Regulation of the effective anti-tumor $CD4^+$ T cell immunity by agonistic CD137 antibody

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CONTENT

1 INTRODUCTION

As the number of cancer cases worldwide increases at an alarming rate, the effects are tremendously challenging to the public health systems and to the afflicted individual alike. The oncoviral research, conducted during the previous few decades, has determined that up to 20% of the cancer cases can be attributed to viral infections (105). In particular types of cancer, some viruses indisputably facilitate the initiation and the development of the disease. For instance, up to 80% of hepatocellular carcinomas (HCCs) are associated with the human hepatitis B virus (HBV) and human hepatitis C virus (HCV) while in almost all cases of cervical carcinomas the Human papillomavirus (HPV) has been identified as the disease catalyst (53).

1.1 History of oncoviral research

(Adapted, unless otherwise mentioned, from Cann (16) and Coffin (22)).

1908: Vilhelm Ellerman and Oluf Bang first demonstrated that avian leukosis virus could be transmitted after cell-free filtration to new chickens, causing leukemia.

1911: Peyton Rous reported cell free transmission of sarcoma in chicken and isolated the infectious agent Rous sarcoma virus (RSV) (134).

1933: Richard Edwin Shope discovered cottontail rabbit papillomavirus or Shope papillomavirus, the first mammalian tumor virus.

1936: John J. Bittner discovered mouse mammary tumor virus as an "extrachromosomal factor" (i.e., virus) transmitted among laboratory strains of mice by breast feeding (10).

1951: Ludwig Gross observed vertical (germ line) transmission of cancers.

1957: The Friend murine leukemia virus discovered by Charlotte Friend provided an animal model system for the study of erythropoiesis and the multistep nature of cancer.

1960's: Temin and Baltimore simultaneously demonstrated that retrovirus particles contain a ribonucleic acid (RNA)-dependent desoxyribonucleic acid (DNA) polymerase-reverse transcriptase (Nobel prize awarded to Baltimore and Temin, 1975).

1964: Anthony Epstein, Bert Achong and Yvonne Barr identified the first human cancer virus from Burkitt lymphoma cells. This virus is formally known as human herpesvirus 4 but more commonly called Epstein-Barr Virus or EBV (35).

Mid-1960s: Baruch Blumberg first physically isolated and characterized Hepatitis B, co-receiving in 1976 Nobel Prize in Medicine or Physiology (Nobelprize.org).

1969: Huebner and Todaro proposed the viral oncogene hypothesis - the transmission of viral and oncogenic information as genetic elements. This explained the vertical (germ line) transmission of 'cancers', first observed by Gross, 1951.

1981: Gallo and co-workers discovered Human T-cell leukemia virus, which was the first pathogenic human retrovirus to be identified.

1983: Barré-Sinoussi et al discovered Human immunodeficiency virus (HIV) - the causative agent of Acquired immunodeficiency syndrome (AIDS).

1984–86: Harald zur Hausen, together with Lutz Gissman, discovered first HPV16 and then HPV18, responsible for approximately 70% of cervical cancers. For the discovery that HPVs cause human cancer, zur Hausen won a Nobel Prize in 2008 (Nobelprize.org).

1994: Patrick S. Moore and Yuan Chang isolated Kaposi sarcoma-associated herpesvirus (KSHV). This search was driven by the work of V. Beral, T. Peterman and H. Jaffe who showed that this cancer must have another infectious cause besides HIV itself (9). Subsequent studies revealed that KSHV is responsible for the epidemiologic patterns of Kaposi’s sarcoma (KS) and related cancers (6).

2008: A method developed by Chang and Moore (digital transcriptome subtraction) was used to isolate DNA fragments of Merkel cell polyomavirus from a Merkel cell carcinoma and it is now believed that this virus causes 70–80% of these cancers (37). This is the first polyomavirus to be well-established as the cause of a human cancer.

1.2 Oncogenic viruses

1.2.1 General aspects of viral oncogenesis

The infectious nature of oncogenic viruses sets them apart from other carcinogenic agents. Therefore, the viral infection’s pathogenesis and the host response should both be extensively researched in order to acquire a thorough understanding of the resulting cancer. Most oncogenic viruses share some common features, although

they often belong to different virus families and use various strategies to contribute to cancer development. One such feature is their ability to infect, but not destroy, their host cell. In contrast to many disease-causing viruses, the oncogenic viruses tend to establish long-term persistent infections. As a result, they have developed advanced host immune response evasion strategies which would otherwise eradicate the virus. In spite of the viral etiology of several cancers, it seems the viruses may often contribute to, but are insufficient for, carcinogenesis; in fact, the majority of tumor virus-infected individuals do not develop cancer. Even for those patients that do develop cancer, several years can pass between the initial infection and the tumor progression. In addition, a variety of co-factors might also play a significant role in the oncogenesis. Examples of such factors include host immunity and chronic inflammation. In short, the long-term interactions between a virus and its host are essential features of the oncogenic viruses, as they initiate a variety of molecular events that may contribute to subsequent virus-mediated tumorogenesis (23).

1.2.2 Classification of oncogenic viruses

Oncogenic viruses are distributed into two major classes, viruses having DNA as a genome (DNA viruses) and those containing RNA as a genome (RNA viruses). Table 1.1 lists the known oncogenic viruses.

Table 1.1: Examples of human oncogenic viruses

Family	Disease	Tumor	Associated virus
DNA viruses			
Papillomaviridae	Warts, including sexual transmitted genital warts	Cervical carcinoma	Human papillomaviruses (multiple types)
Herpesviridae		Kaposi's sarcoma	Human herpesvirus-8
	Infectious mononucleosis	Burkitt's lymphoma	Epstein–Barr virus
Hepadnaviridae	Infectious hepatitis	Hepatocellular carcinoma	Hepatitis B virus
Poxviridae	Smallpox	Malignant-type smallpox	Vaccinia virus
Adenoviridae	Upper respiratory	Adenocarcinomas	Human adenovirus

	tract infection	(cancer of glandular epithelial tissues)	
Polyomaviridae	Progressive multifocal leukoencephalopathy and others	Merkel cell carcinoma	Human polyomaviruses
RNA viruses			
Retroviridae		T cell leukaemia or lymphoma	Human T cell leukaemia virus-1
Flaviviridae	Non-A, non-B hepatitis, hepatic steatosis	Hepatocellular carcinoma	Hepatitis C virus

1.2.3 Mechanisms of oncogenesis

Tumor-associated viruses often lead to malignancies with a prolonged latency and in conjunction with other environmental or host-related cooperating events, including nutritional status, environmental factors, parasitic or viral infection, chronic inflammation, and immunosuppression. The oncogenic mechanisms used by different viruses differ significantly. However, there are some common mechanisms that are used by viruses to induce cellular transformation.

Table 1.2: Mechanisms of oncogenesis and related viruses

Mechanisms	Viruses
Perturbation of signaling pathways	EBV, KSHV, HPV
Deregulation of cell cycle	HPV, HTLV-1
Escape of apoptosis	EBV, HPV
Immortalization of cells	KSHV, HPV
Induction of genetic instability	HCV, HPV, HTLV-1
Insertional mutagenesis	HBV, HPV
Induction of chronic inflammation	HBV, HCV

EBV - Epstein-Barr Virus; **HBV** - human hepatitis B virus; **HCV**- human hepatitis C virus; **HTLV** - Human T-cell leukemia virus 1; **HPV** - Human papillomavirus; **KSHV** - Kaposi sarcoma-associated herpesvirus

Perturbation of signaling pathways is comprised of a mimicking of the signaling ligands and the cellular signaling receptors as well as an activation of the cell surface receptors. These pseudo-signals are passed on, integrated, furcated and interpreted by intracellular signaling networks for cellular transformation (102, 128).

Oncogenic viruses have developed multiple levels of regulation to promote cell cycle progression and cell proliferation, a mechanism referred to as **deregulation of the cell cycle**. At the heart of this process is a signal to the cell cycle's regulatory system that leads to the unchecked entry of the cell into phase S (the phase, where the cell cycle proceeds autonomously, and independent of extracellular signals).

The oncogenic viral infection imposes a significant amount of stress on cells that would induce apoptosis under normal conditions. To avoid premature cell death, these viruses apply several tactics to counteract apoptosis. The **apoptosis escape** comprises the inactivation of the tumor suppressor gene p53. This gene's mutation occurs in more than 50% of all human cancers (51). For examples, HPV E6 (protein from an early region of HPV DNA-genome (157)) suppresses the normal functions of p53 through inhibition of p53 DNA-binding activity or by sequestering p53 in the cytoplasmic compartment (149). This suppression, carried out together with other mechanisms used by the virus, leads to the neoplastic processes in infected individuals.

Immortalization of the cells is another method to promote tumor development. Normal mammalian cells have limited propagation potentials, averaging about 60-70 cell divisions. This limited ability of cells is due to an autonomous cell generation counting device known as a telomere, which can be replenished by telomerase and therefore increase the number of cell divisions. Several oncogenic viruses target the telomerase to immortalize their infected cells. Through this way, the cancer cells obtain unlimited multiplication potentials (51).

One of the main hallmarks of cancer cells is the intrinsic genetic instability resulting in gene mutations. The **induction of genetic instability** leads to the elevated mutability of cancer cells that provides them with a significantly higher chance of gathering enough mutations to reach the stage of malignant transformation (51).

The integration of proviral DNA into the host genome is an integral part of the retroviral life cycle. This integration may occur in the vicinity of or inside important cellular genes, leading to their mutations. This mechanism of tumor development is called **insertional mutagenesis** and will be addressed in further detail in the Retroviruses section.

Chronic inflammation in response to viral infection can also lead to cancer development. For cancer development the inflammatory response sustains and provides the initiated tumor cells with an environment rich in growth/survival factors, activated stroma and DNA-damaging agents, which collectively promote cell proliferation as well as neoplastic transformation (24).

1.3 Anti-tumor immune response

McFarlane Burnet and Lewis Thomas introduced the tumor **immunological surveillance** theory in the 1950s (13, 14). It described how the host's immune system recognizes newly arising tumors' antigens and eliminates these tumors before they become clinically evident. Progressive cancer was understood as an infrequent event in which the tumor cell evaded the immune system's effective control. Now it is reasonably clear that the immune system cannot entirely control tumor development or protect against its progression and metastasis. Nevertheless, the host immune system, including both innate and adaptive mechanisms, remains the main native protection in tumor control, especially with a clear etiological factor (viral).

1.3.1 The tumor-draining lymph nodes

The lymph nodes (LNs) are highly organized lymphoid organs composed of different types of immune cells. The latter are strategically positioned throughout the body and they play an essential role in any immune response. The tumor-draining lymph nodes (drLNs) lie immediately downstream of tumors where they undergo profound alterations due to the presence of the upstream tumor. They are currently emerging as an essential part of tumor immunology. Structurally, the drLNs form the site which tumor antigens first drain and tumor-derived DC initially migrate to. These cells display the antigen to recirculating T lymphocytes, which they also assist in activating. B cells, which encounter antigens as they migrate through the lymph node, are also arrested and activated with the help of some of the activated T cells.

The antigen-specific lymphocytes leave the lymph nodes as effector cells once they have undergone a period of proliferation and differentiation (67).

The drLN is the physical location where cells of the naïve immune system first encounter the tumor antigens. This encounter is the moment of critical decision between initiation of activation and immune tolerance, when the immune system ignores, or fails to react to an antigen. Therefore, the local microenvironment in drLNs turns out to be a key factor in determining the course of the consequent anti-tumor immune response (97).

1.3.2 Innate immunity

The innate immune system is the first intrinsically present line of defense against invading organisms. Innate immunity comprises various innate immune cells, including macrophages, dendritic cells (DCs), neutrophils and natural killer cells (NK) cells and various components of the complement system. In addition, the innate immune system has anatomical features functioning as barriers to infection. Pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), on the cell surface of macrophages and dendritic cells (DCs) recognize the conserved pathogen-derived molecules or the pathogen associated molecular patterns (PAMPs). Binding of PAMPs to TLRs leads to production of pro-inflammatory cytokines and chemokines.

NK cells belong to the innate immune system as they are able to respond to pathogens immediately in a non-specific manner. NK cells are activated in response to interferons or macrophage-derived cytokines. They are also cytotoxic; small granules in their cytoplasm contain special proteins such as perforin and proteases known as granzymes. The release of these components leads to the induction of apoptosis in the target cells.

Another important innate defense mechanism is the ingestion of extracellular particulate material by phagocytosis. Most phagocytosis is conducted by specialized cells, such as macrophages/monocytes, and granulocytes (especially neutrophils); which can engulf and kill pathogens in phagosomes. DCs can also contribute to the process of phagocytosis. Theirs main function is the recognition and degradation of the pathogens. Then, DCs present pathogen with the help of the major histocompatibility complex (MHC) molecules to naïve T cells and consequently launch the adaptive immune response (67).

Several observations suggest that innate immune cells play an important role in the host's defense against tumors. For instance, in humans the Chediak-Higashi syndrome – an autosomal recessive disorder – is associated with impairment in neutrophils, macrophages, and NK cells, and an increased incidence of lymphomas (143).

1.3.3 Adaptive immunity

The term 'adaptive' refers to the differentiation of self from non-self and the adjusting of the response to the particular foreign pathogen. The adaptive immune response comprises B-lymphocytes that produce antibodies, often called the *humoral response* and T-lymphocytes that recognize and eventually kill infected or tumor cell, called the *cell-mediated response*.

Adaptive immunity is triggered when a pathogen evades the innate immune system and generates a threshold level of antigen. The major functions of the adaptive immune system include:

- The recognition of specific foreign antigens during the process of antigen presentation
- The generation of responses that are tailored to maximally eliminate specific pathogen-infected or tumor cells
- The development of immunological memory, where each pathogen is 'remembered' by a signature antibody

T cell maturation

Progenitor T cells from the earlier sites of hematopoiesis begin to migrate from the bone marrow where they generated to the thymus. Thymus is the main source of all T cells. In thymus T cells diversify and then are shaped into an effective primary T cell repertoire by the selection processes. One of these, **positive selection**, permits the survival of only those T cells whose T cell receptors (TCRs) are capable of recognizing self-MHC molecules. It is thus responsible for the creation of a self-MHC-restricted repertoire of T cells. The other, **negative selection** eliminates T cells that react too strongly with self-MHC or with self-MHC plus self-peptides. It is an extremely important factor in generating a primary T cell repertoire that is self-tolerant. There are two main T cell subsets produced after being maturated in thymus: $CD4^+$ and $CD8^+$ T cells (43).

T cell activation and differentiation

Recognition of the pathogens by DCs leads to their activation and maturation. Mature DCs have increased expression of MHC class I and class II. DCs are also known to express different types of T cell co-stimulatory molecules such as CD80 (B7.1), CD86 (B7.2) and etc. on their surface. The receptor for B7 molecules on the T cells is CD28, a member of Ig superfamily like B7 molecules. Macrophages and B cells similarly become activated to express B7 molecules on their surface. DCs, macrophages and B cells are professional antigen-presenting cells even though DCs are much stronger antigen presenting cells (APCs) than macrophages and B cells. DCs migrate from the periphery through afferent lymphatic channels to draining lymph nodes (drLNs). Pathogen-activated DCs present these pathogen-derived antigens to T cells and stimulate the differentiation of naïve T cells into various subtypes to start the adaptive immune response. T cells express the TCR that can recognize tumor antigen on MHC molecules on tumor cells and APC (34, 135). There are two major subtypes of T cells based on the expression of two different coreceptors. T cells that co-express CD8 recognize peptides from intracellular pathogens that are passed to the APC surface by major histocompatibility complex (MHC) class I molecules. Peptide presentation to $CD8^+$ T cells results in increase in numbers in the presence of B7 molecules expressed on APCs to produce cytotoxic T cells (CTLs) that can kill transformed tumor cells via production of cytotoxic molecules. $CD4^+$ T cells recognize peptide antigens processed from pathogens multiplying in intracellular vesicles and those derived from ingestion of extracellular bacteria and toxins are carried to the cell surface by MHC class II molecules and presented to $CD4^+$ T cells, which then receive a second signal from DCs provided by molecules of the B7 family to differentiate into the different subtypes of helper T cells (Th; type Th1 and Th2) (143).

Co-stimulatory and co-inhibitory molecules

T cells require two signals for activation: an antigen specific signal, involving the recognition of a peptide/MHC protein complex by the T cell receptor (described earlier), and additional costimulatory signals. Only when the T cell recognizes both the antigen and a costimulatory signal on APC an immune response is initiated. There are two main families of co-stimulatory molecules: B7/CD28 family, which belongs to larger superfamily of immunoglobulin proteins (120) and tumor necrosis

factor (TNFR)/TNF receptor (TNFR) family (82) (summarized in Table 1.3). Unlike the B7/CD28 family, that contains both co-stimulatory and co-inhibitory proteins, the members of TNF/TNFR family have only co-stimulatory function.

Table 1.3: Co-stimulatory and co-inhibitory molecules in T cell function

Receptor	Expression	Ligands (expression)	Function in immune system
B7/CD28 superfamily			
CD28	Constitutive T cells	B7.1 (CD80), B7.2 (CD86), Activated APC	Immune stimulation: provides signal 2for initial survival and proliferation of naïve T cells
ICOS	Activated T cells	B7RP1 (LICOS), ICOSL, on activated APC and some non-lymphoid tissues	Immune cell differentiation: enhances T helper cytokine production , antibody class switch, germinal center formation
CTLA-4	Activated T cells	B7.1 (CD80), B7.2 (CD86)	Inhibitory: downregulates immune response
PD-1	Activated T and B cells	PD-L1, PD-L2, lymphoid and non-lymphoid tissues	Inhibitory
TNFR superfamily			
CD137 (4-1BB)	Activated T cells, activated DCs, activated NK cells	CD137L (4-1BBL), on activated APCs	Immune cell activation and survival, $CD8^+$ T cell memory
CD134 (OX-40)	Activated T cells (mainly $CD4^+$ T cells)	CD134L (OX-40L), on activated T, B, DC, vascular endothelial cell	$CD4^+$ T cell memory
CD40	B cells, DC, Macrophages	CD40L (CD154), on activated T cells, mainly $CD4^+$ T cells	B cell and DC activation

APC - antigen presenting cell; **B7RP1** – B7-Related protein 1; **CTLA-4** – cytotoxic T lymphocyte associated antigen-4; **DC** – dendritic cell; **ICOS** – inducible co-stimulator; **L** – ligand; **NK** – Natural killer cell

The B7/CD28 superfamily is responsible for initial T cell expansion and short term survival (79). The TNFR superfamily is a group of receptors involved in enhancing T cell proliferation, cytokine production, and particularly survival (122). The lack of positive co-stimulatory ligands or the presence of inhibitory ligands has been suggested to contribute to poor anti-tumor T cell efficacy (31). Detailed function as well as *in vivo* manipulation of co-stimulatory and co-inhibitory molecules, which improves the ability of tumor-specific T cells to control and subsequently eradicate tumor, is described in the "Cancer immunotherapy" part.

$CD8^+$ T cells

$CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTL), belong to a sub-group of T lymphocytes and represent the most critical effector cell of adaptive immunity. $CD8^+$ T cells are recognized as cytotoxic after they become activated. To develop into activated effector CTL that can combat tumor cell, naïve $CD8^+$ T cells need to recognize peptide antigens presented by MHC I molecules on activated APCs. MHC class I molecules are also expressed by non-classic APCs including all nucleated cells. This is very crucial as oncogenic viruses can infect almost all nucleated cells. CTLs expand under the influence of IL-2 over several hundred folds during a primary immune response and constitute a very efficient antigen-specific pool of effectors that are able to kill several targets leaving healthy cells untouched (67).

Cytotoxic $CD8^+$ T cells are armed with special cellular mechanisms and molecules that cause tumor cell destruction. After forming the immunological synapse between $CD8^+$ T cell and its target, CTLs utilize one of the two main mechanisms to kill the tumor cell. The first pathway is a **calcium-dependent** release of specialized cytotoxic granules, such as perforin and granzymes, called **perforin/granzyme pathway**. These lytic granules transported to the tumor cell as one compound and are all required for effective cell killing. Immediately after CTL-target synapse formation, perforin (described originally for its pore forming properties) and granzyme are released from the granules by exocytosis into the junctional space between the two cells. As the perforin contact the target cell membrane, it undergoes a conformational change, polymerizes in the presence of calcium to form cylindrical pores, mediating

granzyme entry inside the target. There are different types of granzyme molecules that promote apoptosis of the target cell. Many target cells have a molecule known as the 6-phosphate receptor on their surface that also binds to granzyme B. Granzyme B/mannose 6-phosphate receptor complexes are then internalized and appear inside vesicles. In this case, perforin is necessary for releasing granzyme B from the vesicle into the cytoplasm of the target cell. Once it enters the cytoplasm of the target, granzyme B cleaves and activates caspases (especially caspase-3). Caspase-3 activates a caspase proteolytic cascade, which eventually activates the caspase-activated deoxyribonuclease (CAD). This nuclease is believed to be the enzyme that degrades the DNA. Other granzymes (A; K; N and etc.) are thought to promote apoptosis by targeting different cellular components (43, 67).

The second pathway for CTL killing is the **calcium-independent** Fas ligand/ Fas-mediated apoptosis **(FasL/Fas)**. In this pathway FasL (CD95L), expressed on the CTLs, binds to their cognate receptor, Fas (CD95), expressed on the target tumor cells. The FasL/Fas engagement causes the activation of an initiator caspase in the target cell, which ultimately leads to DNA cleavage and cell death in a non-inflammatory manner (43).

$CD4^+$ T cells

$CD4^+$ T cells, also called as helper cells, are subpopulation of T lymphocytes, which main function is to provide supporting signals to other immune cells, such as B cells, $CD8^+$ T cells and macrophages either through secretion of cytokines or via direct interaction. Naïve $CD4^+$ T cells recognize antigens presented by MHC class II molecules on activated APCs. APCs activation, mediated by PRR signaling, leads to upregulation of the MHC class II on their surface, as well as co-stimulatory molecules (CD80, CD86) and pro-inflammatory cytokines (such as tumor necrosis factor (TNF), IL-2, IL-1, IL-6 and IL-12) (131). As soon as the activated APCs migrate to tumor-drLNs, they prime naïve $CD4^+$ T cells, which in turn differentiate and polarize into effectors with different functions (Figure 1.1).

