cells [63].
Besides in pancreatic adenocarcinoma, MBT therapy was tested in pheochromocytoma (PHEO) mouse models, which again resulted in reduced tumor growth and increased survival. Studying SCID mice having PHEO showed the importance of innate and adaptive immunity when it comes to reducing the development of tumor. SCID mice are severe combined immunodeficient mice, which means they lack in B and T lymphocytes. In general, there was an increased number of $CD45^+$ leukocytes after the MBT therapy, which was investigated using flow cytometry analysis and immunohistochemistry staining. Moreover, it was found out that the main leukocytes within the tumor after MBT treatment were $CD3^+$ T lymphocytes. With immunohistochemistry staining it was possible to localize different immune cells and it turned out that immune cells were able to penetrate the whole microenvironment of the tumor. The combination of MBT therapy and anti-CD40 antibody yielded to an overall improvement of mouse survival as well as to a total elimination of PHEO tumor cells in most of the treated mice. In addition, retransplantation of tumor cells showed that the adaptive immune system including its immune memory was involved [65].
Similar to the pancreatic adenocarcinoma study, a bilateral PHEO tumor model was also tested. It turned out that after MBTA therapy was applied, there was a reduction of tumor development and an improved survival, similar to PHEO mice, was observed. However, no mouse was totally healed after the treatment. When analyzing the microenvironment including the immune memory of the bilateral PHEO mouse model, the MBTA therapy resulted in lower efficiency including increased burden. Immune cells were analyzed using flow cytometry and it turned out that it was possible for the innate and the adaptive immune cells to penetrate distal or non-injected tumors, except for neutrophils, which were not found in non-injected tumors. In addition, an effective penetration of both cytotoxic $CD8^+$ and helper $CD4^+$ T lymphocytes was observed in

non-injected as well as in MBTA-injected tumors, whereas in the tumors $CD4^+$ T lymphocytes are the most common ones. But the proportion between effector/effector memory $CD8^+$ T lymphocytes and effector memory $CD4^+$ T lymphocytes was significantly increased. The evaluation of central memory T lymphocytes resulted in the fact, that their number only rose in the spleen. Furthermore, the activation of T lymphocytes was verified due to the reduction of naive T lymphocytes in the spleen. From another experiment it was observed that immune memory after a MBTA immunotherapy was carried out by $CD4^+$ T lymphocytes [65].

Colon Cancer The MBTA immunotherapy was additionally tested in colon cancer and it turned out that it activated strong adaptive immune reactions that were specific to tumors. These reactions regulated and reduced the development of tumor cells. Additionally, it was observed that recurring antigens that were related to colon cancer were successfully destroyed, which means that MBTA therapy effectively induced memory over a longer period. When evaluating the adaptive immune cells it was observed that after MBTA treatment the number of B and $CD8^+$ T lymphocytes was significantly higher in the tumor microenvironment. In addition, $CD8^+$ T lymphocytes expressed both TNFα and IFNγ, which rose the cytotoxicity of them [61, 62].
When studying the MBTA therapy in bilateral colon cancer, the development of cancer was reduced, and a longer survival of treated mice was observed. The development of both tumors was completely decreased in around 30% of all mice that were treated with MBTA therapy. Additionally, it was seen that the number of both monocytes and dendritic cells rose in the distal tumor too and only the number of neutrophils was increased in the MBTA-administered tumor [63].

7.3 Reasons for Not Using Intratumoral MBTA Therapy

Since intratumoral administration of MBTA may have some technical difficulties, an alternative form of delivery of the therapy is being developed, which does not rely on intratumoral injections [61]. It is based on irradiated cells that are mixed with MBTA therapy in vitro, which enables mannan-BAM to bind tumor cells. Now, it is possible to inject this mixture subcutaneously. This therapy promotes presenting tumor antigens,

eliminating tumor cells as well as stimulating adaptive immune reactions that are specific to tumors. Up to now, the therapy has not been tested in patients. It was only administered in murine syngeneic GBM tumor models consisting of SB28 and GL261 cells. In addition, it was tested in an immunological cold tumor model, namely in 4T1 breast tumor. Therefore, in further studies the combination of MBTA with irradiated cells will be used to treat tumors that are difficult to reach [69, 70].

The first step of this MBTA vaccine therapy is the isolation and expansion of the patient's tumor tissue via cell culture. Before boosting tumor cells with mannan-BAM, TLR ligands and anti-CD40 antibodies the cancer cells are irradiated. The next step is injecting the treated cells subcutaneously into a distant peripheral site. This causes a local innate inflammatory reaction and innate effectors like neutrophils, macrophages and dendritic cells are recruited. Now inflammasomes are produced and irradiated tumor cells are destroyed. This results in creating native immunogenic neoantigens that are treated by antigen-presenting cells. As a result, adaptive immunity is activated and these anti-tumor reactions are memorized by $CD4^+$ T lymphocytes [62, 63].