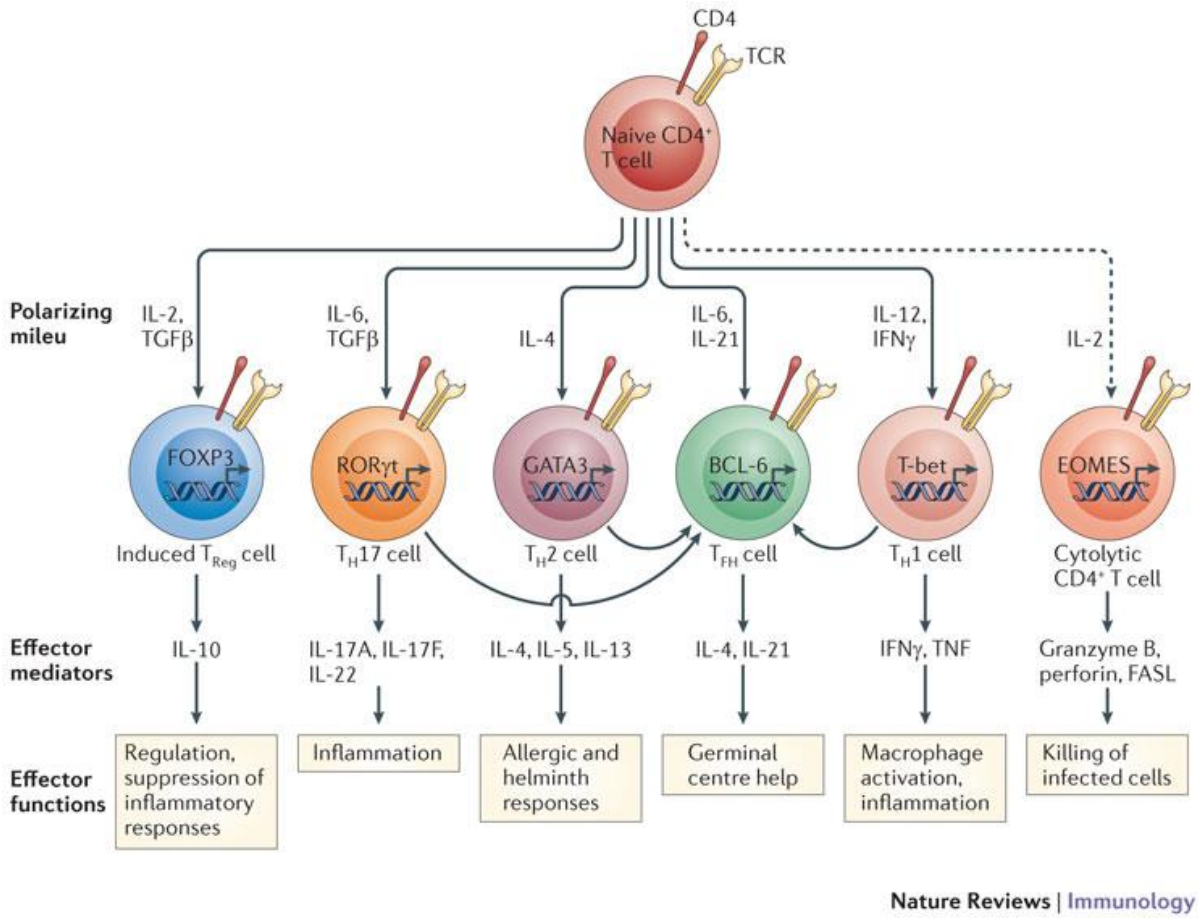

Figure 1.1: Different subsets of CD4$^+$ T cells

BCL-6 B cell lymphoma 6; **EOMES** -eomesodermin, **FASL** - FAS ligand; **FOXP3** - forkhead box P3; **GATA3** - GATA-binding protein 3; **RORγt** - retinoic acid receptor-related orphan receptor-γt; **TCR** - T cell receptor; **TGFβ** - transforming growth factor-β; **TNF** - tumor necrosis factor; adapted from (130).

The differentiation of polarized effector CD4$^+$ T cells is under the control of exclusive sets of transcription factors. The expression of these factors is highly depended on the signals provided by different cytokines. The original source of cytokines is from APCs. Some of the cytokines are produced by already differentiating CD4$^+$ T cells and subsequently create a positive loop, which in turn enhances ongoing differentiation.

T helper 1 (Th1)

Th1 cells are implicated in host defense against intracellular viral and bacterial pathogens. By secreting such cytokines as interferon-gamma (IFN-γ), interleukin-2 (IL-2), IL-10 and tumor necrosis factor-alpha/beta (TNF-α/β), these cells are responsible for maintaining pro-inflammatory T cell-mediated immunity. Pro-inflammatory cytokines promote macrophage activation, cytotoxic CD8$^+$ T cell

proliferation, nitric oxide production, leading to the phagocytosis and destruction of the pathogens.

The master regulator for Th1 differentiation, the T-box transcription factor (T-bet), is involved in activating set of genes to promote differentiation of a particular phenotype. IL-27 signaling induces signal transducer and activator of transcription 1 (STAT1)-dependent expression of T-bet, which promotes expression of IFN-γ and IL-12 that then in turn stimulate STAT4-dependent IFN-γ production and further Th1 differentiation. Excessive Th2-type immune responses have been implicated in the development of autoimmune diseases (148).

T helper 2 (Th2)

Th2 cells are involved in mediating non-inflammatory adaptive immune response. These cells are important for humoral immunity by promoting IgA, IgE and IgG immunoglobulins production by B cells. Th2 cells secrete following cytokines: IL-4, IL-5, IL-6, IL-10, and IL-13 (83). Th2 differentiation occurs in the presence of IL-4 and either IL-2, IL-7. Exposure of activated naive $CD4^+$ T cells to IL-4 induces STAT6-dependent expression of GATA-3 and Growth Factor Independent-1 (GFI-1). GATA-3, the master transcriptional regulator of Th2 cells, promotes IL-5 and IL-13 expression, and along with GFI-1 stimulates the expansion of Th2 cells, while suppressing the differentiation of other T cell subtypes, in particularly Th1 cells. In addition to IL-4-induced activation of GATA-3, IL-2, IL-7 is required during Th2 differentiation to activate STAT5, which cooperates with GATA-3 to promote T cell production of IL-4. IL-4 regulates clonal expansion of Th2 cells, and along with IL-13, promotes B cell production of IgE and alternative macrophage activation. Exaggerated Th2-type immune responses have been associated with the development of chronic allergic inflammation and asthma (83).

Cytotoxic $CD4^+$ T cell

In addition to potent helper function, which $CD4^+$ T cells provide to B cells and $CD8^+$ T cells, there is a sufficient evidence to suggest that effector $CD4^+$ T cells have strong anti-viral and anti-tumor roles that are independent of their helper activities. Strong immune protection mounted by $CD4^+$ T cells. Interestingly, the cytotoxic activity of $CD4^+$ T cell effectors does not depend on Th1 polarization, and expression of transcription factor Eomesodermin (Eomes), but not T-bet, may be crucial in

driving the development of cytotoxic $CD4^+$ T cells *in vivo* (109). Eomes also a member of the T-box transcription factor family, which is important in regulating cytotoxic $CD8^+$ T cells development and functions. Additionally, Eomes has been shown to be required for the upregulation of cytotoxic molecules (e.g. granzymes) in Th1 cells responding to staphylococcal enterotoxin A in the presence of agonist antibodies CD134 (OX40) and CD137 (4-1BB) (109). There are two distinct mechanisms through which cytotoxic $CD4^+$ T cells promote tumor clearance. The release of pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-2, by $CD4^+$ T cells help to coordinate an anti-viral and anti-tumor state in infected tissue. Additionally, cytotoxic $CD4^+$ T cells can directly attack and destroy infected and tumor cells through diverse mechanisms, which are Fas/FasL-dependent and perforin/granzyme-dependent pathways (described earlier). Therefore, $CD4^+$ T cells that acquire cytotoxic function can be considered as a separate $CD4^+$ T cell subset.

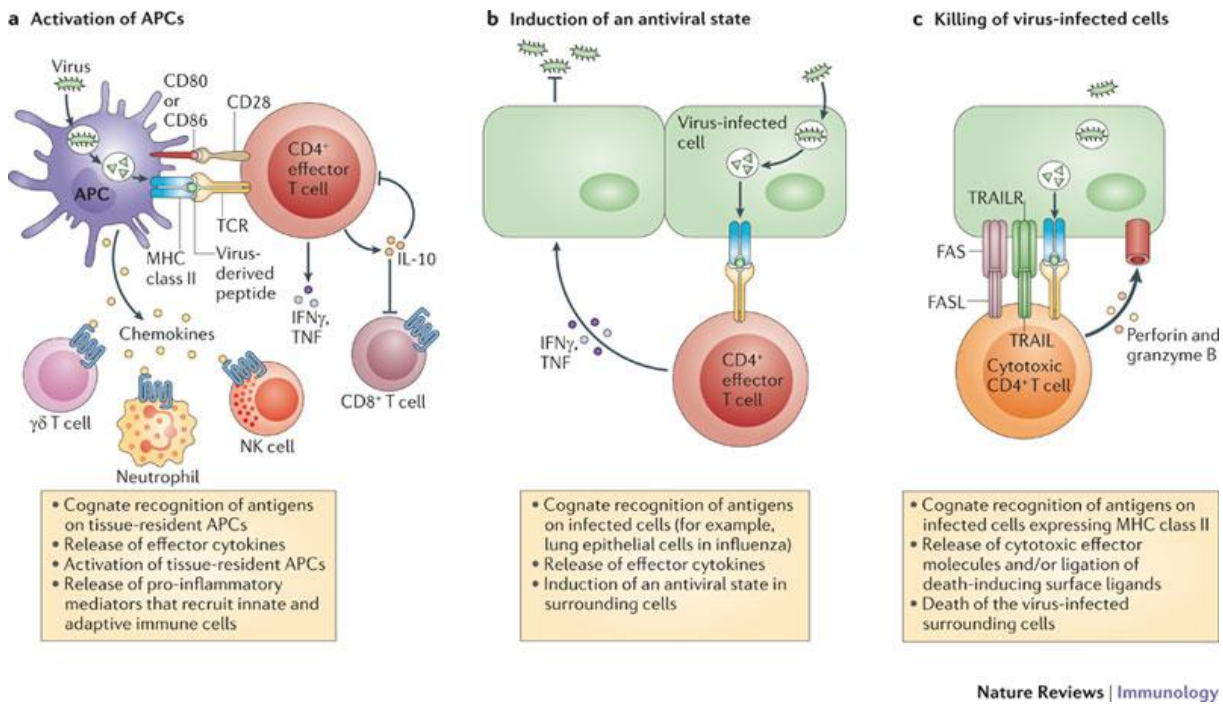

Figure 1.2: Antiviral functions of $CD4^+$ T cells that are independent of their lymphocyte helper functions

FASL - FAS ligand; **NK** – natural killer; **TRAIL** – TNF-related apoptosis-inducing ligand; **TCR** - T cell receptor; **TRAILR** – TRAIL receptor; (130).

T helper 9 (Th9)

Th9 cells are important for host defense against parasitic helminth infections, but may also have detrimental effects including contributing to the development of

chronic allergic inflammation, airway remodeling, and autoimmune disease. Th9 cells preferentially secrete high levels of IL-9, CCL17, CCL22, and IL-10. Master transcription factors that regulate distinct fate of Th9 cells are IRF4 and PU.1 (44, 110). TGF-β was found to avert the differentiation of Th2 towards the development of Th9 cells (136).

T helper 17 (Th17)

Th17 cells contribute to host defense against extracellular bacteria and fungi. Th17 cells develop from naive $CD4^+$ T cells in the presence of TGF-β and IL-6. These cytokines induce the STAT3-dependent secretion of IL-21, IL-23 R, and the transcription factor, ROR gamma t. IL-21 and IL-23 regulate the formation and clonal expansion of Th17 cells, while ROR gamma t-induced gene expression leads to the secretion of IL-17, IL-17, and IL-22. Cytokines secreted by Th17 cells stimulate chemokine secretion by resident cells, leading to the recruitment of neutrophils and macrophages to sites of inflammation. These cells, in turn, produce additional cytokines and proteases that further impair the immune response. Persistent secretion of Th17 cytokines promotes chronic inflammation and may be implicated in the pathogenesis of inflammatory and autoimmune diseases (83).

Regulatory T cells (Tregs)

Tregs are specialized subpopulation of $CD4^+$ T cells, which are naturally present in the immune system as 10-15% of $CD4^+$ T cells in mice. The main function of Tregs, also known as suppressor T cells, is to maintain immune homeostasis. It implicates suppression of successful immune responses and keeps in check of self versus non-self recognition. Failure of the latter results in autoimmune destruction of host cells and tissue (112). Like other T cells, one subset of Tregs matures in the thymus where they are characterized by the variable expression of CD4, CD25 and Foxp3. These are the natural Tregs. Natural Tregs express IL-2Rα chain (CD25) and the transcriptional repressor forkhead box protein 3 (Foxp3). The importance of Foxp3 is defined by genetic mutations in this molecule which result in a fatal autoimmune disorder known as immune dysregulation, Polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome, which develops early in infancy (7). Additionally, natural Tregs are characterized by surface expression of Neuropilin-1 (Nrp-1), which was found to be selectively expressed on natural Tregs (152). Tregs that arises in the periphery are called inducible Tregs. This cell population is generated from naïve $CD25^+$ or $CD25^-$

T cells in the periphery upon antigen presentation by semi-mature DCs and under the influence of IL-10, transforming growth factor β (TGF-β) and possibly IFN-α (94).

Higher numbers of $CD4^+$ Tregs in cancer patients compared to normal healthy controls have been reported in recent years for many cancers, including head and neck, hepatocellular, gastric, breast, ovarian, lung, melanoma, renal cell, and pancreatic cancer (150). Studies investigating $CD4^+CD25^+$ cells show that in cancer patients, Tregs constitute 13% to 52% of the total number of $CD4^+$ T cells (150). Increased numbers of Tregs have also been observed in numerous human and animal studies of chronic viral infections, including oncogenic viruses, in Hepatitis C virus (HCV) (11), HIV (5), EBV (87) and herpes simplex virus (HSV) (129) infections. The biological significance of these higher peripheral numbers of Tregs is a matter of debate. In EBV infected humans, induction of Treg inhibits $CD4^+$ T cell responses to EBV proteins supporting viral persistence and promoting the induction of EBV-associated tumors (94).

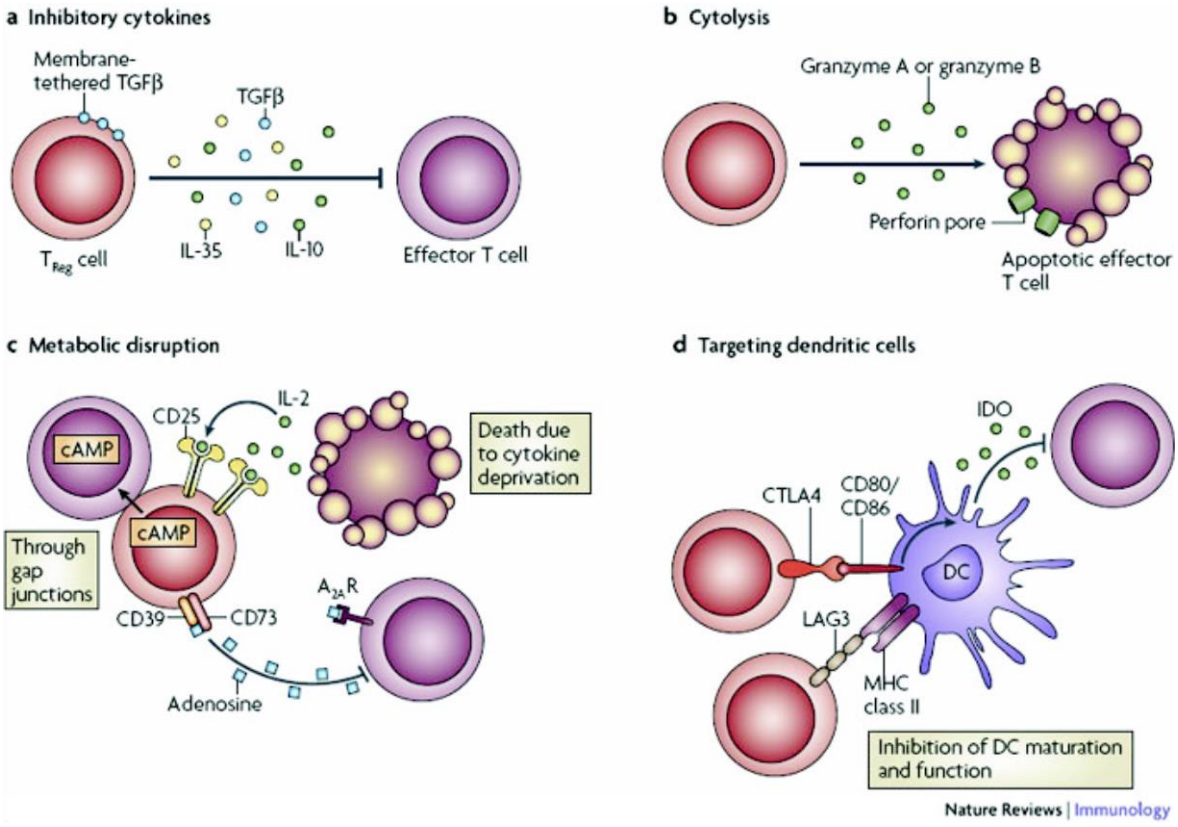

Figure 1.3: Basic mechanisms used by Tregs (139)

There are four suppression mechanisms, which are utilized by Tregs (Figure 1.3). Tregs can srelease **inhibitory cytokines**, such as IL-10, TGF-β, and IL-35, and apply these soluble factors as a main mechanism of suppression (Figure 1.3a). Recent studies showed that Tregs may use **perforin/granzyme-mediated cytotoxicity**, which leads to apoptosis of effector T cells (Figure 1.3b). **Metabolic disruption** includes high-affinity CD25 dependent cytokine-deprivation-mediated apoptosis, cyclic adenosine monophosphate (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A ($A_{2A}R$)-mediated immunosuppression (Figure 1.3c). Mechanisms **targeting DCs** contain the modulation of maturation and function of DCs through lymphocyte-activation gene 3 (LAG-3) –MHC-class-II-mediated suppression of DC maturation, and CTLA4–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which itself is an immunosuppressive molecule made by DCs (Figure 1.3d) (139).

Plasticity of $CD4^+$ T cells

Although the production of the specific cytokines , such as IFN-γ, IL-4 and IL-17 is commonly used to ascribe subsets (Th1, Th2 and Th17, respectively) to corresponding $CD4^+$ T cells, accumulating evidence suggests that $CD4^+$ T cells, are more plastic than previously estimated. Additionally, certain cytokines (for example, IL-10) can be produced by subpopulations of cells within multiple effector subsets. They all are capable of mediating direct anti-viral and anti-tumor functions, of providing help for B cells, of regulating immunopathology and of mediating cytotoxic killing of virus-infected or transformed cells. Certain $CD4^+$ T cell subsets — particularly Tregs — are often defined less by their cytokine profile and more by their functional attributes. Foxp3 is an essential transcription factor required to manifest the Tregs phenotype and function (described earlier). Plasticity, in regards to Tregs, determines whether a Treg cell changes its functional capability and still maintains its fundamental $Foxp3^+$ Tregs identity (113). Induced Tregs can readily switch to other T helper cell programs under certain cytokine conditions. For instance, induced Tregs can become IL-17-producing cells upon stimulation of IL-6 and IL-21. Th17 cells in turn can also convert into IFN-γ-producing Th1 cells or IL-4-producing Th2 cells when stimulated by IL-12 or IL-4, respectively. However, it is still unclear, whether natural Tregs can be converted to the effector $CD4^+$ T cells and change their function. The effector T cells had been thought to be terminally differentiated lineages, but it now appears that there is considerable plasticity to gain different phenotype (158).

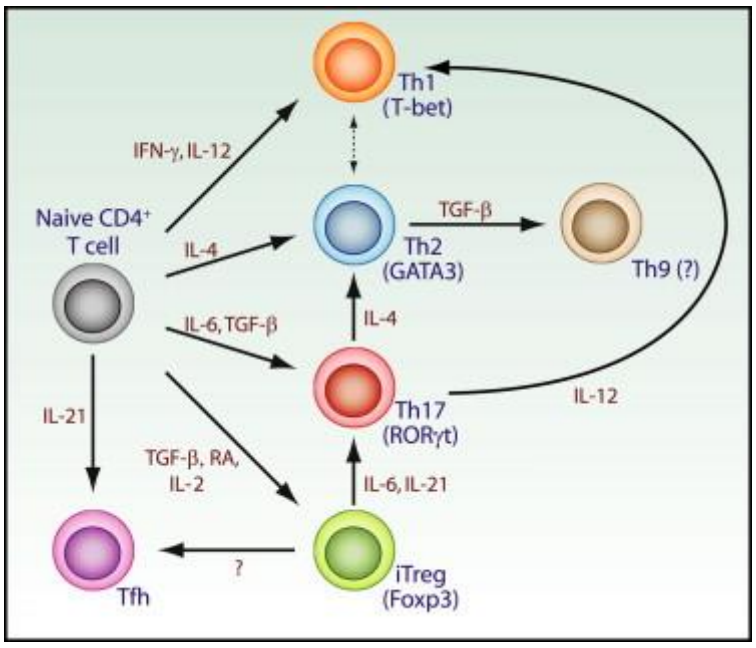

Figure 1.4: The cytokine milieu determines CD4$^+$ T cell differentiation and conversion (158)

1.4 Cancer immunotherapy

Cancer immunotherapy represents the most promising tumor-treatment approach since the development of the first chemotherapies in the late 1940s. It works through enhancing the innate powers of the immune system to locate and attack cancer.

The prevailing techniques of cancer immunotherapy can be divided into two major clusters known as non-specific and antigen-specific therapies.

1.4.1 Specific immunotherapy

Specific immunotherapy can be attained by either adoptive transfer or vaccination. Adoptive transfer means the actual components of the immune system, which are already capable of producing a specific immune response, are transferred into the patients. Vaccination, on the other hand, involves the administration of a certain antigen to induce a specific immune response.

1.4.2 Non-specific immunotherapy

Non-specific immunotherapy refers to therapies that can stimulate the immune system by using a substance that activates or boosts immune cell function regardless of their antigen specificity.

Cytokines

A number of scientific studies have demonstrated the use of cytokines in immunotherapy that can lead to the destruction of tumors through one of following two general mechanisms: first, a direct antitumor effect or second, an indirect enhancement of the antitumor immune response. In the first method/mechanism, cytokines, such as the TNF-α, IFN- α, IFN- β, IL-4, and IL-6 interact directly with tumor cells to induce the latter to either commit suicide or to stop further growth. Although these cytokines are effective when considered as single agents, the administration of a cytokine cocktail can be even more potent as an anticancer agent due to the various synergistic effects stemming from the different cytokines. However, some cytokines can have hazardous side effects. For instance, TNF- α and IL-6 are able to suppress the growth of some tumors while actually promoting the growth of others. Therefore, any immunotherapeutic use of cytokines involves a careful calculation of the potential positive effects and the drawbacks of the procedure (78).

Immune system checkpoints blockade

An instrumental part of any immune system is its ability to keep itself from attacking other healthy cells in the body. To do so it uses ‘checkpoint’ molecules expressed on immune cells which need to be either activated or inactivated to launch an immune response. Cancer cells sometimes manage to bypass these checkpoints and avoid being targeted by the immune system.

- **CTLA-4**

Cytotoxic T lymphocytes antigen-4 (CTLA-4) is a member of the CD28 superfamily and is expressed on T cells after CD28 binding and activation (114). It normally acts as a type of “off switch” that helps to keep T cell in check and prevents the latter from attacking other cells in the body. The ligands for CTLA-4 – B7-1 and B7-2, are the same as for CD28 but with higher affinity (75). CTLA-4 competes with CD28 and inhibits T cell proliferation and signaling.

Ipilimumab is a monoclonal antibody that attaches to CTLA-4 and obstructs its ability to downregulate the immune system. This drug is used to treat malignant cancers, such as melanoma, which have been deemed to be inoperable or have disseminated and metastasized to other organs. This drug has demonstrated a survival benefit. However, the number of patients responding is limited. In addition, the severe side

effects from CTLA-4 blockade, such as diarrhea, skin rash, itching skin and heartburn, limit the drug usage even further (141).

- **PD-1/PDL-1**

The accumulation of experience in targeting CTLA-4 increased the interest in investigating additional immunologic checkpoints, such as the programmed cell death 1/ programmed cell death ligand 1 (PD-1/PD-L1) pathway. PD-1 is an inhibitory receptor expressed on activated T cells, B cells, and natural killer cells (39). Compared to CTLA-4, it seems to be found more often in T cells in inflamed tissues and tumors, where it helps contain the immune response under control. It does this by attaching itself to PD-L1 (B7-H1), which is expressed on hematopoietic cells and in peripheral tissues including tumor, and to PD-L2 (B7-DC), whose expression is more restricted to hematopoietic cells (30). Tumor expression of PD-L1 acts as a protection against T cell-mediated killing through promoting T cell exhaustion. Notably, some cancer cells have large amounts of PD-L1 on their surface, which helps them evade immune attacks (160).

The anti-PD-1 antibodies with the longest follow-up data are nivolumab and pembrolizumab. These drugs have demonstrated a high response rate in patient with melanoma (31, 32) as well as measurable superiority to chemotherapy (32).

The tumor necrosis factor receptor family as a target for cancer immunotherapy

In the past few decades, the generation of monoclonal antibodies targeting immune-stimulating receptors has led to an increase anti-tumor immunity in cancer-bearing hosts resulting in therapeutic responses. The general principle regarding this therapy is that the function of T cells in cancer-bearing hosts is suppressed by the tumor microenvironment. Therefore, the immune stimulating antibodies help to overcome this immune suppression by promoting the function of APCs and T cells, which eventually results in tumor regression. (96). Many of the tumor necrosis factor receptor (TNFR) family members have been proposed as a potential immunotherapy targets.

- **CD137/CD137L**

The first of the TNFR family members that was identified as a possible immunotherapy target was CD137, also known as 4-1BB (92). CD137 is absent on

naïve T cells, but it is upregulated and continually expressed following T cell activation. It is also expressed on NK cells, and on various activated cells of hematopoietic as well as on non-hematopoietic cells including endothelial cells of some tumors. CD137 binds to its ligand, CD137L, which is expressed predominantly on activated APCs, such as macrophages, DCs, and B cells, but it can also be found on non-hematopoietic cells at sites of inflammation. This ligation results in activation of classic and non-classic nuclear factor kB pathways that promotes the higher production of anti-apoptotic molecules such as Bcl2 and Bcl-xl and protects tumor antigen specific cells from activation-induced cell death. CD137/CD137L interaction also improves proinflammatory cytokines expression, such as IL-2, TNF-α, lL-6, and IL-12 (144). More recently, a connection between CD137 signaling and the T cell master transcription factor Eomes has been revealed from observations of high expression levels of Eomes in $CD4^+$ and $CD8^+$ tumor-infiltrating T cells following CD137 agonist therapy in a mouse melanoma tumor model (28). Eomes, member of the T-box family of transcription factors, have been implicated as critical determinants of effector T cell activity to various infectious and tumors.

In addition to CD137's role in promoting proliferation and survival of antigen specific T cells, CD137 can be expressed on tumor cells and vascular endothelium (144). This suggests the agonist CD137 antibodies could promote direct tumor cell death via antibody dependent cell mediated cytotoxicity (ADCC) and tumor cell phagocytosis (4).

Clinical trials are exploring the importance of anti-CD137 therapy in patients with different malignancies. A fully humanized anti-CD137, PF-05082566, is currently being tested in clinical trials as either a single agent in patients with advanced cancer or in combination with rituximab in patients with Non-Hodgkin's lymphomas (NCT01307267) (21).

- **CD134/CD134L**

CD134, also known as OX-40, is expressed on T cells, NK cells and NKT cells, and neutrophils. The ligand, CD134L, is predominantly expressed on activated DCs, as well as on macrophages, B cells, Langerhans cells, smooth muscle cells, endothelium and mast cells. CD134/CD134L interaction delivers strong co-stimulatory signal, leading to an enhanced cell proliferation, survival, effector function and migration (46). The importance of CD134 signaling was demonstrated in tumor-

bearing mice where up to 80% of the animals were cured depending on the tumor model (145). $CD134^+$ T cells were present in a wide variety of human malignancies (138), which promoted translation of anti-CD134 therapy to the clinic.

- **CD40/CD40L**

CD40 was mainly considered a molecule essential for humoral immunity, since it was initially discovered on B cells and DCs. However, CD40/CD40L axis turned out to be also important for development of T cell response. It promotes functional maturation leading to an increase in antigen presentation and cytokine production, and a subsequent increase in the activation of antigen specific T cells. Targeting CD40 with an agonist monoclonal antibody has been tested in patients with both hematological cancers and solid tumors (8, 15).

1.5 Retroviruses

Most retroviruses are RNA viruses that can cause either leukemia (malignancy of lymphoblasts, myeloblasts, or erythroblasts) or sarcoma (solid tumors that can metastasize in any organ of the body).

The retrovirus genome consists of two molecules of RNA, which are physically linked by hydrogen bonds. In addition, there is a specific type of transfer RNA (tRNA) present in all particles that is required for replication. The genomes of simple retroviruses encode the virion capsid/nucleocapsid (*gag*) proteins, that compose the core of the virus; the enzymes needed for genome replication: reverse transcriptase, that transcribes the RNA genome into its DNA component; and integrase; that catalyzes the integration of the double-stranded DNA copy into the host genome, *Pol/In*; and the envelope proteins (*env*) that bind the cell surface molecules used for virus entry.

Retroviruses generally replicate by binding to a cellular receptor and causing transcription of genomic RNA into proviral DNA and integration of proviral DNA into chromosomal DNA. Latency may be established at this point, or transcription may occur to produce new genomes and mRNA. The virus is released by budding, usually without cytopathology (85).

1.5.1 Mechanism of retroviruses' oncogenesis

The mechanisms by which oncogenic retroviruses induce tumor formation vary. Tumor formation often involves activation of a cellular oncogene and down-regulation of tumor suppressor genes. There are three major classes of oncogenic retroviruses, the non-acute transforming viruses, the acute transforming viruses and the trans-acting viruses.

Table 1.1.4: Major categories of oncogenic retroviruses (101)

Category	Occurance (incubation period)	Mechanism of transformation (clonality)	Replication competence	Examples
Non-acute transforming	In nature, more common (years)	Insertional up-regulation of cellular proto-oncogenes (clonal)	Competent	F-MuLV, ALV, FeLV
Acute transforming	In nature, uncommon (weeks)	Action of viral oncogenes (polyclonal)	Defective, requires helper virus	ASV, MSV, FeSV, SFFV
Trans-acting transforming	In nature, uncommon (years)	Transactivation by viral accessory genes (oligoclonal)	Competent	HTLV-1, BLV

ALV - Avian leukemia virus; **ASV** - Avian sarcoma virus; **BLV**- Bovine leukemia virus; **FeLV** - Feline leukemia virus; **FeSV** - Feline sarcoma virus; **HTLV** - Human T-cell leukemia virus; **F-MuLV** – Friend murine leukemia virus; **SFFV** – Spleen focus forming virus; **MSV** - Murine sarcoma virus.

Non-acute transforming retroviruses: insertional mutagenesis

Tumorigenesis results from mutations caused by either promoter/enhancer insertion or by insertional mutagenesis. During this process viruses can activate cellular proto-oncogenes by inserting a viral long terminal repeat (LTR) close to the oncogenes to induce tumor. Transformation due to the effect of the LTR can be effected in two ways – promoter insertion and enhancer activation. In promoter insertion, the DNA provirus is integrated upstream and in the same orientation to a proto-oncogene (a normal cellular gene that can influence cell growth). Transcription is initiated in an

LTR of the provirus and reads through the downstream cellular gene, increasing its rate of transcription. If the cellular gene that is up-regulated has an influence on cellular growth, then transformation may occur. The formed tumors are typically clonal but the transformed cells will depend upon the genomic site of insertion (Fig 1.2 a). For enhancer activation - the provirus DNA is oriented to read away from the cellular gene, the enhancer sequences in the provirus may bind cellular factors that "open" condensed DNA and enhance transcription of neighboring cellular genes regardless of their orientation (Fig 1.2 b). If the gene influences cellular growth, it results in transformation. Since enhancer activation can occur at more sites in the cellular genome, it is a more common phenomenon than promoter insertion (101).

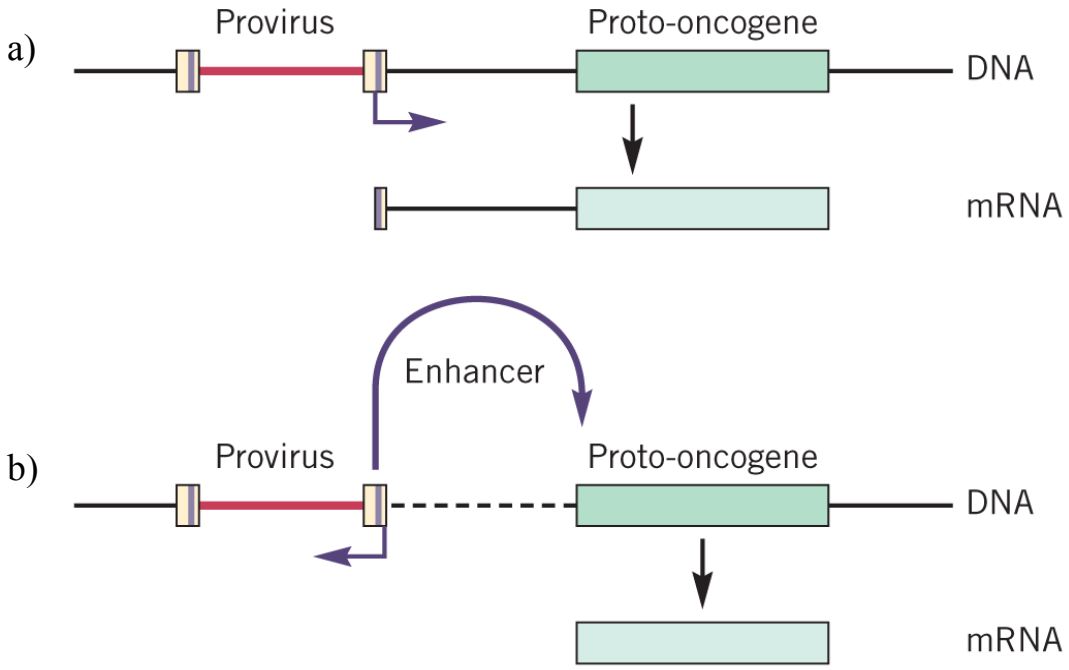

Figure 1.5: Mechanisms of transformation (101)

a) Promoter insertion, b) Enhancer activation

Acute transforming retroviruses: viral oncogenesis

Acute-transforming retroviruses are typically replication defective as they have lost their genes such as *gag* and *env* during an ancestral recombination event, and they rapidly induce tumors because of the viral oncogenes that they carry (*v-onc*). These oncogenes possess a specific transforming activity at a high level. Most *v-onc* genes are derived from proto-oncogenes (*c-onc*). Hence, they can grow only in the presence of a replication-competent non-transforming retrovirus (101).

Trans-acting retroviruses: viral accessory genes

These retroviruses encode several accessory genes that are not only essential for their replication but also play a role in their transforming activity. However, the mechanism of oncogenesis is complex and there is also the requirement of subsequent non-viral genetic events for tumor formation. HTLV-1 is the best studied example trans-acting retroviruses (101).

1.5.2 Friend virus complex as a model to study immunity to retroviruses

Friend virus-induced disease

Charlotte Friend discovered the Friend viral erythroleukemia in 1956 and it has since become a reliable model for understanding host genetic barriers to retroviral diseases. It has assisted in finding mouse genes that control susceptibility to virus-induced cancer and has also been a good model for testing anti-retroviral drugs. Friend virus (FV) belongs to the family of retroviruses. FV is a complex of two viruses: Friend murine leukemia virus (F-MuLV), a replication-competent helper virus that is nonpathogenic in adult mice; and spleen focus-forming virus (SFFV), a replication-defective virus that is the pathogenic component (70). SFFV cannot produce its own particles because its *gag* gene is defective; therefore, it spreads by being packaged in F-MuLV-encoded particles produced in cells co-infected by both viruses. Pathology in susceptible adult mice is characterized by a polyclonal proliferation of erythroid precursor cells, which results in massive splenomegaly. Tumor development is a three-step process that is well characterized on a molecular level:

A false proliferative signal induced by the binding of SFFV gp55 envelope glycoproteins to erythropoietin receptors on nucleated erythroid cells causes splenomegaly (80). In this way, the infection produces a massively expanded population of actively dividing susceptible to viral infection cells. Ultimately, SFFV genomes integrate into two specific sites common to FV-induced erythroleukemias: Spi1 and p53 (70). Insertional mutagenesis-mediated deregulation and overexpression of the Spi1 proto-oncogene, in combination with integration-induced inactivation of the p53 tumor suppressor gene, result in fully malignant erythroleukemias in susceptible mice. The resistant strains of mice are those with a certain MHC background (C57BL/6) that mount immune responses with sufficient

potency and speed to prevent the accumulation of transformed cells due to their antigen-presenting cells can present immunodominant T cell epitopes of FV (52).

FBL-3 model and immune response

FBL-3 cells are an FV-induced tumor cell line of C57BL/6 origin (41). FBL-3 cells do not produce infectious FV, but represent a highly immunogenic FV-transformed tumor cell line that expresses immunogenic FV antigens (18, 64). The antigens that are therefore recognized by protective immune cells are viral antigens expressed by virus-transformed tumor cells and are presented by MHC molecules.

Previous studies used this model, where mice were inoculated intraperitoneally (i.p.) with FBL-3 cells and died 1-2 weeks later with progressive ascites, splenomegaly, and lymphadenopathy (47). The work on this model has demonstrated that: **(1)** both $CD4^+$ and $CD8^+$ T cells can independently promote tumor elimination in the absence of the contribution by T cells of the other phenotype; **(2)** the major antitumor effect of the $CD4^+$ T cells results from the recruitment and activation of other effector cells, particularly macrophages; **(3)** the induction of tumor-specific antibody responses following T cell transfer is not an essential component of tumor eradication; **(4)** NK cells are not important in tumor elimination (48).

However, at that time nothing was known about the Tregs and therefore, population of $CD4^+$ T cells was not divided into the different compartments and the role of those cells was not determined in the FBL-3 model. Only in 2001 Iwashiro et al. demonstrated that mice persistently infected with FV develop approximately twice the normal percentage of splenic $CD4^+CD25^+$ T cells and lose their ability to reject subcutaneous (s.c.) implantation of FBL-3 cells (64) due to a dysfunction of antigen-specific $CD8^+$ T cells (154). In contrast, in uninfected mice after s.c. implantation of FBL-3 cells, the tumor grows locally and subsequently regresses in a $CD8^+$ T cell dependent manner over a time period of 20 days (64). Later on it has been shown that during FBL-3 tumor rejection Tregs suppress cytotoxic $CD4^+$ T cells when $CD8^+$ T cells are not active (2).Previously we showed that in the absence of $CD8^+$ T cells, $CD4^+$ T cells could not control tumor development whereas additional ablation of Tregs enabled these cells to eliminate the tumor. This dual treatment augmented production of cytokines and cytotoxic molecules by $CD4^+$ T cells and increased cytotoxic $CD4^+$ T cell responses. Therefore, the capacity of tumor-reactive $CD4^+$ T cells to reject tumors largely depends on the regulatory effect of $CD4^+$ Tregs (2).

2 AIM AND SCOPE OF WORK

Oncogenic viruses, such as retroviruses, hepatitis viruses and papilloma viruses are associated with 15% to 20% of all human malignancies. In spite of advances in the development of immune therapeutic strategies to combat cancer, the underlying mechanisms of immunotherapies against virus-induced cancer are not well understood. In the current study, we have used the highly immunogenic FV-induced FBL-3 tumor cell line as a model to examine the immunological mechanisms of tumor control in the course of costimulatory immunotherapy. CD137 (4-1BB) is an activation-induced costimulatory molecule on T cells that upregulates survival genes, enhances cell division and induces cytokine and cytotoxin production by tumor-specific T cells. $CD8^+$ T cells are important in the immune control of many tumors. In our model, complete rejection of FBL-3 tumor cells in C57BL/6 mice also depends on $CD8^+$ CTLs. $CD4^+$ T cells play an important role in facilitating help for effector $CD8^+$ T and B cell responses against tumor antigens. Additionally, $CD4^+$ T cells can also play a direct protective role in tumor rejection. However, regulatory T cells (Tregs) are known suppressors of anti-tumor immune responses and are often recruited by tumor cells to evade T cell mediated destruction. In regard to the FBL-3 tumor model, Tregs inhibit effector $CD4^+$ T cell responses during tumor rejection. Interestingly, some Tregs retain a degree of plasticity, and under certain conditions may change ("reprogram") to adopt a pro-inflammatory phenotype. It has been described that in addition to being expressed on activated effector T cells, CD137 is also expressed on Tregs. It is still unclear, however, what the effect of activation of the CD137 signaling pathway in Tregs is.

Thus, the focus of this PhD study was to investigate the influence of CD137 agonist antibody on both the $CD4^+$ T cell and Tregs compartments during virus-induced tumor formation. For this purpose, it was important to determine the phenotype acquired by $CD4^+$ T cells following CD137 agonist therapy by analyzing the expression of various activation, differentiation, and proliferation markers. Moreover, it was of interest to determine the cytotoxicity of those cells. Furthermore, it was important to define the origin of the expanded Tregs after αCD137 treatment, as knowledge on this topic will provide new concepts to therapeutically interfere with Tregs expansion.

Therefore, our study contributes to the existing knowledge on CD137 agonist immunotherapy of cancers by exploring the significance of effector $CD4^+$ T cells and by providing new important information about possible Tregs plasticity.

3 MATERIALS

3.1 Laboratory animals

3.1.1 Wild-type mice

C57BL/6 (B6)	Resistance genotype H-2b/b, Fv1b/b, Fv2r/r, Rfv3r/r Harlan Winkelmann GmbH, Borchen, Germany.

3.1.2 Congenic mice and transgenic mice

CD45.1-congenic B6	B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ, Inbred at Central Animal Laboratory, University Hospital Essen, Germany.
B6NU-F	B6Cg/NTac-Foxn1^{nu}NE10. Foxn1nu mutation backcrossed to the C57BL/6NTac inbred strain. The deficiency in T cell function allows athymic mice to accept and grow xenografts as well as allografts of normal and malignant tissues. Taconic company.
MHC class II knockout	B6.129S2-H2$^{dlAb1\text{-}Ea}$/J. Mice that are homozygous null for MHC class II genes *H2-Ab1, H2-Aa, H2-Eb1, H2-Eb2, H2-Ea.* A dramatic decrease is observed in the number of CD4 positive T cells in thymus, spleen and lymph nodes. This strain should serve as a suitable recipient of xenogenic Class II MHC transgenes allowing the engineering of mouse models of human MHC Class II-associated diseases.
DEREG mice	Created by Dr. Tim Sparwasser's group (Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, Munich, Germany) and maintained at animal facilities of University Hospital Essen. DEREG (**de**pletion of **reg**ulatory T cell) mice were generated from bacterial artificial chromosome (BAC)

technology. These mice express a diptheria toxin receptor (DTR) enhanced green fluorescent protein (eGFP) fusion protein under the control of the *foxp3* locus. Usage of DEREG mice allows both detection and inducible depletion of $Foxp3^+$ Treg cells.

All mice used were sex-matched and were 8-16 weeks of age at the beginning of experiments. The central animal laboratory kept the mice under specific pathogen-free (SPF) conditions which were maintained for the entire experimental phase. The mice had free access to drinking water and standard food.

3.2 Cell line

FBL-3 is an FV-induced tumor cell line derived from a C57BL/6 mouse (41). FBL-3 cells were maintained in complete RPMI medium supplemented with 10% FCS and 0.5% Penicillin/Streptomycin.

3.3 Equipment and materials

The equipment and materials used in this study are listed in Tables 3.1 and 3.2 below.

Table 3.1: Equipment

Item	**Manufacturer**
Biofuge fresco	Heraeus, München
Centrifuge 5415 C	Eppendorf, Hamburg
CO_2 incubator	Thermo, Dreieich
Freezer	LIEBHERR, Ochsenhausen
Heating block	Grant, QBC
Infrared lamps	Phillips, Amsterdam
Laminar flow	KOJAIR®, Meckenheim
LSRII flow cytometer	Becton Dickinson, Heidelberg
Megafuge 1.0R	Heraeus, München
Neubauer cell counting chamber	Becton Dickinson, Heidelberg
Reflected-light microscope CK 2	Hund, Wetzlar

Refrigerator	LIEBHERR, Ochsenhausen
Single channel pipettes (10, 20, 100, 200, 1000 µl)	Eppendorf, Hamburg
Sorvall centrifuge fresco	Thermo, Dreieich

Table 3.2: Materials

Material	**Manufacturer**
Beakers	Schott, Mainz
Cannulae (G23; G25; G27)	Becton Dickinson, Heidelberg
Cell culture flasks (T25; T75; T175)	Greiner bio-one, Frickenhausen
Cell culture plates, sterile (6; 24 and 96 well)	Greiner bio-one, Frickenhausen
Cell microstrainer (70 µm)	Falcon BD, Heidelberg
Disposable syringes (5 ml; 10 ml)	B. Braun, Melsungen
Erlenmeyer flasks	Schott, Mainz
FACS tubes	Becton Dickinson, Heidelberg
Forceps, pointed and curved	Oehmen, Essen
Microtest™ cell culture plates, 96 wells	Falcon BD, Heidelberg
Parafilm	American National Can, Chicago
Plastic pipettes (sterile; 1 ml; 5 ml; 10 ml; 25 ml)	Greiner bio-one, Frickenhausen
PP screw-cap tubes (15 ml; 50 ml)	Greiner bio-one, Frickenhausen
Reaction tubes (1,5 ml; 2 ml)	Eppendorf, Hamburg
Scissors, large and small	Oehmen, Essen

U-shaped microplates (96 wells)	Greiner bio-one, Frickenhausen

3.4 Chemicals and media

Unless not otherwise mentioned, the following chemicals listed were procured from the companies Applichem, Merck, Roth and Sigma.

autoMACS run and wash buffer (Miltenyi Biotec), bovine serum albumin (BSA), brefeldin A (BFA), calcium chloride, dextran, dimethyl sulfoxid (DMSO), ethanol, ethylendiaminetetraacetic acid (EDTA), FACS Clean (BD Bioscience), FACS Flow (BD Bioscience), FACS Rinse (BD Bioscience), fetal calve serum (FCS) (Biochrom), Ficoll (GE Healthcare), 37 % formaldehyde, formalin, incidine 8%, isopropanol, L-Glutamine, penicillin-streptomycin (PenStrep), phosphate buffered saline (PBS) (Gibco), RPMI-1640-Media (Gibco), trypan blue, trypsin-EDTA, Cytofix/Cytoperm [BD Pharmingen], CFSE Cell Proliferation Kit [Invitrogen], Foxp3 staining kit [eBioscience].