These reactions are demonstrated in Figure 13 [62].

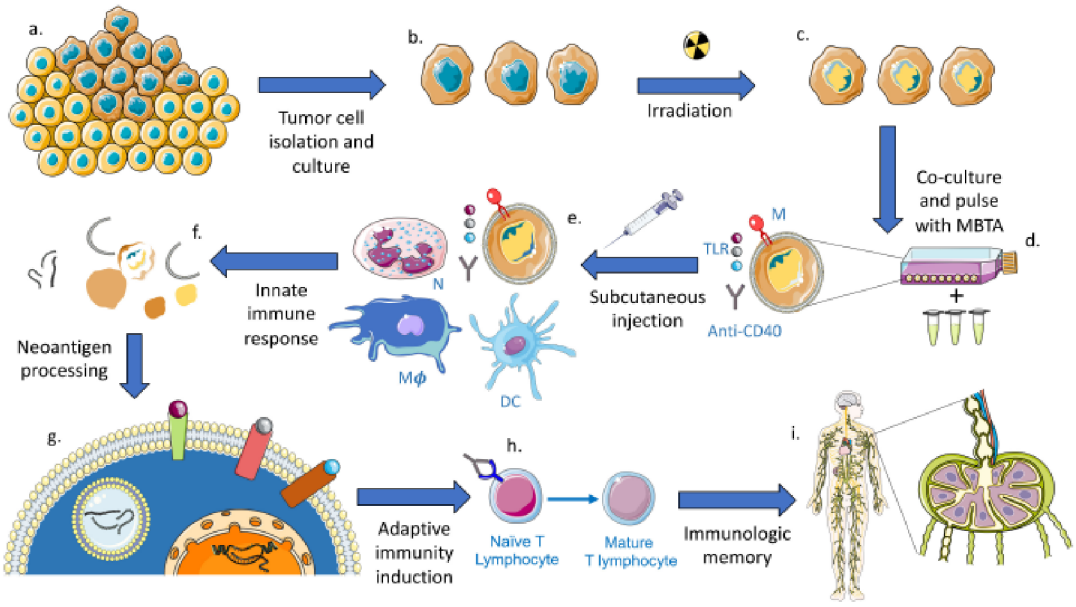

Figure 13: MBTA Therapy combined with irradiated cells [62].

The cancer cells are destroyed due to initiation of apoptosis, stop of the cell cycle and mitotic disaster [62].

8 Conclusion

The main aims of this thesis were to summarize the current knowledge regarding regulatory T lymphocytes (Tregs) and intratumoral MBTA immunotherapy as well as to suggest future steps to analyze and target these cells, which may be further used to increase the efficiency of the therapy.

As described in chapter 7.2, the efficiency of MBTA immunotherapy is decreasing within advanced primary tumors as well as in bilateral tumor models mimicking distal metastasis with higher tumor burden in pancreatic adenocarcinoma or pheochromocytoma [65]. One of the reasons for the low efficiency might be caused by suppressive properties of Tregs [16, 29, 39, 40]. There is no direct focus on the role of Tregs during MBTA therapy, with one exception. In order to improve the MBTA immunotherapy in bilateral pancreatic adenocarcinoma mouse model, it was combined with anti-CTLA-4 antibody. One reason of using anti-CTLA-4 antibody was the expectation that regulatory T lymphocytes are decreased by the antibody which subsequently promotes immune reactions. However, this approach did not lead to successful improvement of the therapy [68]. A reason why this combination did not work might be the fact, that the anti-CTLA-4 antibody was administered only intratumorally. In addition, the amount of anti-CTLA-4 antibody during the whole therapy was lower compared to other research groups where the systemic application allowed a higher dose. Another limitation is that authors used anti-CTLA-4 antibody only in three injections on days 20, 27 and 34 from the beginning of the therapy when tumors were already in advanced stadium [68, 71, 72]. Therefore, in further experiments it will be interesting if the efficiency of MBTA therapy combined with anti-CTLA-4 antibody could be increased when using an increased antibody concentration, combining intratumoral administration of MBTA immunotherapy with systemic transfer of anti-CTLA-4, or only timing must be modified.

In addition, the number of Tregs increases with tumor growth and thus, the bigger the tumor the higher the inhibition of immune reactions leading to decreased immune responses during the MBTA immunotherapy [23, 30]. Moreover, from the treatment of bilateral pancreatic adenocarcinoma it was observed that only the treated tumor responded to the MBTA therapy whereas no immune reaction in the distal non-treated tumor could be observed [68]. Again, the reason for the lacking immune reactions in

the distal tumor might be the higher amount of regulatory T lymphocytes within the microenvironment of the tumor leading to inhibition of immune reactions [21, 23, 29, 30, 33, 40]. Similar to the results of MBTA therapy applied in bilateral pancreatic adenocarcinoma, the success in completely healing bilateral colon cancer is rather low [63]. Again it is supposed that the suppression through regulatory T lymphocytes might be the reason for these disappointed results [21, 23, 29, 30, 33, 40].