3.5 Antibiotics

Ampicillin (Sigma)

Penicillin / Streptomycin (Gibco)

3.6 Buffers and Media

All solutions and buffers (unless otherwise mentioned) were prepared using double distilled water (see Table 3.3).

Table 3.3: Buffers and media

Description	Composition
Culture medium	500 ml RPMI 1640 (Gibco) 10% FCS (Gibco) 0.5% Penicillin/Streptomycin mixture
FACS buffer	PBS 0.02% Na-azide 0.5% BSA

Freezing mediums	40% FCS 10% DMSO 50% RPMI medium
MACS buffer	PBS supplemented with 0.5 % BSA and 2 mM EDTA
PBBS	1 l Phosphate buffered saline (PBS) 1.0 g glucose

3.7 Antibodies

Table 3.4: Antibodies for flow cytometry

Antibodies	**Clone**
Surface antibodies	
CD11a –PE; rat anti-mouse antibody [eBioscience]	M17/4
CD11b (Mac-1) -FITC; rat anti-mouse antibody [eBioscience]	WT.5
CD137-PE; monoclonal rat anti-mouse [BD Pharmingen]	1AH2
CD25-perCP-Cy5.5; monoclonal rat anti-mouse antibody	PC61
CD28 Purified anti-mouse antibody	37.51
CD43-FITC monoclonal rat anti-mouse antibody	1B11
CD43-PE; monoclonal rat anti-mouse antibody [eBioscience]	1B11
CD45.1-APC; monoclonal rat anti-mouse antibody [eBioscience]	A20
CD45.1-FITC; monoclonal rat anti-mouse antibody [eBioscience]	A20
CD45.2- eFluor® 450; monoclonal rat anti-mouse antibody [eBioscience]	104
CD4-AF700; monoclonal rat anti-mouse antibody [eBioscience]	RM4-5
CD4-eFluor 605; monoclonal rat anti-mouse antibody [BioLegend]	RM4-5
Fc block; Affinity purified anti-mouse CD16/CD32 [eBioscience]	93

CD8a-PerCP; monoclonal rat anti-mouse antibody [eBioscience]	53-6.7
KLRG-1-Brilliant Violet 421; monoclonal rat anti-mouse/human antibody [BioLegend]	2F1
Neuropilin-1-APC; monoclonal rat anti-mouse antibody [R&D sytems]	761705
Surface antibodies	
CD154 –PE; monoclonal rat anti-mouse antibody [eBioscience]	MR1
Eomes- eFluor 450; monoclonal rat anti-mouse antibody [eBioscience]	Dan11mag
Eomes- PerCP-eFluor® 710; monoclonal rat anti-mouse antibody [eBioscience]	Dan11mag
Foxp3-APC; monoclonal rat anti-mouse antibody [eBioscience]	FJK-16S
Foxp3-FITC; monoclonal rat anti-mouse antibody [eBioscience]	FJK-16S
Foxp3-PE; monoclonal rat anti-mouse antibody [eBioscience]	NRRF30
Granzyme B–APC; monoclonal anti-human antibody [Invitrogen]	GB12
Helios–eFluor 450; anti-mouse/human antibody [BioLegend]	22F6
IFN-γ-FITC; monoclonal rat anti-mouse antibody [eBioscience]	XMG1.2
IL-2–eFluor 450; anti-mouse antibody [eBioscience]	JES6-5H4
Ki67-PE-Cy7; monoclonal rat anti-mouse/human antibody [eBioscience]	SolA15
T-bet-PE-Cy7; monoclonal mouse anti human/mouse antibody [eBioscience]	eBio4B10
TNF-α–Pe-Cy7; rat anti-mouse antibody [eBioscience]	MP6-XT22

3.8 Fluorochromes

The antibody-coupled fluorochromes and their absorption- and emission maxima are listed in Table 3.5.

Table 3.5: Characteristics of fluorochromes

Flurophore	Abbreviation	Absorption (nm)	Emission (nm)
Alexa flour 488	AF488	488	519
Alexa flour 647	AF647	650	647
Alexa Fluor 700	AF 700	633	723
Allophycocyanin	APC	633	660
APC-cyanine7	APC-Cy7	650	774
Brilliant violet 421	BV421	407	421
Brilliant violet 605	BV605	405	605
eFlour 650	eF650	407	650
Eflour 780	eF780	633	780
eFluor 450	eFluor 450	405	450
Fluorescein isothiocyanate	FITC	488	518
Green fluorescent protein	GFP	509	395/475
PE-cyanine5	PE Cy5	660	670
Peridinin-chlorophyll-protein complex	PerCP	488	675
Phycoerythrin	PE	488	575
Phycoerythrin–Cy7	PE Cy7	488	785

3.9 Standard kits

Table 3.6: Standard kits

Kit	Manufacturer
Cytofix/cytoperm intracellular staining kit	BD Pharmingen, Heidelberg, Germany
Foxp3 staining set	eBioscience, San Diego, USA
Mouse $CD4^+$ T cell isolation kit II	Miltenyi Biotec, Bergisch Gladbach,

	Germany

3.10 Depletion antibodies

$CD8^+$ T cell depletion antibody	Clone 169.4.2.1, produced by YTS 169.4.2.1 hybridoma cell line.
$CD4^+$ T cell depletion antibody	Produced by YTS 191.1 hybridoma cell line

3.11 Treatment reagents

*InVivo*MAb anti m CD137	CD137 antibody, Clone LOB12.3, purchased from BioXcell.
Diphtheria toxin (DT)	Diphtheria toxin, Corynebacterium diphtheria – Calbiochem, purchased from Merck.

3.12 MHC II tetramer

The MHC class-II tetramer was provided by the NIH Tetramer Facility

APC-labelled (I-Ab) MHC class II tetramers loaded with $I\text{-}A^b$-restricted MoMSV-envelope epitope (H19-Env) derived peptide (EPLTSLTPRCNTAWNRLKL) (121). MHC class-II tetramers were provided by NIH tetramer core facility used for the detection of $I\text{-}A^b$ FV envelope specific $CD4^+$ T cells (Emory University, Atlanta, USA).

4 METHODS

4.1 Animal trials

The animal experiments were conducted according to the guidelines of the Federation of European Laboratory Animal Science Association.

4.2 Tumor challenge

Mice were shaved on the right flank and 1×10^7 FBL-3 tumor cells were injected subcutaneously (s.c.) in 100 µl of PBS through a 27-gauge needle on day 0. In order to verify tumor volume by external caliper, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Tumor size based on caliper measurements were calculated by formula: tumor area (cm^2) = $\pi * a * b$, where a = half of length and b = half of width. After 4, 6, 8, 11, 15, and 20 days,On day 6 mice were sacrificed, and drLNs (inguinal) were resected.

4.3 *In vivo* depletion of lymphocyte subsets and antibody treatment

Mice were depleted of $CD8^+$ T cells by intraperitoneal (i.p.) injection of 0.5 ml of supernatant from the hybridoma cell line 169.4 producing a CD8a-specific monoclonal antibody. CD8 depletion was started at day 0 and carried out every other day for the tumor growth analysis until mice were sacrificed due to the progressive tumor growth, or four times (on day 0, 2, 4, 6) for the experiments where mice were sacrificed at day 6 post tumor inoculation. The treatment depleted more than 90% of the $CD8^+$ T cells in lymph nodes.

Depletion of Tregs was done in DEREG mice by i.p. injection of 0,5 µg DT (Merck) diluted in endotoxin-free PBS three times on every third day starting at day -1 . The treatment depleted more than 95% of the $CD4^+$ $eGFP^+$ T cells in lymph nodes of DEREG mice.

To deplete $CD4^+$ T cells, mice were injected i.p. with 0.5 ml supernatant from the hybridoma cell line YTS 191.1 producing a CD4-specific monoclonal antibody. Depletion was carried out every other day starting at day 0. The treatment depleted more than 90% of the $CD4^+$ T cells in lymph nodes.

The mAb YTS191.1 was of immunoglobulin G2b isotype and was produced and used as ascites fluid or culture supernatant fluid. The hybridoma cell line was a kind gift

from Dr. Kim Hasenkrug (Laboratory of Persistent Viral Diseases, Rocky Mountain laboratories, NIAID, Montana, USA), and was stored in liquid Nitrogen chamber for later use.

The αCD137 (LOB 12.3) used *in vivo* were produced by Bioxcell. Dosing per injection was 100 µg administered i.p. every other day from day 0 three times.

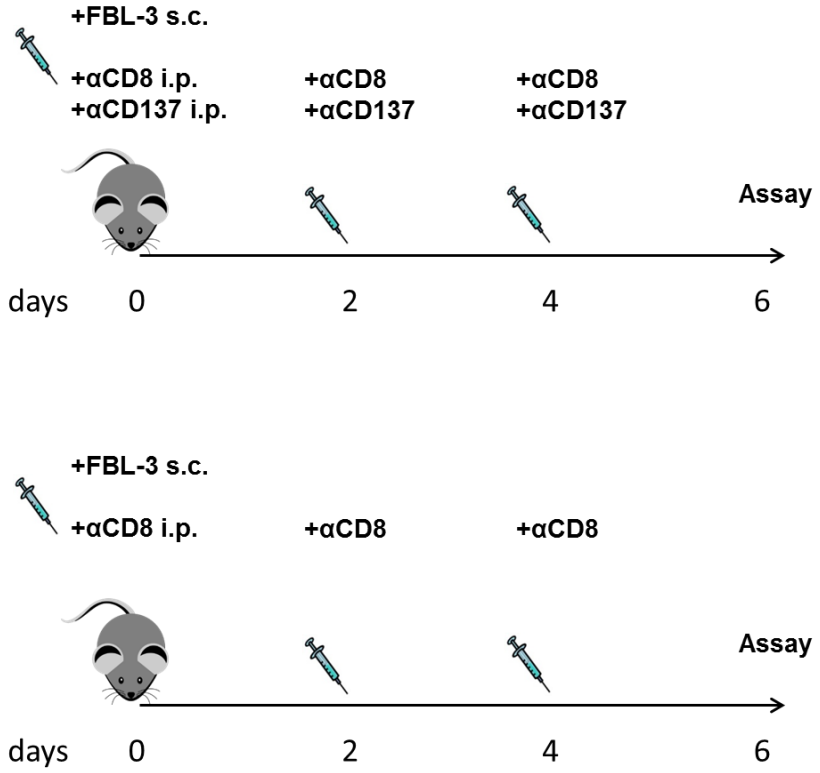

Figure 4.1: Treatment and T cell depletion protocol

4.4 Flow cytometry

Flow cytometry is a method for analyzing expression of cell surface and intracellular molecules, characterizing and defining different cell types. It allows simultaneous multi-parameter analysis of single cells. It is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins or ligands that bind to specific cell-associated molecules.

4.4.1 Methodology of flow cytometry

A flow cytometer includes three main systems: fluidics, optics, and electronics (61) (Fig 4.2).

- ❖ The fluidics system hydrodynamically focuses the cell stream to the laser beam for examination.
- ❖ The optics system includes lasers to light the particles in the sample stream and optical filters, which are used to direct the light signals to the detectors.
- ❖ The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

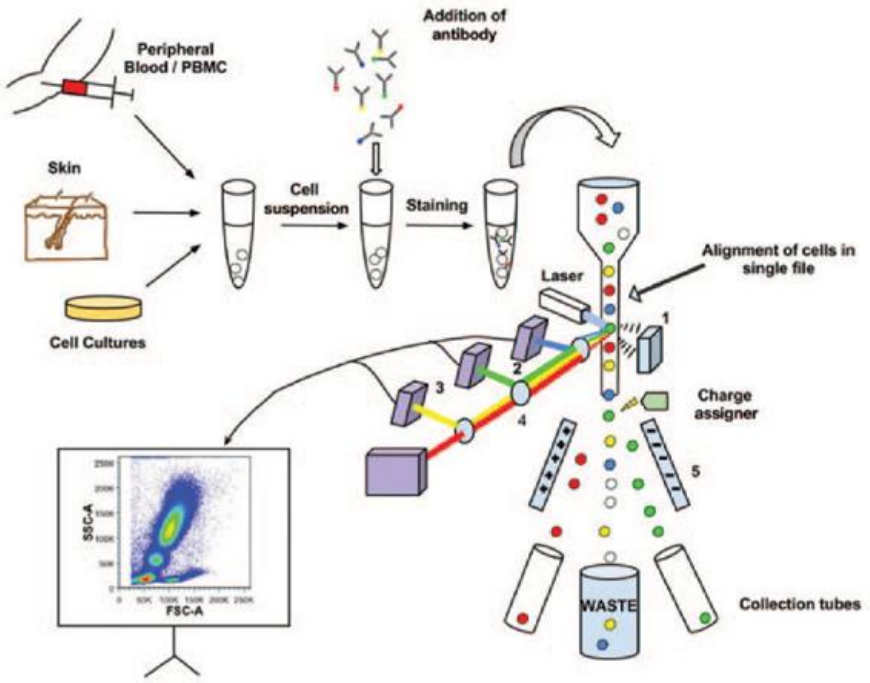

Figure 4.2: Principle of flow cytometry

(1) Forward-scatter detector, (2) side-scatter detector, (3) fluorescence detector, (4) filters and mirrors, and (5) charged deflection plates (66).

Light scattering

Light scattering takes place when a particle deflects incident laser light. Factors that affect light scattering include the cell's membrane, nucleus, and any granularity of the cell. Cell shape and surface topography are also important (65). Forward-scattered light (FSC) is proportional to cell size. FSC is a measurement of mostly diffracted light and is detected just off the axis of the incident laser beam in the forward direction by a photodiode.

Side-scattered light (SSC) is proportional to cell granularity or internal complexity. SSC is a measurement of a light that occurs at any interface within the cell where there is a change in refractive index. SSC is collected at approximately 90 degrees to

the laser beam by a collection lens and then redirected to the appropriate detector (61) (Fig 4.3).

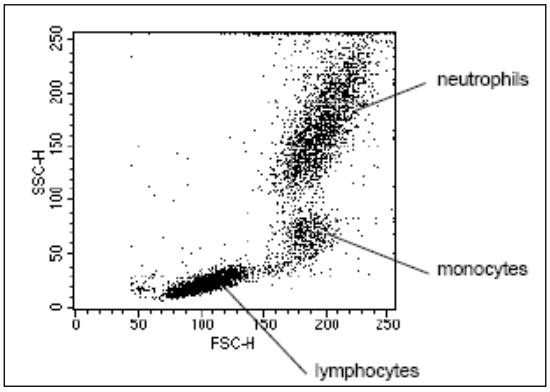

Figure 4.3: Identification of cells based on FSC v/s SSC

(61)

Fluorescence

A fluorescent compound (fluorochrome) absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light results in an electron in the fluorescent compound rising to a higher energy level. The excited electron quickly goes back to its ground level, emitting the excess energy as a photon of light. This transition of energy is called fluorescence (61). These methods enable a quantitative investigation of the surface molecules. The basis for this is an antigen antibody reaction conducted with fluorescently-marked antibodies which are aimed at particular surface molecules. The single cell suspensions are analysed by flow cytometry (Fig 4.4).

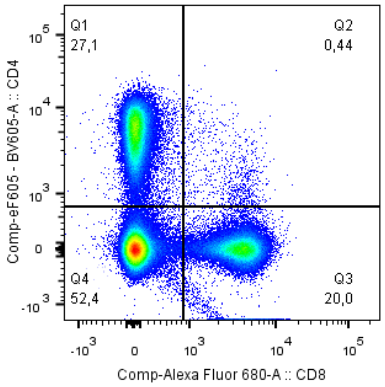

Figure 4.4: Specific binding of fluorochrome-labelled antibodies to cell surface antigens

Depicts cell characteristics with respect to expression profile of 2 molecules**,** e.g., in this blot with CD8 on the y-axis and CD4 on the x-axis.

Signal detection

Light signals are generated as particles pass through the laser beam in a fluid stream. These light signals are converted to electronic signals (voltages) by photodetectors and then assigned a channel number on a data plot. A voltage pulse is created when a particle enters the laser beam and starts to scatter light or fluorescence. Once the light signals, or photons, strike one side of the photomultiplier tube (PMT) or the photodiode, they are converted into a proportional number of electrons that are multiplied, creating a greater electrical current. The electrical current travels to the amplifier and is converted to a voltage pulse. The highest point of the pulse occurs when the particle is in the center of the beam and the maximum amount of scatter or fluorescence is achieved. As the particle leaves the beam, the pulse comes back down to the baseline (61) (Fig 4.5).

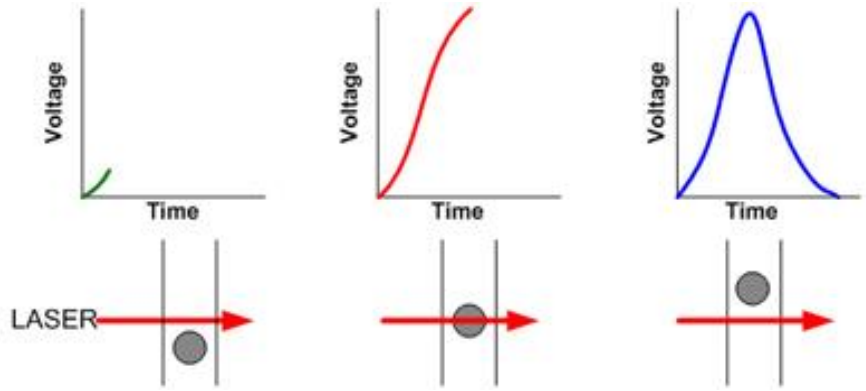

Figure 4.5: Formation of a voltage pulse

4.5 Preparation of single cell suspensions of lymph nodes

For the removal of lymph nodes, required number of mice were anaesthetised with Isofluran® and killed by cervical dislocation. Lymph nodes were collected in 6-well plates kept on ice. Each lymph node was homogenised using a sterile sieve to make single cell suspension of lymph node cells using a sterile plunger of a 5 ml syringe. The thicker components were removed and the single cell suspension was transferred into a 50 ml tube. The lymph node suspension was filled up to 5 ml with PBBS. An aliquot was taken from each suspension to count viable cells by Trypan blue exclusion assay. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the

membrane is damaged. Thus, all the cells, which are not colored are viable. An aliquot of cells was diluted with 0.4% Trypan blue stain and 10 µL of the diluted cell solution was transferred onto the Neubauer cell counting chamber and cover-slipped to be examined under microscope (at 10x) for cell counting. Formula for counting number of viable cells for the total cell concentration:

Numbers of cells/ml = numbers of cells over a large square X dilution factor

Meanwhile, the suspensions in the tubes were centrifuged at 300 x g at 10°C for 8 minutes. The cell pellet was re-suspended with PBBS to a desired final concentration of lymph node cells of 1 x 10^8 cells/ml.

4.6 Staining of cells and FACS analysis

1x10^6 cells from each tissue were suspended in FACS buffer, centrifuged for 5 min with 300 x g at room temperature and the supernatant was removed. The cell pellet was re-suspended in 100 µl FACS buffer containing 0.5 - 2 µl conjugated antibodies to various surface molecules and incubated in the dark for 30 min at 4°C. After a double rinse in FACS buffer, the cells were re-suspended with 400 µl FACS buffer. Data were acquired from 250,000 to 500,000 lymphocyte-gated events per sample.

In order to check intracellular production of IFN-γ, TNF-α and IL-2 cells from lymph nodes, samples were stimulated with plate-bound CD3 antibody in the presence of 2 µg/ml of CD28 antibody and 2 µg/ml of brefeldin A for 5 h at 37^0 C. Anti-CD28 was used to obtain the full range of T-cell activity in challenged mice. The cells were then stained for surface expression of different markers, fixed and permeabilized with Cytofix/Cytoperm solution (BD). The cells were then washed, permeabilized, and incubated with Fc blocking anti-mouse CD16/CD32. After that cells were labelled with monoclonal antibodies specific for IL-2, IFN-γ, TNF-α and anti-CD154. Foxp3, Eomes, T-bet expression as well as granzyme B production was detected by intranuclear, intracellular staining using an anti-mouse/rat Foxp3 antibody and the Foxp3 staining kit. Helios expression was measured by intracellular staining using an anti-mouse/human Helios antibody and the Foxp3 staining kit.

After a double rinse in FACS buffer, the cells were re-suspended in a volume of 400 µl with FACS buffer and analyzed in detail. Data were acquired from 250,000 to 500,000 lymphocyte-gated events per sample. The fluorescently-stained characteristics of the cells were measured on LSRII flow cytometer and evaluated with FlowJo software.

4.7 Tetramer and tetramer staining

Tetramer staining is a gold-standard method for T cell analyses and isolation of a very small number of antigen-specific cells. This method established in 1996 by a group of scientists Davis, McHeyzer-Williams and Altman was mainly considered to label T cells in an antigen-specific manner. Schumacher´s laboratory in Holland developed mouse (I-A^b) MHC class II tetramers in which the MHC heterodimers are expressed with a genetically fused peptide in insect cells. Heterodimers of the extracellular domains of the MHC class II alpha and beta chain are produced in insect cells associated with the T cell epitope attached to the beta chain. Four identical biotin-containing pMHC complexes are bounded to fluorescently labelled streptavidin for binding to TCRs (Fig 4.7). Tetramers bind to T cells that express T cell receptors specific for the cognate peptide – MHC complex and can then be utilized to track Ag-specific T cells by flow cytometry. MHC–II tetramer positive cells are effector $CD4^+$ T cell populations.

The (I-A^b) class-II tetramers were used to detect the I-A^b FV envelope specific $CD4^+$ T cells. 1 x 10^6 nucleated lymph node cells were incubated with APC-labelled I-A^b tetramers for 2-3 hrs at 37^0C and later stained with surface molecules to quantify the population of tumor-specific $CD4^+$ T cells by flow cytometry. After washing, cells were stained with anti-CD4 antibody and anti-monocytes CD11b for 8 minutes at 4^0C. Cells were washed, re-suspended in buffer containing PI and analyzed by Flow cytometry and 200,000 to 500,000 lymphocyte gated events per sample and analysis were recorded.

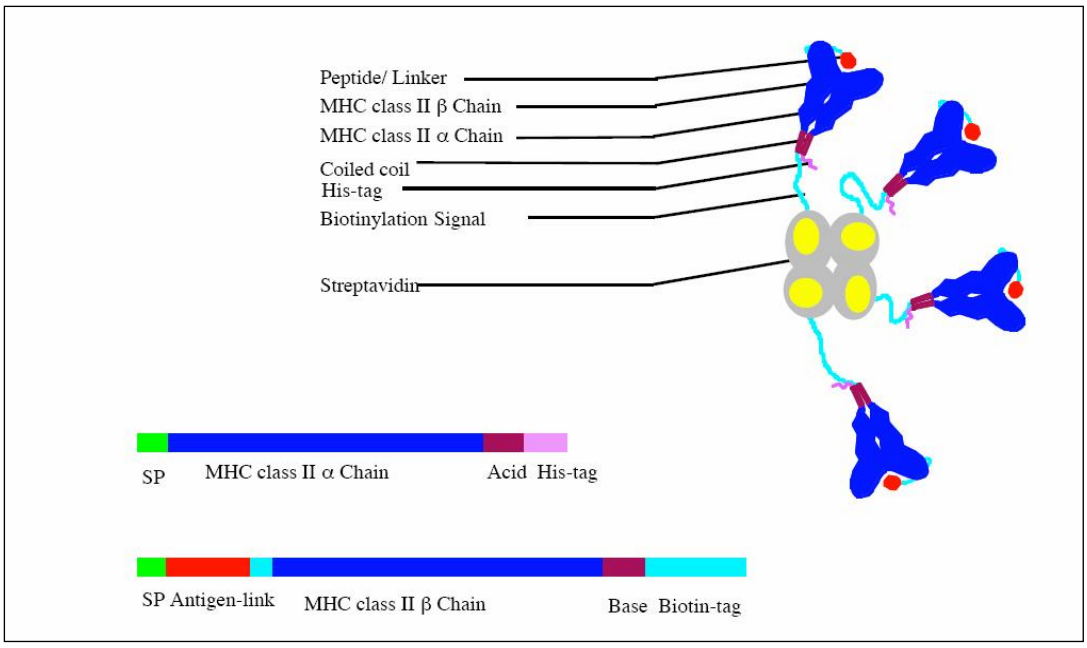

Figure 4.6: Schematic representation of MHC class-II tetramer components (60)

4.8 *In vivo* cytotoxicity assay

This assay allows determining the cytotoxic function of T cells in different groups of tumor-bearing mice (Fig. 4.6). Lymphocytes were isolated from lymph nodes and spleens from CD45.1 mice (donor mice), which were depleted for $CD4^+$ and $CD8^+$ T cells two times before being sacrificed. This depletion allowed to enrich $MHCII^+$ cells by exclusion of all T cells, as only $MHCII^+$ cells can serve as targets for $CD4^+$ T cell-mediated killing. Single cell suspensions were prepared and the cells were washed with 40 ml of PBS. Mononuclear cells from the spleens were separated additionally by Percoll density gradient centrifugation. Splenocytes were suspended in 20 ml of RPMI 1640 medium and slowly overlaid on a 20 ml Percoll layer in 50 ml Falcon tube. After centrifugation (300×g, 10 min, without break) the lymphocytes containing middle layer were transferred to the fresh tube. Consequently, cells were washed twice in 50 ml of PBS. Cell suspensions from lymph nodes and Percoll-separated spleen cells were mixed and divided into equal volumes of 15 ml of RPMI medium into two tubes. The cells from one tube were loaded with the class II-restricted peptide recognized by $CD4^+$ T cells for 1,5 h at 37°C and afterwards were stained with 40nM CFSE (carboxyfluorescein succinimidyl ester) dye for 10 min at 37°C and then for 5 min on ice (target cells, experimental). The phenotype of these cells would be $CD45.1^+CFSE^+$. The unloaded cells remained intact and would be separated from the target cells as $CD45.1^+CFSE^-$ (control cells). Peptide loaded and unloaded cells

were counted using Trypan blue exclusion microscopy and suspended in sterile PBS in the ratio 1:1. 1.0×10^7 cells of each population (per mouse) were injected intravenously (i.v.) into tumor-bearing mice. 20 hrs after i.v. injection of the donor cells, recipient mice were sacrificed and surface staining with CD45.1 antibody of the cells from drLNs was performed. Subsequently, in vivo killing activity was quantified in single-cell suspensions from the drLN of each tumor-bearing mouse.

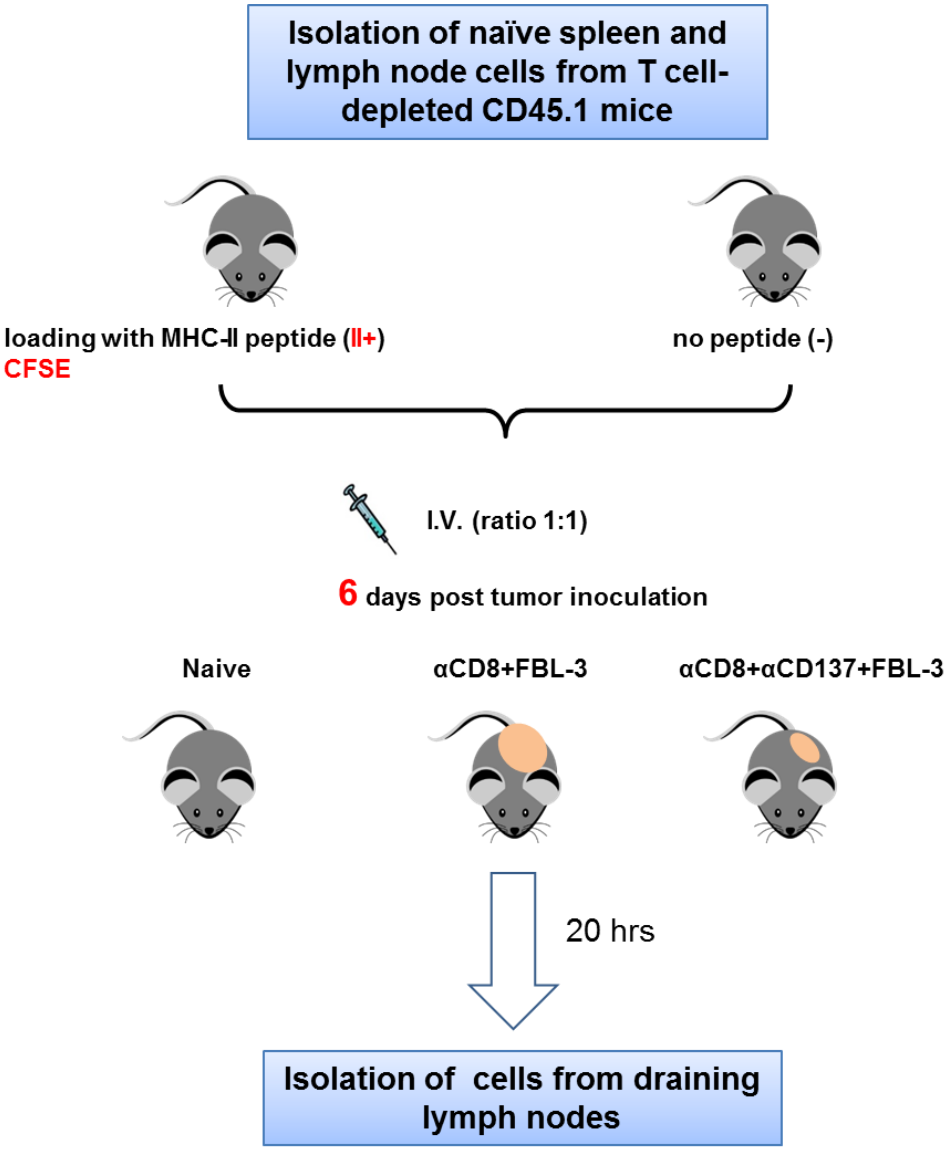

Figure 4.7: Scheme of the in vivo cytotoxicity assay to show the killing of peptide-loaded cells by $CD4^+$ CTL in draining lymph nodes

Cytotoxicity was calculated using the following formula: cytotoxicity = 100% × (1-((unloaded/loaded)$_{control}$ / (unloaded/loaded)$_{experimental}$)).

Tumor target lysis assay was performed using 2 ×10^5 FBL-3 cells per mouse labeled with 10 µM of CFSE. FBL-3 cells were injected i.p. into naïve CD45.1 mice. Additionally, mice received sorted $CD4^+GFP^+$ cells from either αCD8, or αCD8+αCD137 tumor-bearing or naïve mice. The mice were sacrificed 48 hrs later and intraperitoneal lavage was performed with 10 ml PBS to obtain cells. Cells were washed once, re-suspended in buffer containing fixable viability dye to exclude dead cells and analyzed by flow cytometry for $CFSE^+$ signal.

4.9 Cell isolation with the MACS technology

MACS technology is a fast method for the isolation of viable and functionally active cells by magnetic labeling. It is grounded on MACS MicroBeads in combination with manual MACS separators and columns. When MACS columns are placed in MACS separator, the MACS columns matrix provides a magnetic field strong to retain cells labelled with the magnetic beads. The magnetically labelled cells are retained in the column, while unlabeled cells pass through. The retained cells are eluted from the MACS column after removal from the magnet.

The isolation of $CD4^+$ T cells was conducted using the mouse $CD4^+$ T cell isolation kit II, which provides only labeling of non-$CD4^+$ T cells. With this negative isolation the pre-activation of $CD4^+$ T cells is prevented. Briefly, the cells were re-suspended in MACS buffer (40µL buffer per 1x10^7 cells). Afterwards biotinylated antibody cocktail was added to couple all non-$CD4^+$ T cells (10µL buffer per 1x10^7 cells). The cell suspension with the biotinylated antibody cocktail was incubated for 10 minutes at 4°C in the dark. Ten minutes later Macs buffer was added (30µL buffer per 1x10^7 cells). Then anti-biotin magnetic Microbeads were added (20µL buffer per 1x10^7 cells). After the incubation period of 20 minutes cells were washed by the centrifugation for 10 minutes at 520 xg. Next, supernatant was discarded and cells were re-suspended in 700µL MACS buffer per 1x10^7 cells. The LS columns were equilibrated in the magnetic field of the MACS separator. LS columns were washed with MACS buffer three times before being used. Each sample was passed through the magnetic columns and un-labeled $CD4^+$ cell population was collected in the

separate tubes. Collected cells were centrifuged and the pellets are re-suspended with 1 ml PBS.

FACS analysis of the 10.000 cells was always performed for proving the $CD4^+$ T cell purity.

4.10 Sorting of GFP^+ Tregs and adoptive cell transfer

A fluorescence-activated cell sorter is used to retrieve populations of interest from a heterogeneous population for further study. If a cell can be specifically identified by its physical or chemical characteristics, it can be separated using a flow sorter. Briefly, when the specific cells pass through a laser beam they are monitored. Droplets containing single cells are given a positive or negative charge, based on whether the cell has limited the fluorescence or not. Droplets containing a single cell are then detected by an electric field into collection tubes according to their charge

For the transfer experiments of GFP^+ $CD4^+$ T cells, pre-enriched $CD4^+$ T cells (MACS technology) from DEREG mice were sorted on the basis of GFP fluorescence. Separation of the GFP^+CD4^+ from GFP^-CD4^+ T cells was performed on a FACSDiVa cell-sorter (Becton Dickinson, San Jose, CA). GFP was excited with a 488 nm laser wavelength and fluorescence was measured through a 585/42 nm bandpass filter. For each experiment 200.000–330.000 cells were sorted by flow cytometry.

After the separation of the cells the purity of the separated cell populations was always analyzed again by flow cytometric analysis of 10.000 cells. To >95% pure populations of $CD4^+GFP^+$ or $CD4^+GFP^-$cells was achieved.

Between 1 and 3 $x10^6$ $CD4^+GFP^-$ cells and 1-5 $x10^5$ $CD4^+GFP^+$ cells were transferred into CD45.1 recipients by i.v. injection in 0.5 ml PBS on day 0 following FBL-3 challenge and antibodies administration.

4.11 Exclusion of dead cells in flow cytometry

The exclusion of dead cells and cellular debris in flow cytometry was performed using the dye propidium jodide (PI). PI has a high DNA binding constant and is efficiently excluded by intact cells. It is useful for DNA analysis and dead cell discrimination during flow cytometric analysis. When excited by 488nm laser light, PI fluorescence is detected in the far red range of the spectrum (562-588nm band-pass filter). In healthy cells, the intact cell membrane prevents the fast access of PI and therefore it is only slowly absorbed by the healthy cells. However, should a cell be apoptotic or is

damaged or killed in the measuring process, then the cell membrane lacks its protective function and PI rapidly diffuses into the cell. There, it is taken up in the cell nucleus and DNA with which it interacts. The cell is thus marked as “dead” and can be detected by the flow cytometer. PI in amount of 1 µl was added to the stained cells in 400 µl FACS buffer and the sample was immediately analysed.

For the exclusion of dead cells in intracellular staining, Fixable Viability Dye (FVD) was used. FVD is a viability dye that can be used to label dead cells prior to fixation and/or permeabilization procedures. FVD stain is based on the reaction of a fluorescent reactive dye with cellular proteins (amines). These dyes cannot penetrate live cell membranes, so only cell surface proteins are available to react with the dye, resulting in dim staining. The reactive dye can infuse the damaged membranes of dead cells and stain both the interior and exterior amines, resulting more intense staining. FVD eF780 was added in amount of 1 µl per 1 mL of cells together with the surface staining antibodies.

4.12 Statistical analyses

Statistical analyses and graphical presentations were computed with Graph Pad Prism version 5. Statistical differences (p-value) between two groups were performed using unpaired t test. Statistical differences (p-value) between the different parameters were performed testing with the Kruskal-Wallis one-way analysis of variance on ranks and Newman-Keuls multiple comparison tests. The p-value is a probability with a value ranging from zero to one. In this work, all p-values ≤ 0.05 were determined significant.

5 RESULTS

Despite of robust tumor immunity, tumor growth is not controlled in cancer patients. Consequently, this can lead to metastasis and death. One approach of cancer immunotherapy in the animal model has been the stimulation of the Tumor Necrosis Factor Receptor (TNFR) superfamily member CD137 (also known as 4-1BB), which is involved in T cell activation and function (122) including expansion, survival, and cytokine production of effector T cells (27, 73). This work set out to investigate the effector function of $CD4^+$ T cells and Tregs in the course of CD137 co-stimulation during virus-induced tumor formation.

The experiments described in the current work were performed with C57BL/6 (B6) mice. The tumor size was measured and the role of different immune cell subsets was identified. As lymph nodes play a central role in anti-tumor immunity, our main focus in the FBL-3 model was on tumor draining lymph nodes (drLN). FBL-3 cells (1×10^7) were injected into the right flank of B6 mice and after 6 days post tumor challenge (ptc), T cell responses were determined.

5.1 CD137 agonist therapy in $CD8^+$ T cell depleted mice promotes FBL-3 tumor cell rejection through a CD4-dependent mechanism

T cell immune responses against tumor cells play a pivotal role in the arrest of tumor growth. Prior studies have shown that $CD8^+$ T cells are essential in controlling FBL-3 tumor progression (64, 153). As others have seen in this model, we observed no tumor control in mice depleted for $CD8^+$ T cells and animals had to be euthanized in average 15 days after tumor infusion (Fig. 5.1B). To define the influence of a co-stimulatory CD137 antibody on the $CD4^+$ T cell compartment in tumor protection, $CD8^+$ T cell depleted tumor-bearing mice were treated every second day with the CD137 antibody starting at day 0 (described in Methods). Strikingly, despite the absence of $CD8^+$ T cells, the co-stimulation of $CD4^+$ T cells with CD137 agonist restored anti-FBL-3 tumor immunity and injected FBL-3 cells were completely rejected after in average 22 days of tumor challenge (Fig. 5.1C). This is only a slight delay of 7 days in tumor rejection compared to only FBL-3 challenged mice, in which $CD8^+$ T cells mediate tumor rejection (Fig. 5.1A). This experiment suggests that even in the absence of the cytotoxic $CD8^+$ T cell compartment $CD4^+$ T cells are capable of controlling tumor progression. In order to prove that tumor rejection was due to a

αCD137 induced effector $CD4^+$ T cell responses, tumor growth was analyzed in mice lacking both $CD4^+$ and $CD8^+$ T cells, and administrated with a αCD137 antibody. Additional depletion of $CD4^+$ T cells reversed the effects on tumor rejection - in the absence of these T cell compartments no control of tumor growth was observed (Fig. 5.1D), indicating that $CD4^+$ T cells can mediate anti-tumor immunity when stimulated with CD137 antibody.

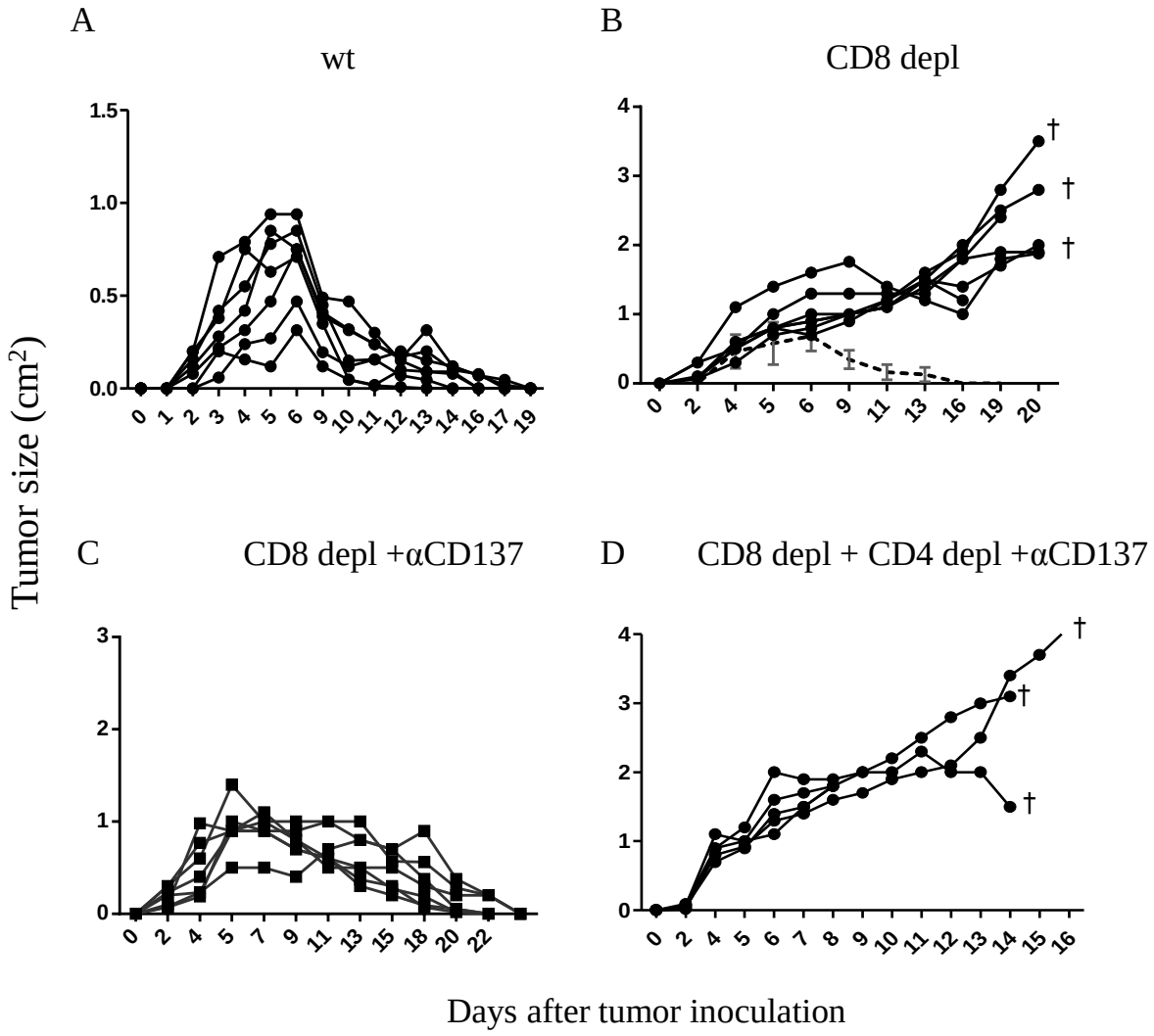

Figure 5.1: Influence of different cell populations and αCD137 therapy on tumor formation

Effects of depletion of $CD8^+$ T cells (B), depletion of $CD8^+$ T cells and αCD137 treatment (C), depletion of $CD8^+$ T cells, $CD4^+$ T cells and αCD137 treatment (D). A, no depletion (injected with PBS). B6 mice were injected s.c. with 1×10^7 FBL-3 cells (1×10^7) and tumor size was measured. Mice were depleted for their $CD8^+$, $CD4^+$ T cells as described in methods. Each line represents tumor progression in an individual mouse. Dragger symbol: mice were euthanized due to progressive tumor growth.

5.2 CD137 ligation increases the numbers of activated tumor-specific CD4$^+$ T cells in draining lymph nodes of FBL-3 challenged mice

To understand the underlying immunbiology of the described phenomenon, we next characterized differences in the anti-tumor CD4$^+$ T cell responses in mice depleted for CD8$^+$ T cells compared to those that also received CD137 agonist treatment during FBL-3 tumor challenge. The total population of CD4$^+$ T cells showed no differences in numbers between the groups only lacking CD8$^+$ T cells and additionally treated with αCD137 (Fig. 5.2).