Thus, the first important step should be proper detection of Tregs during MBTA therapy. In order to detect Tregs, it will be necessary to perform an experiment involving bilateral Panc02 model. Every mouse will be subcutaneously injected with Panc02 cells into each flank. After development of the tumor (based on Lit. [64] after 12 days), the mice will be randomly divided into two groups, whereas in one group one tumor will be treated with MBTA therapy and the other group, which is the control group, will be treated with a phosphate-buffered saline (PBS) solution. After approximately 15 days from the beginning of the therapy the mice will be sacrificed and samples of treated and distal tumor as well as spleen (or other lymphatic nodes) will be collected [64]. For detection of Tregs one half of the taken samples will be prepared for flow cytometry analysis and the other half will be analyzed using immunohistochemistry staining. Using these two methods it will be possible to determine and localize not only Tregs but also other different immune cells. In order to detect Tregs using flow cytometry the transcription factor FoxP3 is made visible using anti-FoxP3 antibodies that are connected to fluorochromes. However, fluorochrome panels to target not only FoxP3 but other immune cell markers, such as CD45 for all leukocytes, CD3 for T lymphocytes, CD4 for helper T lymphocytes and CD8 for cytotoxic T lymphocytes, will have to be prepared exactly for the flow cytometry machine which will be used. Such fluorochrome panel will be dependent on lasers and filters which might be different in each machine [45].

Besides flow cytometry, IHC staining for FoxP3 will be included as additional method to study the localization of Tregs. Based on preliminary literature research anti-FoxP3 rabbit antibody (clone D6O8R) from Cell Signaling (CST, #12653) or from Abcam (clone 236A/A7) is required. Ideally, hematoxylin and eosin staining will be also included to stain cell nuclei and extracellular matrix [40, 46].

Based on the results from the suggested experiment described above, additional experi-

ments can be performed to target Tregs.

Besides the decreased success in treating bilateral tumors or tumors which already are in an advanced growing stage, a better prognosis in patients with lower amount of Tregs is possible [23]. Therefore, it is important to decrease the number of Tregs considerably. That can be done by performing different therapies against Tregs, which are described in more detail in chapter 5.5. Due to the fact that also other immune cells have the same receptors as Tregs, it is difficult to target only Tregs. Therefore, in order to improve the specificity, near infrared photoimmunotherapy might be a proper method to localize regulatory T lymphocytes. In addition, intratumoral injection of anti-CD25 immunotoxin 2E4-PE38 might also be an effective therapy, that enables the reduction of regulatory T lymphocytes only in the tumor and has the advantage that Tregs in the spleen are not disturbed due to the low concentration of the toxin [43].

Furthermore, it will be important to perform an experiment with a bilateral Panc02 mouse model, that is treated with a combination of MBTA therapy and a higher dose of anti-CTLA-4 antibodies. For that, Panc02 cells will be injected subcutaneously in both flanks of each mouse. After 12 days the mice will be randomly divided into five groups, whereas every group consists of six mice. The first group will be treated with MBTA into both flanks, the second with MBTA in the right and PBS in the left flank, the third group will be treated with MBTA in the right and anti-CTLA-4 antibody in the left flank, the fourth group with PBS in the right and anti-CTLA-4 antibody in the left flank and the fifth group will be treated with PBS in both flanks. The MBTA therapy will be administered intratumorally 12 times (based on Lit. [68] on day 0, 1, 2, 8, 9, 10, 16, 17, 18, 24, 25 and 26) and the anti-CTLA-4 antibody will be administered on day 0, 1, 2, 16, 17 and 18. In addition, the concentration of anti-CTLA-4 antibody will be, based on Lit. [72], 10 µg anti-CTLA-4 antibody in 50 µL PBS per mouse and administration. In order to get significant results, it will be necessary to repeat the experiment at least four times with different amount of administrations as well as different concentrations of the anti-CTLA-4 antibody. In addition, it will be important to change the mode of administration from intratumorally to intraperitoneally or subcutaneously [68, 72].

Furthermore, for treating Tregs only in the microenvironment of the tumor, it will be necessary to carry out an experiment including a bilateral Panc02 mouse model. Therefore,

every mouse will be subcutaneously injected with Panc02 cells into both flanks. The mice will be randomly divided into two groups, whereas each group will consist of seven mice. Five and nine days after tumor cell transplantation, one group will be treated with the anti-CD25 immunotoxin 2E4-PE38 into both, left and right flank and the other group will be treated with the immunotoxin only in the right flank. Each administration will consist of 10 µg immunotoxin. The number of Tregs in the microenvironment after the therapy will be analyzed using flow cytometry. However, the disadvantage of this therapeutic method is that the used immunotoxin 2E4-PE38 is not available ready-to-use, which means it has to be produced beforehand [73].