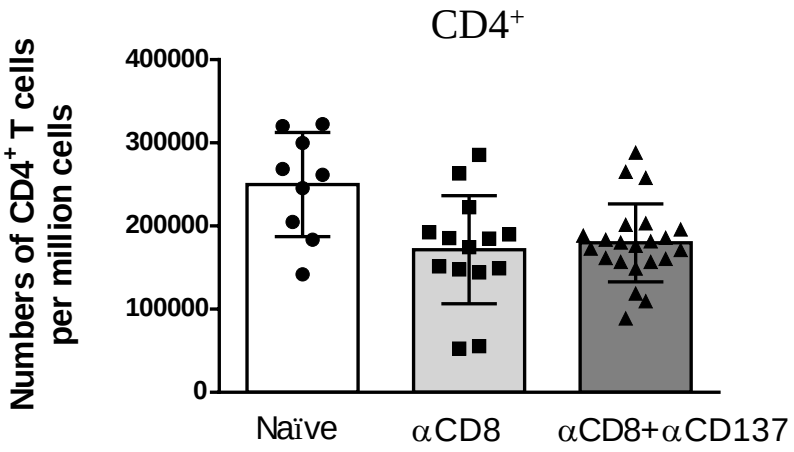

Figure 5.2: αCD137 therapy leads to the expansion of CD4$^+$ T cells in draining lymph nodes

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and depleted for CD8$^+$ T cells. Simultaneously with tumor inoculation, some mice also received αCD137 treatment as described in methods. At day 6 post tumor challenge (ptc) draining lymph nodes were analyzed for total numbers of CD4$^+$ T cells by flow cytometry. The experiment was repeated three times with comparable results.

However, the expression of the proliferation-associated antigen Ki67 on CD4$^+$ T cells from draining lymph nodes was significantly enhanced in mice treated with CD137 agonist antibody and depleted for CD8$^+$ T cells in comparison to mice only lacking CD8$^+$ T cells or in naïve animals (Fig. 5.3A). One possible explanation for the unaltered CD4$^+$ T cell numbers despite increased cell proliferation was that these cells might leave the drLN and migrate into the tumor microenvironment. It has previously been demonstrated that in FBL-3 tumor challenged mice CD4$^+$ T cells infiltrate the tumor bed and constitute a significant part of the tumor infiltrating

lymphocytes (2). In addition, we analyzed expression of KLRG-1 (killer cell lectin-like receptor subfamily G member 1, a marker for terminally differentiated effector T cells) (56) on $CD4^+$ cells from different groups of mice. Significantly higher proportions of $CD4^+$ T cells expressed KLRG-1in mice following co-stimulatory therapy than in the control groups (Fig. 5.3B).

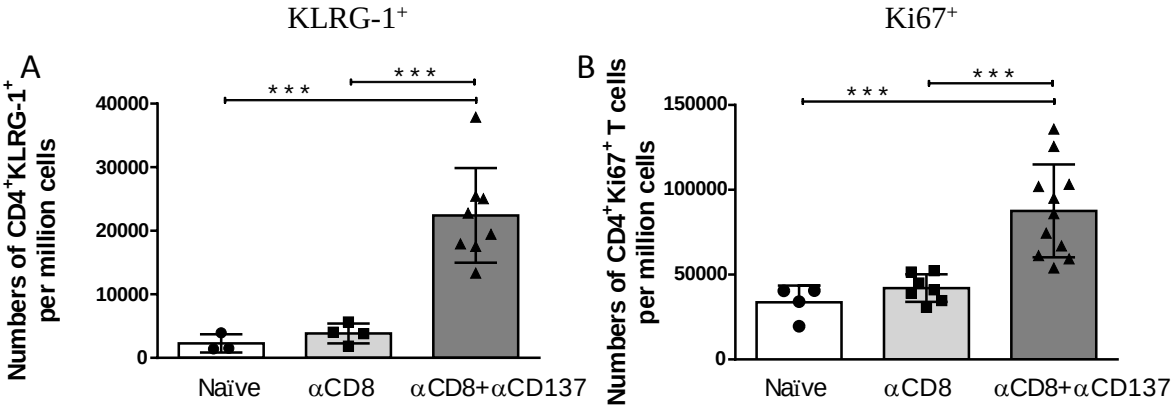

Figure 5.3: αCD137 therapy leads to the activation of CD4+ T cells in draining lymph nodes

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and additionally treated with αCD8. Some mice also received αCD137 treatment as described in methods. At day 6 ptc draining lymph nodes were analyzed for expression of different molecules by flow cytometry. A, the maturation profile was detected by surface expression of KLRG-1. B, the proliferation of $CD4^+$ T cells was measured by the intracellular expression of Ki67. Each dot represents an individual mouse and the mean numbers are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (****P<0.0005*). The experiment was repeated three times with comparable results.

Next, we focused on the specific characteristics of $CD4^+$ T cells in mice depleted for $CD8^+$ T cells following CD137 therapy. Significantly enhanced numbers of $CD4^+$ T cells co-expressed the activation markers CD43 (the activation-induced glycosylated isoform) (69) and CD11a (a co-stimulatory signaling molecule upregulated upon antigen recognition) (90) in mice following CD137 therapy and $CD8^+$ T cell depletion than in mice only depleted for $CD8^+$ T cells or in naïve animals (Fig. 5.4A). It has been shown that FBL-3 tumor cells expresses FV antigens that can be recognized by $CD8^+$ and $CD4^+$ T cells (18, 63). To analyze the tumor-specific immune response after CD137 treatment we used the tetramer technology (as described in Materials

and Methods) to stain lymphocytes from the drLN of FBL-3 challenged mice with MHC class-II tetramers loaded with the I-A^b-restricted MoMSV-envelope epitope (H19-Env) peptide-encoding (EPLTSLTPRCNTAWNRLKL) sequence (115). At 6 days ptc αCD137-treated mice had significantly more tetramer$^+$ $CD4^+$ T cells than only $CD8^+$ T cell depleted mice that did not receive treatment (Fig. 5.4B) showing that αCD137 signaling enhances tumor-specific $CD4^+$ T cell immunity. These data demonstrate that targeting the CD137 molecule with an agonist antibody in tumor-bearing mice depleted for $CD8^+$ T cells leads to the expansion of activated and highly differentiated tumor-specific $CD4^+$ T cells.

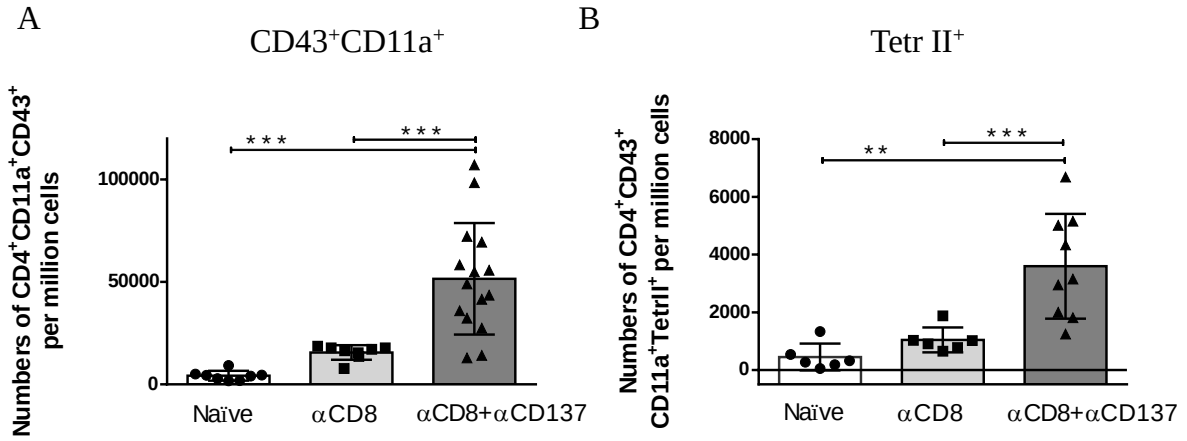

Figure 5.4: αCD137 therapy leads to the activation of $CD4^+$ T cells in draining lymph nodes

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and additionally treated with αCD8. Some mice also received αCD137 treatment as described in methods. At day 6 ptc draining lymph nodes were analyzed for expression of different molecules by flow cytometry. B, the activation of $CD4^+$ T cells was analyzed by surface expression of CD11a and CD43 (double positive). Numbers of leukemia specific $CD4^+TetII^+$ T cells reactive with I-A^b MHC class-II tetramers specific for the FV-Env epitope (B). All tetramer positive T cells expressed cell-surface activation marker CD43 and CD11a. Each dot represents an individual mouse and the mean numbers are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (**P<0.005, ***P<0.0005*). The experiment was repeated five times with comparable results.

5.3 αCD137 therapy leads to expansion of activated CD4$^+$ T cells with T helper phenotype

To test whether the anti-tumor effect following CD137 therapy was driven by improved CD4$^+$ T helper cell activity, we performed functional studies of FBL-3-induced CD4$^+$ T cells by analyzing T-box transcription factor (T-bet) (109) expression as well as production of the key pro-inflammatory cytokines IFN-γ, TNF-α and IL-2. To characterize the total population of CD4$^+$ T helper cells that was activated during tumor growth and rejection, we used the marker CD154 (CD40L) (17). In order to exclude Tregs from the conventional CD4$^+$ T helper cells (Tcon) we also stained for intracellular expression of the transcriptional factor forkhead box P3 (Foxp3). Following CD137 agonist therapy in tumor-bearing CD8$^+$ T cells-depleted mice, we observed a significantly enhanced number of T-bet expressing conventional CD4$^+$ T cells than in the groups only depleted for CD8$^+$ T cells or naïve mice (Fig. 5.5A). However, there was no significant increase of CD154 expression in CD137 agonist treated mice in comparison to only CD8$^+$ T cell-depleted animals (Fig. 5.5B).

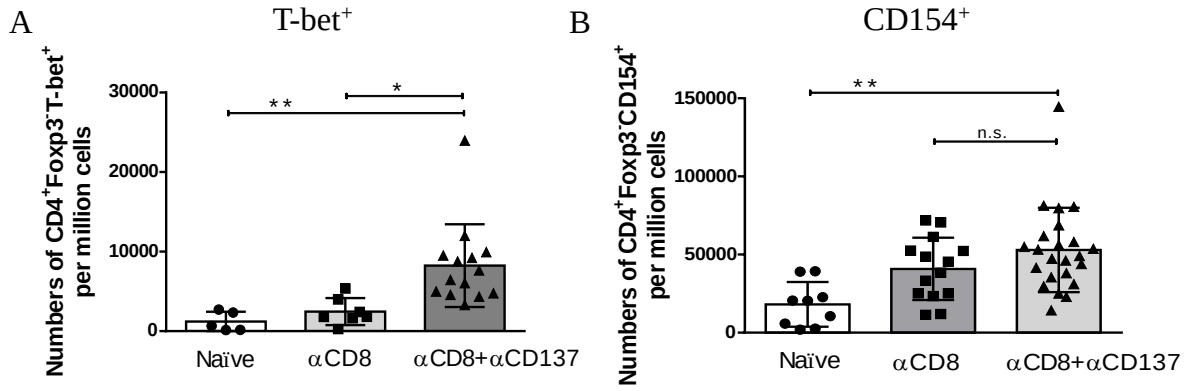

Figure 5.5: αCD137 ligation results in functional reactivation of CD4+ T cells in draining lymph nodes of tumor bearing mice

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and additionally treated with αCD8. Some mice also received αCD137 treatment as described in methods. At day 6 ptc draining lymph nodes were analyzed for expression of different molecules by flow cytometry. Intracellular marker Foxp3 was used to exclude Treg cell population from the analysis. (A) Flow cytometry was used to determine the numbers of CD4$^+$ T cells expressing intracellular transcription factor T-bet (A). Numbers of activated (positive for CD154) CD4$^+$Foxp3$^-$ T cells (B) in different treatment of mice. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given

in the figures (*P<0.05, **P<0.005, n.s. – not significant). The experiments were repeated at least five times with comparable results.

CD154 is a marker of activation, which is transiently expressed on the surface of $CD4^+$ T cells activated through ligation of their T-cell receptor. Tumor-bearing mice depleted for $CD8^+$ T cells show an activated phenotype of $CD4^+$ T cells by CD154 expression, although tumor control was abolished (Fig. 5.1B). In this group $CD154^+$ T cells were weak producers of pro-inflammatory cytokines. Using intracellular cytokine staining after stimulation with αCD137, we found significantly more $CD4^+CD154^+$ T cells producing the cytokines IFN-γ, TNF-α and IL-2 (Fig. 5.6). Notably, while CD154 expression was significantly increased in $CD8^+$ T cell deficient animals, the frequency of $CD4^+CD154^+$ T cells producing one of the three cytokines was not improved in the same group compared to the naïve control mice. Collectively, the data indicates that in the absence of $CD8^+$ T cells αCD137 therapy induces strong $CD4^+$ T cell responses with T helper phenotypes in the FBL-3 tumor model.

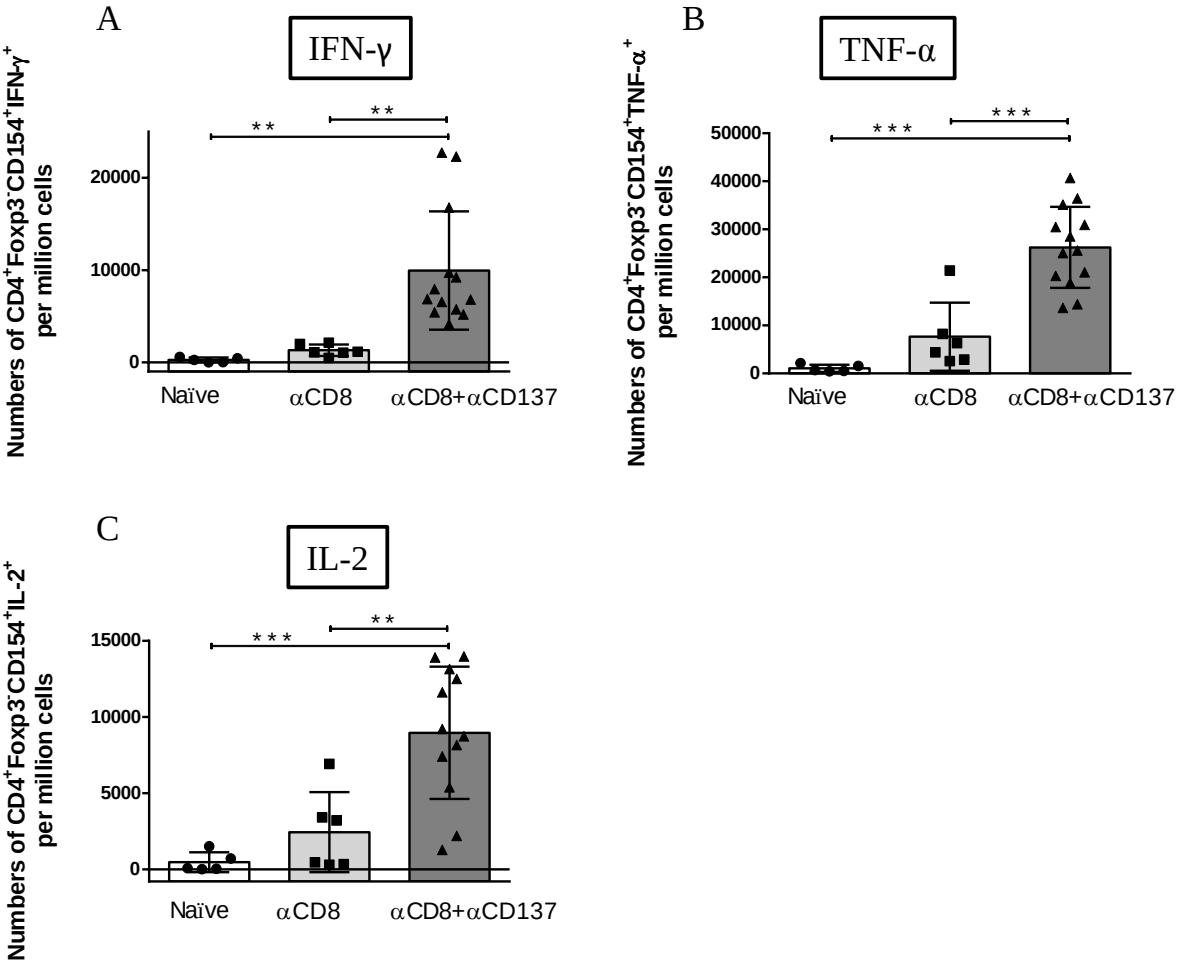

Figure 5.6: αCD137 ligation results in improved production of cytokines in CD4$^+$ T cells from draining lymph nodes of tumor bearing mice

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and additionally treated with αCD8. Some mice also received αCD137 treatment as described in methods. At day 6 post tumor transplantation lymphocytes from draining lymph nodes were analyzed. Intracellular marker Foxp3 was used to exclude Treg cell population from the analysis. Frequencies of CD4$^+$CD154$^+$ T cells producing cytokines (IFN-γ, TNF-α and IL-2) are shown (A-C). Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (***P<0.005,* ****P<0.0005*). The experiments were repeated at least three times with comparable results.

5.4 αCD137 treatment induces tumor-specific cytotoxic CD4$^+$ T cells that can kill FBL-3 cells

It has been demonstrated that after αCD137 therapy T-box transcription factor Eomesodermin (Eomes) mediates the expression of cytotoxicity-associated genes

that are efficient in killing tumor cells (28). Interestingly, the CD4 T cells in CD8-depleted, αCD137-treated mice showed significantly enhanced expression of Eomes (Fig. 5.7A). $CD4^+$ T cells usually express only little Eomes as cytotoxicity is mostly mediated by $CD8^+$ T cells, suggesting that $CD4^+$ T cells may have acquired cytotoxic activity after CD137 signaling. Additionally, significantly higher numbers of $CD4^+$ T cells from αCD137-treated mice produced the lytic molecule granzyme B (Fig. 5.7B), which is known to be important for controlling cancer (25). Indeed, we found that $Eomes^+CD4^+$ T cells from the treated mice showed substantially higher expression of the effector molecule granzyme B (Fig. 5.8). Nine percent of all $conCD4^+$ T cells were double positive for Eomes and granzyme after αCD137 treatment (Fig. 5.8A), which corresponded to 36% of the $Eomes^+CD4^+$ T cells producing granzyme B (Fig. 5.8B). The induction of tumor-specific $CD4^+$ T cells expressing granzyme B suggested that these cells might be responsible for the tumor elimination in αCD137-treated, $CD8^+$ T cell depleted mice (Fig. 5.1C).

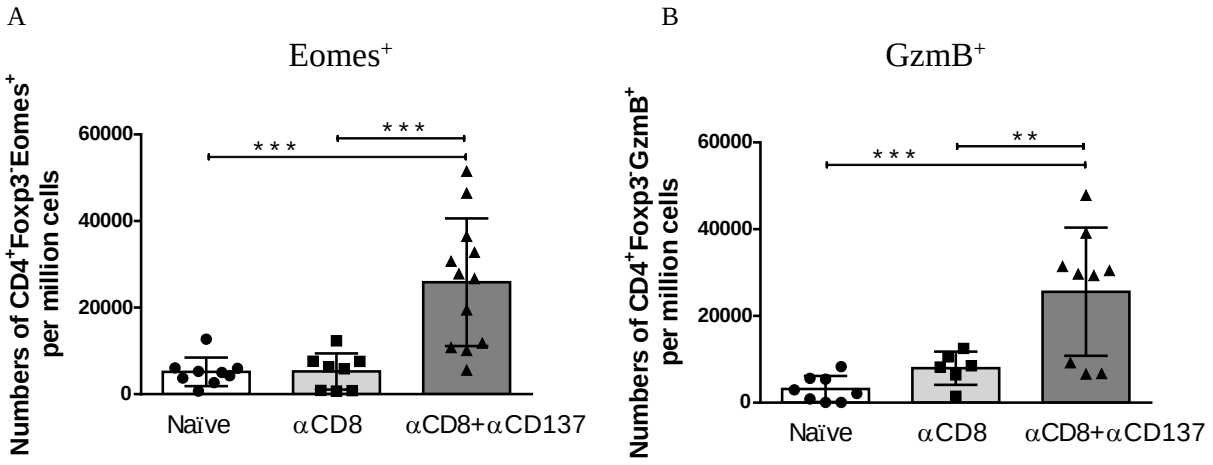

Figure 5.7: αCD137 + αCD8 combination therapy programs cytotoxic $CD4^+$ T cell differentiation

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and additionally treated with αCD8. Some mice also received αCD137 treatment as described in methods. At day 6 ptc $CD4^+Foxp3^-$ ($conCD4^+$) T cells from draining lymph nodes were analyzed. A, intracellular expression of transcription factor Eomesodermin (Eomes) was measured by flow cytometry and numbers of $Eomes^+$ $conCD4^+$ T cells from mice of the different groups are shown. B, numbers of $CD4^+Foxp3^-$ T cells producing granzyme B (GzmB). Each dot represents an individual mouse and the mean percentages are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (**P<0.005, ***P<0.0005*). All experiments were repeated at least three times with comparable results.

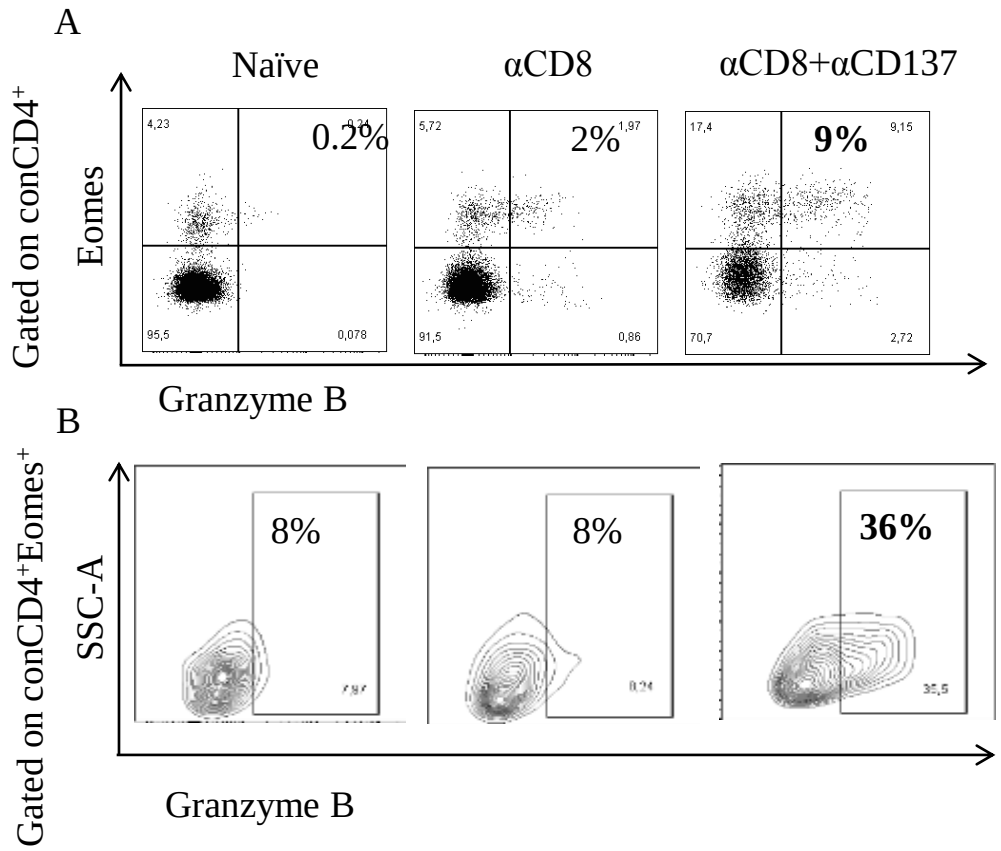

Figure 5.8: αCD137 + αCD8 combination therapy leads to the expansion of granzyme B producing CD4$^+$ T cells

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells on day 0. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete CD8$^+$ T cells and αCD137 (described in methods). At day 6 post tumor transplantation CD4$^+$Foxp3$^-$ (conCD4$^+$) T cells from draining lymph nodes were analyzed. A, representative dot plot of Eomes and GzmB expression (A) from mice of the different groups. B, representative contour plot of GzmB producing conCD4$^+$Eomes$^+$ T cells in different treatment of mice. Data are representative for at least 3 independent experiments.

To analyze whether the increased expression of the cytotoxic molecule granzyme B correlated with improved tumor specific lysis of target cells following CD137 agonist therapy we performed a series of in vivo killing experiments. Tumor-bearing mice were depleted for CD8$^+$ T cells with or without additional αCD137 treatment. Five days after tumor challenge all groups of mice received lymphocytes from naïve CD45.1 donor mice that were loaded with a MHC class II-restricted FBL-3-specific CD4$^+$ T cell epitope peptide (115). These donor lymphocytes served as CFSE-labeled targets. Twenty hours after i.v. injection of CFSE targets (day 6 ptc), mice

were sacrificed and *in vivo* killing activity was quantified in single-cell suspensions from the drLN of each tumor-bearing mouse. The *in vivo* cytotoxicity assay revealed that $CD4^+$ T cell killing was at the detection limit of the assay in only $CD8^+$ T cell depleted tumor-bearing mice, whereas the additional αCD137 treatment resulted in an average of more than 30% target cell killing (Fig. 5.9). Moreover, when peptide-loaded targets from $MHCII^{-/-}$ mice were used in the assay, we found that all killing activity of effector $CD4^+$ T cells was lost, confirming the specific TCR-MHC-II recognition in $CD4^+$ T cell depended killing. Collectively, these data suggest that tumor-specific $CD4^+$ T cells can gain cytotoxic activity against FBL-3 tumor cells when $CD8^+$ T cells are not active but this activity is dependent on co-stimulatory signals of the CD137 pathway.

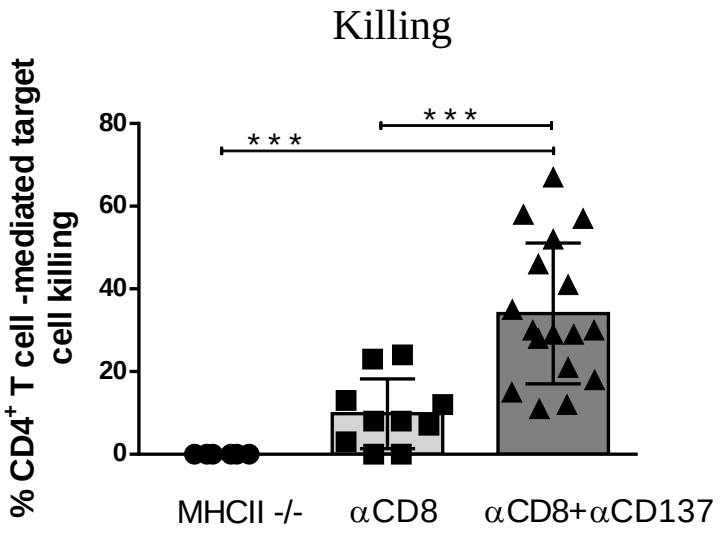

Figure 5.9: αCD137 + αCD8 combination therapy leads to $CD4^+$ T cell killing

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells on day 0. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). Mean percentages of killing *in vivo* CTL assay. Briefly, tumor bearing mice were depleted for their $CD8^+$ T cells and treated or not with αCD137. Target cells from the spleen and lymph nodes of the naive donor mice were labeled with CFSE and pulsed with the specific CD4 epitope peptide. Another cell population was obtained from naïve CD45.1 mice and not pulsed with peptide. In order to verify tumor-specific killing, cells from MHCII-/- were used as donor cells and labeled either with CFSE or with CellTrace Violet. At day 5 after tumor inoculation, both populations of target cells were co transferred intravenously into treated tumor-bearing mice. Cells from MHCII-/- mice were only transferred into mice treated with αCD8 + αCD137. Twenty hours later, lymphocytes were isolated from the draining lymph nodes and analyzed by flow cytometry to determine the percentage of remaining target cells that are either $CFSE^+$,$CD45.1^+$ or CellTrace $Violet^+$.

Each dot represents an individual mouse and the mean percentages are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (****P<0.0005*). All experiments were repeated at least three times with comparable results.

5.5 αCD137 therapy leads to expansion of Tregs

We have previously shown that direct anti-tumor effects of $CD4^+$ T cells were strictly regulated by Tregs (2). In the absence of $CD8^+$ T cells Tregs suppressed effector $CD4^+$ T cell responses and impaired tumor rejection (Fig. 5.10) (2).

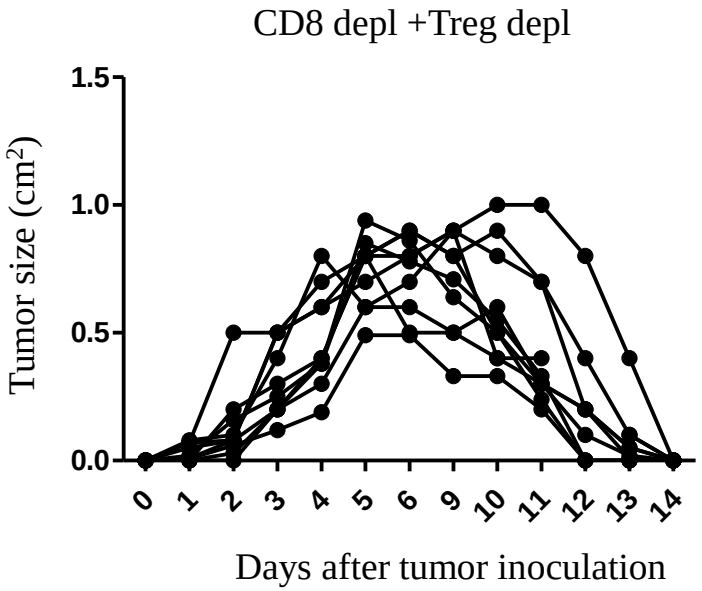

Figure 5.10: Influence of both $CD8^+$ T cell and Treg depletion on tumor formation

DEREG mice were injected s.c. with 1×10^7 FBL-3 cells (1×10^7) and tumor size was measured. Mice were depleted for their Tregs and $CD8^+$ T cells as described in methods. Each line represents tumor progression in an individual mouse.

As we found that αCD137 treatment drives $CD4^+$ T cell mediated cytotoxicity and restores anti-tumor immunity as described above, we next investigated whether αCD137-treatment influences the otherwise suppressive response of Tregs. We first confirmed expression of CD137 on Tregs of FBL-3 challenged mice and found indeed expression of CD137 on Tregs (Fig. 5.11). A subpopulation of Tregs expressed the CD137 molecule on a subpopulation of activated ($CD43^+$) Tregs in FBL-3 challenged mice and therefore might be possible consumers of CD137 antibodies (Fig. 5.11).

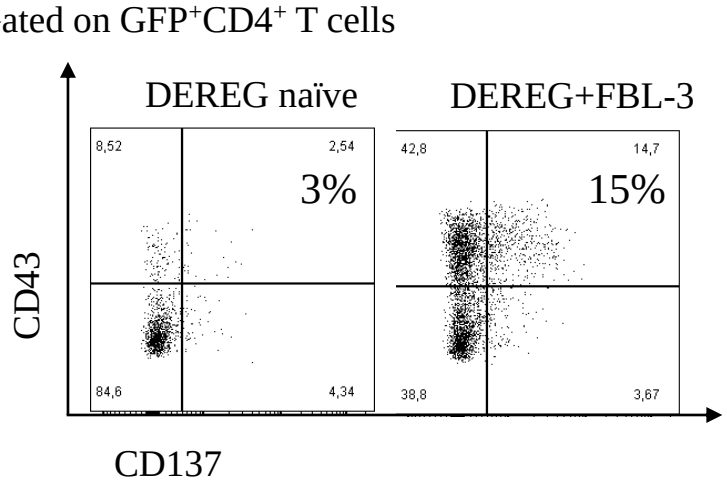

Figure 5.11: Expression of CD137 on Tregs from draining lymph nodes of tumor bearing mice

DEREG mice were inoculated s.c. with 1×10^7 FBL-3 cells on day 0. At day 6 ptc GFP^+ T cells from draining lymph nodes were analyzed. Representative dot plot of CD137 and CD43 expression in naïve and tumor-bearing DEREG mice are shown. Data are representative for at least 3 independent experiments.

It was of interest to investigate the impact of CD137 agonist on the phenotype and function of Tregs during FBL-3 tumor rejection. On day 6 ptc Tregs migrate from lymph nodes to the tumor microenvironment in FBL-3 tumor-bearing mice and in drLNs constitute only 13% of the total $CD4^+$ T cell population (2). In contrast, following αCD137 injection mice lacking $CD8^+$ T cells and challenged with tumor showed a significant expansion of $Foxp3^+$ Tregs comprising up to 30% of all $CD4^+$ T cells in the tumor-draining lymph nodes (Fig. 5.12A).

CD25 expression by $CD4^+$ T cells is widely used as a marker to identify Tregs. However, cells with regulatory properties are also found in the $CD4^+CD25^-$ subset. We therefore assayed which subpopulation of $Foxp3^+$ Tregs ($CD25^+$ or $CD25^-$) was expanded after the agonist CD137 immunotherapy. Interestingly, no enhancement in $CD25^-$ Treg numbers was observed in the group of mice treated with αCD137 in comparison to the control groups (Fig. 5.12B). In contrast, a significant increase in numbers of $Foxp3^+CD25^+$ cells was detected in αCD137 treated mice compared to animals only depleted for $CD8^+$ T cells and challenged with FBL-3 tumor cells (Fig. 5.12B).

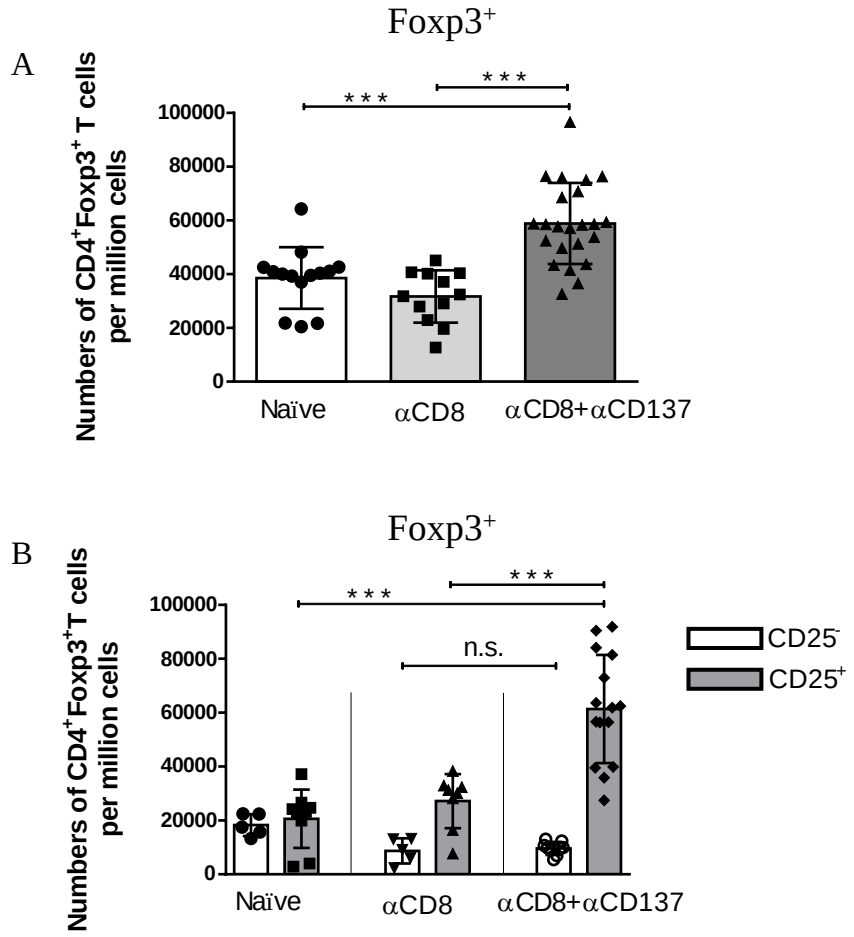

Figure 5.12: αCD137 stimulates expansion of CD25$^+$ Tregs

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete CD8$^+$ T cells and αCD137 (described in methods). At day 6 ptc draining lymph nodes were analyzed for total numbers of CD4$^+$ Foxp3$^+$T cells (A). B, numbers of CD25$^-$ and CD25$^+$ in Foxp3$^+$ T cells. Differences of groups are presented in bars and means are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (***$P<0.0005$, n.s. – not significant). The experiment was repeated at least five times with comparable results.

5.6 CD137 signaling elicits activation, proliferation, and differentiation of nTregs

Next, we investigate the impact of CD137 agonist on the phenotype and function of Tregs during FBL-3 tumor rejection. The αCD137 treatment induced significant activation and differentiation of Tregs, as indicated by expression of the markers

(CD43 and CD11a (Fig. 5.13A, (68)). Significant improvement of these molecules expression by $CD4^+Foxp3^+$ T cells was observed in αCD137 treated mice and depleted for $CD8^+$ T cells than in control groups of only depleted and naïve animals (Fig. 5.13A). The same trend was discernible for tumor-specific $tetramerII^+$ regulatory T cells, although it failed to reach statistical significance (Fig. 5.13B).

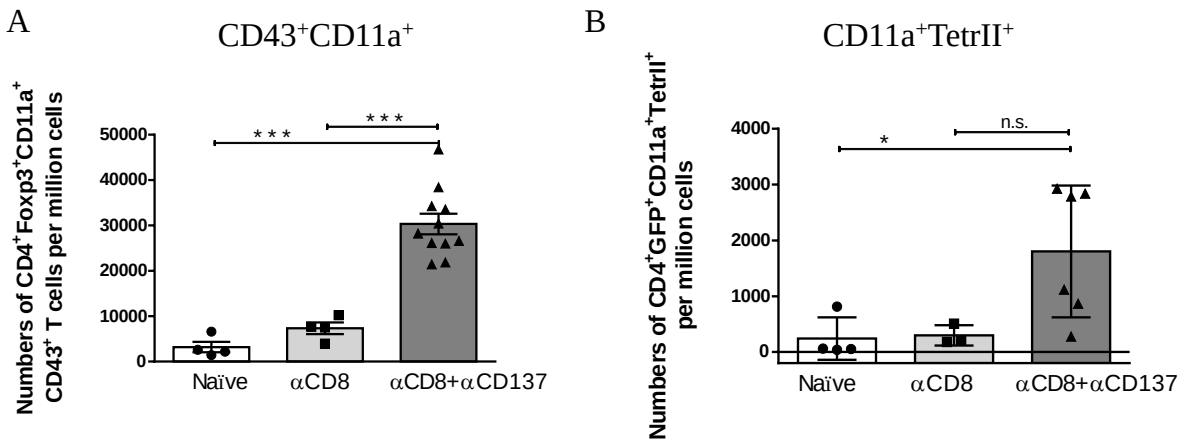

Figure 5.13: αCD137 stimulates Tregs activation

DEREG mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). At day 6 ptc draining lymph nodes were analyzed for expression of different molecules by flow cytometry. A, Total numbers of surface expression of CD11a and CD43 (double positive). Numbers of leukemia specific $CD4^+GFP^+TetII^+$ T cells reactive with $I\text{-}A^b$ MHC class-II tetramers specific for FV-Env epitope (B). All tetramer positive T cells expressed cell-surface activation marker CD11a. Each dot represents an individual mouse and the mean numbers are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (****P<0.0005,* n.s. – not significant). The experiment was repeated three times with comparable results.

We also found a significant increase of recently proliferated Tregs identified by the expression of Ki67 (Fig. 5.14A), which goes in line with the previous findings of the expanded Treg population, comprising up to 30% of all $CD4^+$ T cells in the tumor-draining lymph nodes (Fig. 5.12A). Furthermore, we observed an increased expression of the maturation marker KLRG-1 on Tregs, which have been linked to cytotoxic activity of $CD8^+$ T cells and NK cells (Fig. 5.14B).

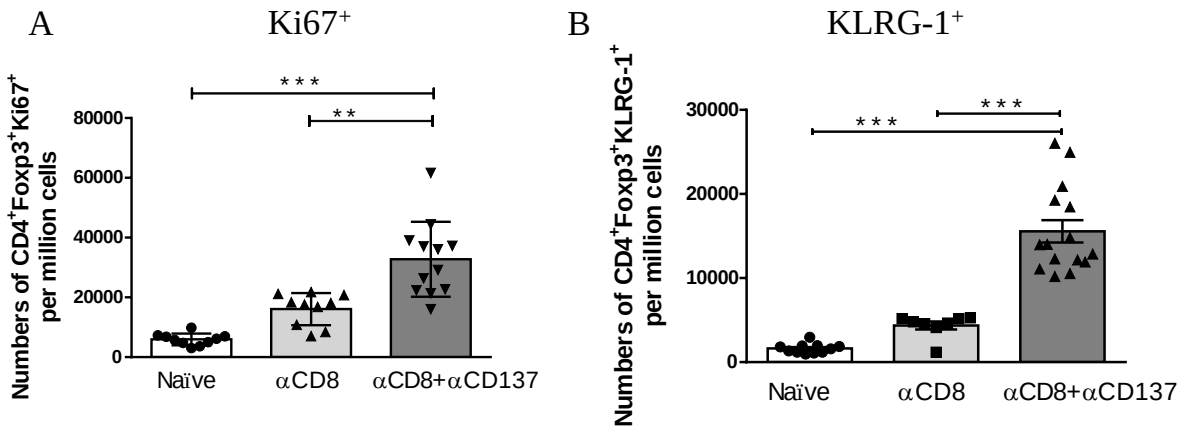

Figure 5.14: αCD137 stimulates Tregs proliferation and differentiation

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). At day 6 ptc draining lymph nodes were analyzed for expression of different molecules on $Foxp3^+CD4^+$ T cells by flow cytometry. A, the proliferation of $CD4^+$ T cells was measured by the intracellular expression of Ki67. B, the maturation profile was detected by surface expression of KLRG-1. Each dot represents an individual mouse and the mean numbers are indicated by a line. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (***P<0.005, ***P<0.0005*). The experiment was repeated three times with comparable results.

Next, it was of interest to determine the origin of Tregs. Tregs can derive from thymus (nTregs) or come from peripherally, induced iTregs. Firstly, Helios was known as a marker of nTregs (127), however recently it was mostly used as marker for Treg activation (3). Neuropilin 1 (Nrp-1), a receptor for ligands of the vascular endothelial growth factor family was identified as a marker for nTregs (12). Interestingly, the vast majority of Tregs expanding after CD137 treatment expressed Nrp-1 (Fig. 5.15A), suggesting that CD137 treatment mostly affected thymic-derived nTregs. This was also supported by the observation that $Foxp3^+$ Tregs expanded in the thymus of αCD8+αCD137 treated tumor-bearing mice (Fig. 5.15B). Thus, αCD137 treatment of CD8 depleted, tumor bearing mice induced strong activation, proliferation, and differentiation of a subset of nTregs.

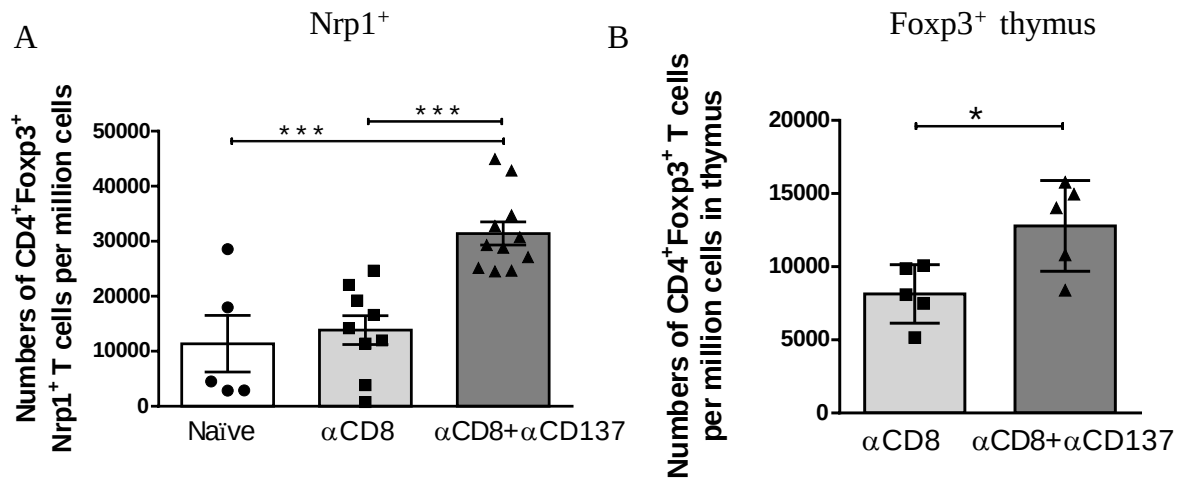

Figure 5.15: Expansion of natural Tregs in the draining lymph nodes and in the thymus of tumor-bearing mice following CD137 agonist therapy

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). At day 6 ptc draining lymph nodes and thymus were analyzed by flow cytometry. Numbers of Treg cells in draining lymph nodes analyzed for expression of surface Neuropilin1 (Nrp1) in differently treated tumor-bearing mice (A). B, total numbers of $CD4^+Foxp3^+$T cells in thymus. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (*P<0.05, ***$P<0.0005$). The experiment was repeated three times with comparable results.