To sum up, a major advantage of MBTA therapy is the stimulation of the two parts, innate and adaptive immune system, resulting in robust anti-tumor response. Moreover, MBTA therapy has the ability to effectively control the progression of multiple different tumors without antigen dependency. However, the effectiveness of MBTA therapy is decreasing in case of metastatic tumor models with higher tumor burden or advanced primary tumors [61]. Therefore, the experiments have been suggested to improve the MBTA outcomes with special focus on Tregs.

In further research it will be necessary to perform the suggested experiments to study the efficiency of the proposed therapies against Tregs during MBTA immunotherapy. Moreover, it will be important to proof the hypothesis that the suppressive properties of regulatory T lymphocytes are the reason for the low success in completely treating especially bilateral types of cancer.

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List of Abbreviations

A2A Adenosine receptor

ACT Adoptive cell therapy

ADCC Antibody-dependent cellular cytotoxicity

APC Antigen-presenting cell

BAM Biocompatible anchor for cell membrane

CAM Cell-surface adhesion molecules

cAMP Cyclic antimicrobial peptide

CAR Chimeric antigen receptor

CCL Chemokine ligand

CCR Chemokine receptor

CD Cluster of differentiation

CLR C-type lectin receptor

CRISPR Clustered regularly interspaced short palindromic repeats

CST Cell signalling technology

CTL Cytotoxic T lymphocyte

CTLA Cytotoxic T-lymphocyte associated protein

DAMP Damage-associated molecular pattern

DC Dendritic cell

DNA Deoxyribonucleic acid

dsRNA Double-stranded ribonucleic acid

Foxp3 Forkhead box p3 protein

GARP Glycoprotein A repetitions predominant

GITR Glucocorticoid-induced TNF-receptor-related proteins

GPCR G-protein-coupled receptor

GrzA/B Granzyme A/B

HIV Human immunodeficiency virus

HLA Human leukocyte antigen

ICOS Inducible T cell co-stimulator

IDO Indoleamine 2,3-dioxygenase

IFN Interferon

Ig Immunoglobulin

IL Interleukin

iMVA Non-replicative inactivated vaccinia virus Ankara

IPEX Immune dysregulation, polyendocrinopathy, enteropathy, X-linked

ITIM Immunoreceptor tyrosine-based inhibitory motif

iTreg Induced or adaptive regulatory T lymphocytes

KO Knock out

LAG Lymphocyte-activation gene

LAP LC3-associated phagocytosis

LC Langerhans cell

LTA Lipoteichoic acid

mAb Monoclonal antibody

MAC Membrane attack complex

MBTA Mannan-BAM, TLR ligands and Anti-CD40 antibody

MDSC Myeloid-derived suppressor cell

MHC Major histocompatibility complex

mRNA Messenger ribonucleic acid

NIR-PIT Near-infrared photoimmunotherapy

NK cell Natural killer cell

NKT cell Natural killer T lymphocyte

NF Nuclear factor

NLR NOD-like receptors

NOD Nucleotide-binding oligomerization domain

NRP Neuropilin

NSCLC Non small cell lung carcinoma

nTreg Natural regulatory T lymphocyte

PAMP Pathogen-associated molecular pattern

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PD Programmed cell death protein

Pfr Perforin

PGL Paraganglioma

PHEO Pheochromocytoma

PI Primary immunodeficiency

PRR Pattern recognition receptor

pTreg Regulatory T lymphocyte developed at peripheral sites

R-848 Resiquimod

RIG Retinoic acid-inducible gene

RLR RIG-I-like receptor

RNA Ribonucleic acid

Rnf20 Ring finger protein 20

SCID mice Severe combined immunodeficient mice

ssRNA Single-stranded ribonucleic acid

TAA Tumor-associated antigen

TAM Tumor associated macrophages

$\mathbf{T}_{conv}$ Conventional T cell

TCR T cell receptor

TGF Transforming growth factor

Th T helper lymphocyte

TIGIT T lymphocyte immunoreceptor with Ig and ITIM domains

TIL Tumor-infiltrating lymphocytes

TIM Triose-phosphate isomerase

TIR Toll-IL-1 receptor domain

TLR Toll-like receptor

TME Tumor microenvironment

TNF Tumor necrosis factor

TNFR tumor necrosis factor receptor

Treg Regulatory T lymphocyte

tTreg Regulatory T lymphocyte developed in thymus

Usp22 Ubiquitin-specific peptidase 22

List of Figures