5.7 A subset of Tregs acquires helper and cytotoxic $CD4^+$ T cell functions after αCD137 treatment

The CD137 agonist antibody augmented Treg responses in tumor bearing mice but this activation did not result in suppression of anti-tumor immunity. We therefore asked, whether αCD137 activated Tregs showed evidence of phenotypic plasticity and acquired anti-tumor effector functions. We examined the *in vivo* induction of CD154 expression, a functional mediator of T cell help (CD40-ligand) (116), on Tregs in drLNs of αCD137 treated mice. Figure 5.16A shows that CD154 was expressed on a subset of $Foxp3^+$ Tregs after treatment. To investigate the functionality of these cells, the expression of proinflammatory cytokines (IL-2, IFN-γ, TNF-α) was measured. The production of these cytokines implies a major alteration in the Treg phenotype, because they are strongly suppressed in the $Foxp3^+$ lineage (147). While $CD8^+$ T cell deletion induced no TNF-α production in $CD4^+Foxp3^+$ from tumor drLNs,

an addition of αCD137 mediated TNF-α production in the subset of CD154/Foxp3 double-positive cells (Fig. 5.16B). Remarkably, these $CD4^+$ T cells only produced TNF-α but not IL-2 or IFN-γ (data not shown).

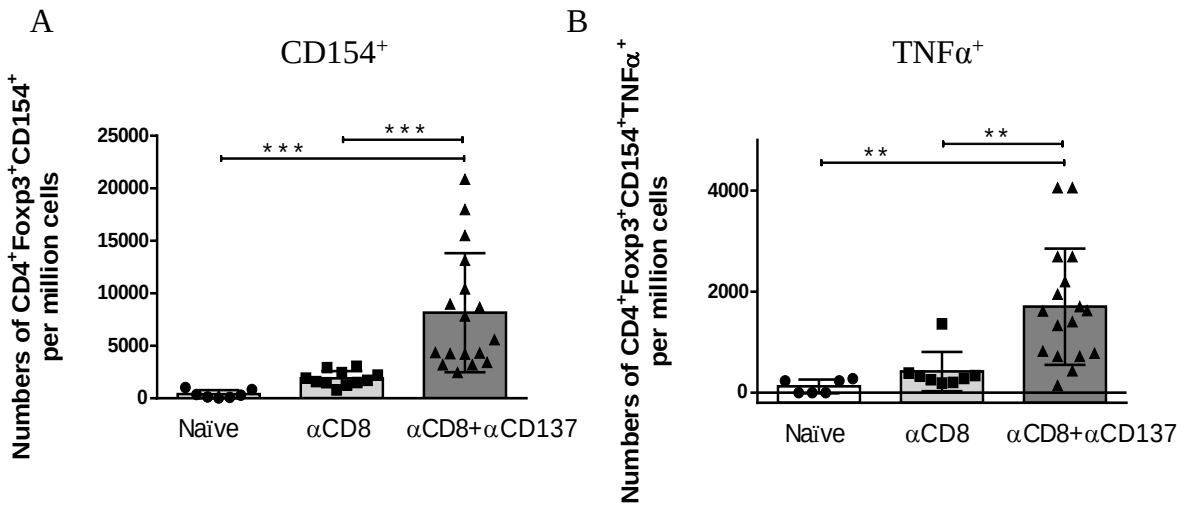

Figure 5.16: Tregs upregulate helper T cell markers after αCD137 treatment

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). At day 6 ptc $Foxp3^+$ Treg cells from draining lymph nodes were analyzed for different characteristics. A, numbers of $Foxp3^+$ T cells expressing CD154 molecule. C, $CD154^+Foxp3^+$ T cells producing TNF-α. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (***P<0.005, ***P<0.0005*). The experiment was repeated three times with comparable results.

To determine if αCD137 therapy also induced markers of cytotoxicity in $Foxp3^+CD4^+$ T cells from FBL-3 challenged mice, we quantified the expression of the T-box transcriptional factor Eomes in these cells, which strongly correlates with T cell cytotoxicity. Strikingly, the number of Eomes expressing $Foxp3^+$ T cells was significantly increased in αCD137 treated mice compared to animals only depleted for $CD8^+$ T cells and challenged with FBL-3 tumor cells (Fig. 5.17A). Only in the presence of αCD137 the Treg cell fraction supported Eomes upregulation, comprising up to 3% of total $CD4^+$ T cells in contrast to non-treated αCD8 received mice (0,1%) or naïve animal (0,1%) (Fig. 5.17B).

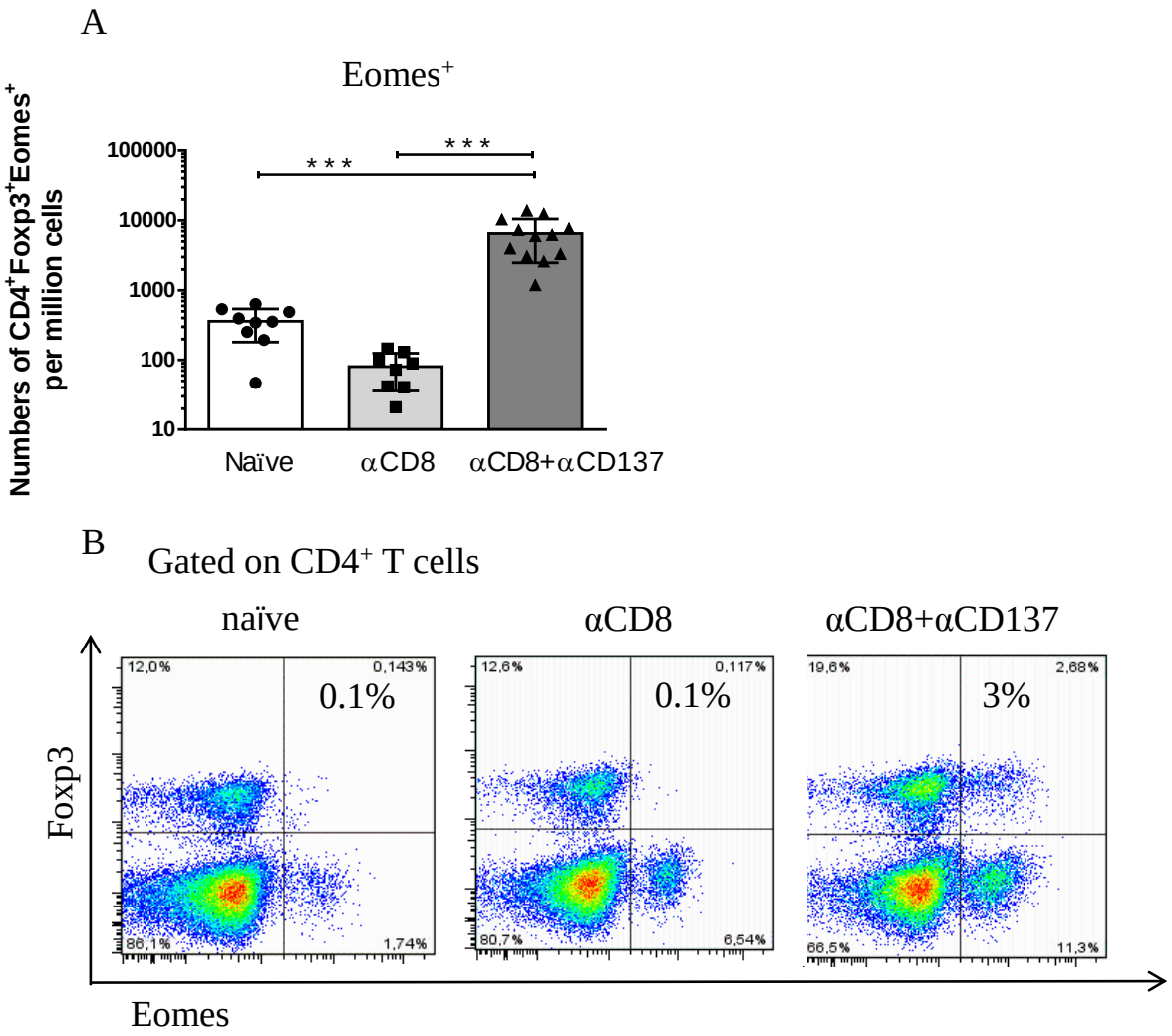

Figure 5.17: Tregs acquire an effector-like phenotype following αCD137 therapy
B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). At day 6 ptc $Foxp3^+$ T cells from drLNs were analyzed for transcription factor Eomes. Numbers of Eomes expressing Treg cells are shown in A. B, representative dot plots of $Eomes^+Foxp3^+$ Treg cells in different treatment of mice are shown. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (****P<0.0005*). The experiment was repeated three times with comparable results.

Up to 14% of the $Foxp3^+CD4^+$ T co-expressed Eomes after αCD137 injection, whereas almost none of these double-positive cells were found before antibody treatment (Fig. 5.18). Most of the $Foxp3^+Eomes^+CD4^+$ T cells also expressed the Treg activation marker Helios (Fig. 5.18).

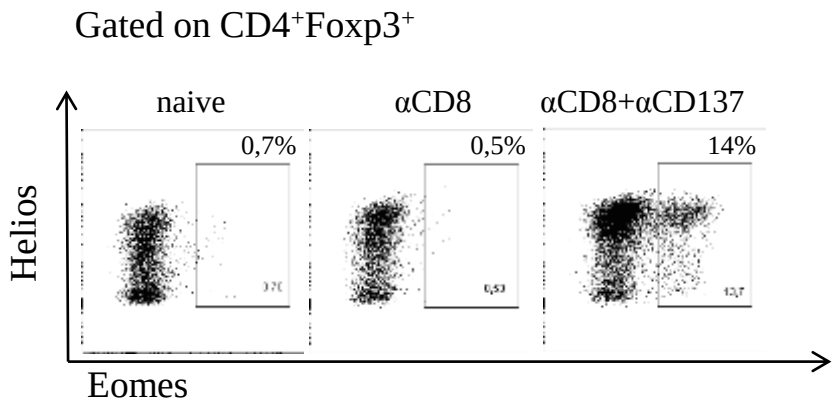

Figure 5.18: Expression of Eomes in Tregs

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells on day 0. At day 6 ptc $Foxp3^+$ T cells from draining lymph nodes were analyzed. Representative dot plots of Eomes and Helios expression in different group of mice are shown. Data are representative for at least 3 independent experiments.

In addition, within the tumor draining lymph nodes $Foxp3^+CD4^+$ T cells started to produce granzyme B following αCD137 therapy, whereas no granzyme B production was found in the cells of mice from the control groups (Fig. 5.19). Taken together, these results demonstrate that CD137 signaling may convert subsets of Tregs into cytokine expressing helper T cells or even cytotoxic killer cells.

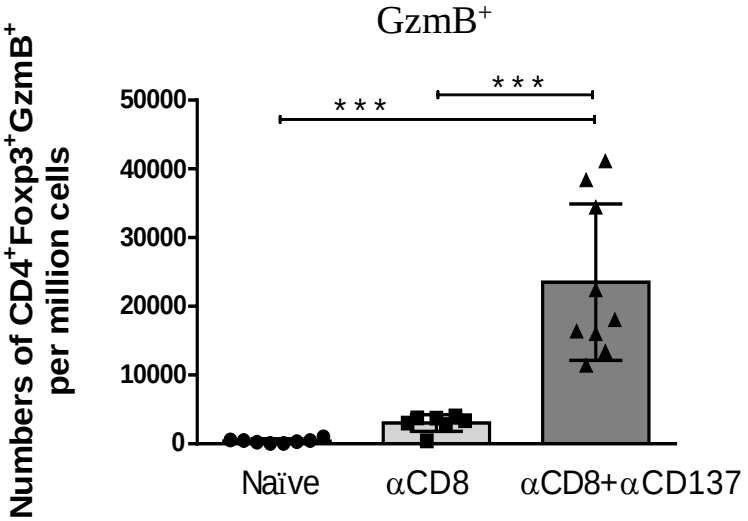

Figure 5.19: CD137 ligation leads to increased GzmB production by Tregs

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells. Simultaneously with tumor inoculation, mice also received monoclonal antibody to deplete $CD8^+$ T cells and αCD137 (described in methods). The numbers of GzmB producing Treg cells in draining lymph nodes at day 6 ptc

are shown. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (****P<0.0005*). The experiment was repeated three times with comparable results.

5.8 CD137 stimulated Foxp3$^+$ CD4$^+$ T cells mediate tumor cell killing

The effector cell characteristics found in CD137 stimulated Foxp3$^+$ CD4$^+$ T cells prompted us to ask whether these reprogrammed Tregs were capable of eliminating FBL-3 tumor cells. We performed a series of *in vivo* killing experiment in DEREG mice, which express a diphtheria toxin (DT) receptor under the control of the *Foxp3* promoter. Intravenous administrations of DT allow to rapidly (within hours) and selectively delete more than 95% of Foxp3$^+$CD4$^+$ T cells (Fig. 5.20).

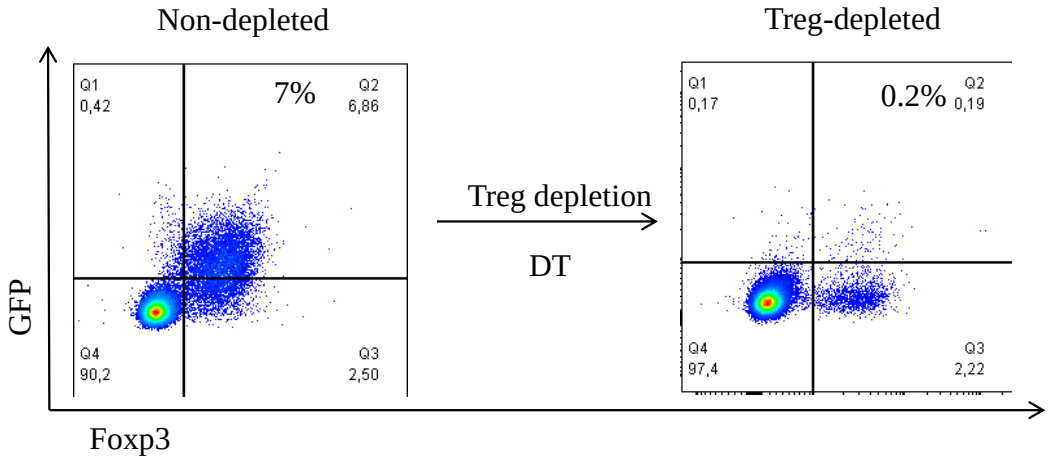

Figure 5.20: Efficacy of Treg depletion in DEREG mice

DEREG tumor-bearing mice were injected with DT i.v. as described in methods. Lymphocytes from drLNs were stained for intracellular Foxp3 production and gated on CD4$^+$ T cells. Loss of Tregs (CD4$^+$Foxp3$^+$GFP$^+$ T cells) in a representative DEREG mouse treated with DT i.v. is shown. Percentages of CD4$^+$Foxp3$^+$GFP$^+$ T cells (upper and lower right quadrant) are given above the dot plots.

This was important because we wanted to study the immediate effect of the Foxp3$^+$ cells on target cells and not the secondary effect that a more sustained Treg depletion has on effector T cell responses. DEREG mice were challenged with tumors, depleted for CD8$^+$ T cells and treated with αCD137 to induce the population Foxp3$^+$CD4$^+$ T cells with cytotoxic effector phenotype. Cell tracer-labeled peptide-loaded (Friend Virus CD4$^+$ T cell epitope peptides that are expressed on FBL-3 tumor

cells) target cells were injected intravenously (i.v.) simultaneously with DT or PBS on day 5 ptc and the *in vivo* killing of the targets was determined 20 hrs later. In PBS control mice the total population of $CD4^+$ T cells, including conventional T cells and the $Foxp3^+CD4^+$ T cells that expressed Eomes mediated a potent *in vivo* killing activity (Fig. 5.21). Strikingly, this activity was significantly decreased after specific Foxp3 ablation due to DT administration (Fig. 5.21), suggesting a potent contribution of the converted Tregs to the total MHC class II restricted killing of peptide-loaded targets.

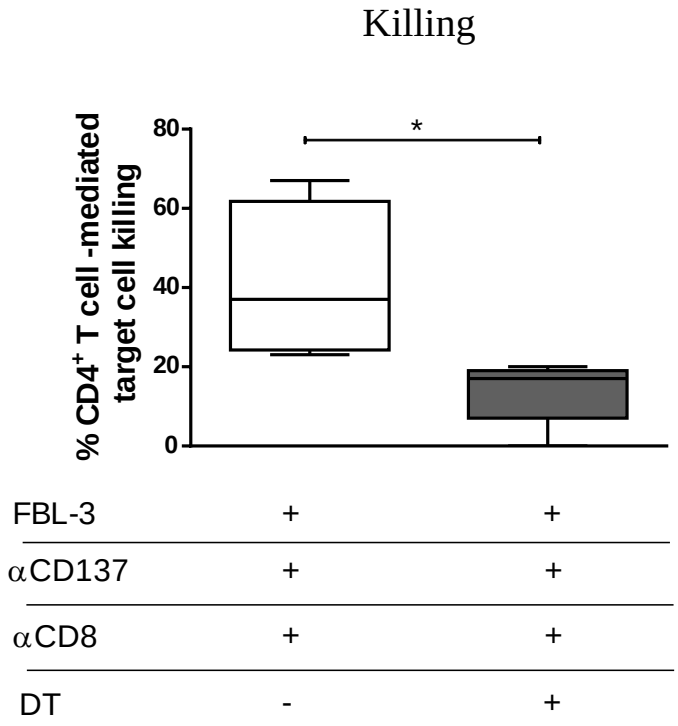

Figure 5.21: Functional plasticity of Tregs after αCD137 treatment leads to FBL-3 tumor elimination

Mean percentages of killing *in vivo* CTL assay (described in methods). FBL-3 tumor bearing DEREG mice were depleted for their $CD8^+$ T cells and treated with αCD137. Target cells from the spleen and lymph nodes of the naive donor mice were labeled with CFSE and pulsed with the specific CD4 epitope peptide to be tested. Additionally naïve cells from CD45.1 mice were isolated and not pulsed with peptide. At day 5 after tumor inoculation, both populations of target cells together with diphtheria toxin (DT) were co transferred intravenously in the same amount into treated DEREG tumor-bearing mice. Twenty hrs later, lymphocytes were isolated from the draining lymph nodes and analyzed by flow cytometry to determine the percentage of remaining target cells that are either $CFSE^+$ or $CD45.1^+$. White bar correspond to FBL-3 challenged and αCD8+αCD137 treated group, whereas grey bar correspond to the mice additionally treated with DT. Differences between the two groups

were analyzed by using t-test. Statistically significant differences between the groups are given in the figures (*P<0.05). Experiment was repeated two times with comparable results.

This killing activity of CD137-stimulated Foxp3$^+$CD4$^+$ T cells was confirmed in another *in vivo* cytotoxicity assay, in which we directly used FBL-3 tumor cells as targets (described in materials and methods). The FBL-3 cells were transferred into the peritoneal cavity of naïve mice together with donor Foxp3$^+$CD4$^+$ T cells from tumor challenged mice either left untreated, treated with αCD8 or αCD8+αCD137 treated. Forty-eight hours later we re-isolated and quantified the remaining FBL-3 cells by a peritoneal lavage. In mice that received Foxp3$^+$CD4$^+$ T cells from αCD8+αCD137 treated animals up to 90% of the tumor cells were eliminated (Fig. 5.22). This FBL-3 cell killing was significantly higher than in groups that received Foxp3$^+$ cells either from non-treated or only αCD8 treated mice tumor-bearing mice (Fig. 5.22). Thus, responding to αCD137 therapy a subset of Tregs obtained an effector phenotype of cytotoxic CD4$^+$ T cell and was able to eliminate FBL-3 tumor cells *in vivo*.

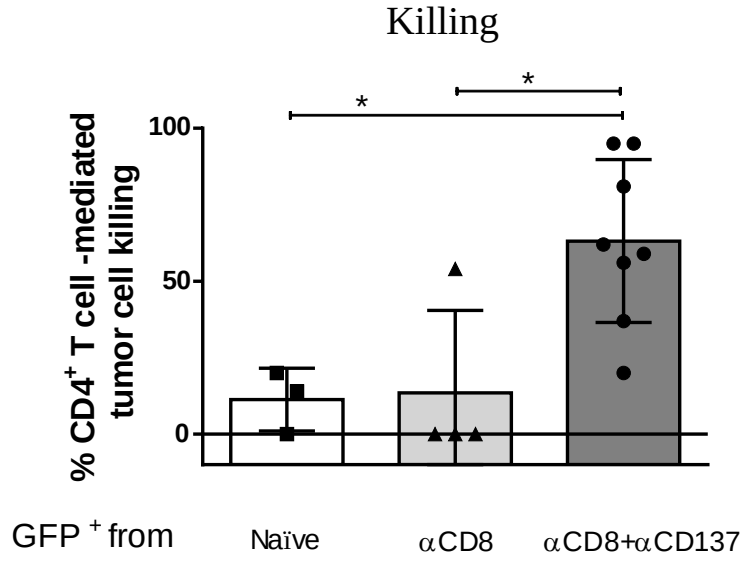

Figure 5.22: Tregs eliminate FBL-3 tumor cells following αCD137 therapy

Mean percentages of killing are presented. Naïve CD45.1 mice received i.p. injection of FBL-3 cells labeled with CFSE. Additionally, mice received sorted CD4$^+$GFP$^+$ cells from either αCD8, or αCD8+αCD137 tumor-bearing or naïve mice. Mice were sacrificed 48 hrs later and intraperitoneal lavage was performed. The results depict mean percentages of FBL-3 cells killing calculated according to control, only FBL-3 challenged mice. Each dot represents an individual mouse. Differences between the two groups were analyzed by using one-way

ANOVA test. Statistically significant differences between the groups are given in the figures (*P<0.05). Experiment was repeated two times with comparable results.

5.9 αCD137 treatment does not convert conventional $CD4^+$ T cells into $Foxp3^+$ $CD4^+$ T cells

The appearance of $Foxp3^+CD4^+$ T cells with cytotoxic activity against tumor cells after αCD137 therapy might be explained by two different pathways. The Tregs convert into cytotoxic effectors or conventional $CD4^+$ T cells with cytotoxic potential may start to express Foxp3 after CD137 signaling. To test these possibilities, an adoptive transfer experiment with $Foxp3^+$ or $Foxp3^-$ donor cells ($CD45.2^+$ cells were from DEREG mice and distinguished based on their GFP expression) into CD45.1 mice that were challenged with FBL-3 cells and treated with αCD8 and αCD137 antibodies. After 6 days the donor cells were re-isolated from the tumor-drLNs and analyzed for their Foxp3 and Eomes expression. None of the conventional $CD4^+$ T cells ($Foxp3^-$ donor cells) that expressed Eomes also expressed Foxp3. However, 14% of the transferred Tregs ($Foxp3^+$ donor cells) started to additionally express Eomes in the recipient mice (Fig. 5.23), supporting our previous results that Tregs can be reprogrammed into $Eomes^+$ effector T cells after CD137 signaling (Fig. 5.17 and 5.18). In contrast, no induction of Foxp3 expression in conventional cytotoxic $CD4^+$ T cells was observed after antibody treatment (Fig. 5.23).

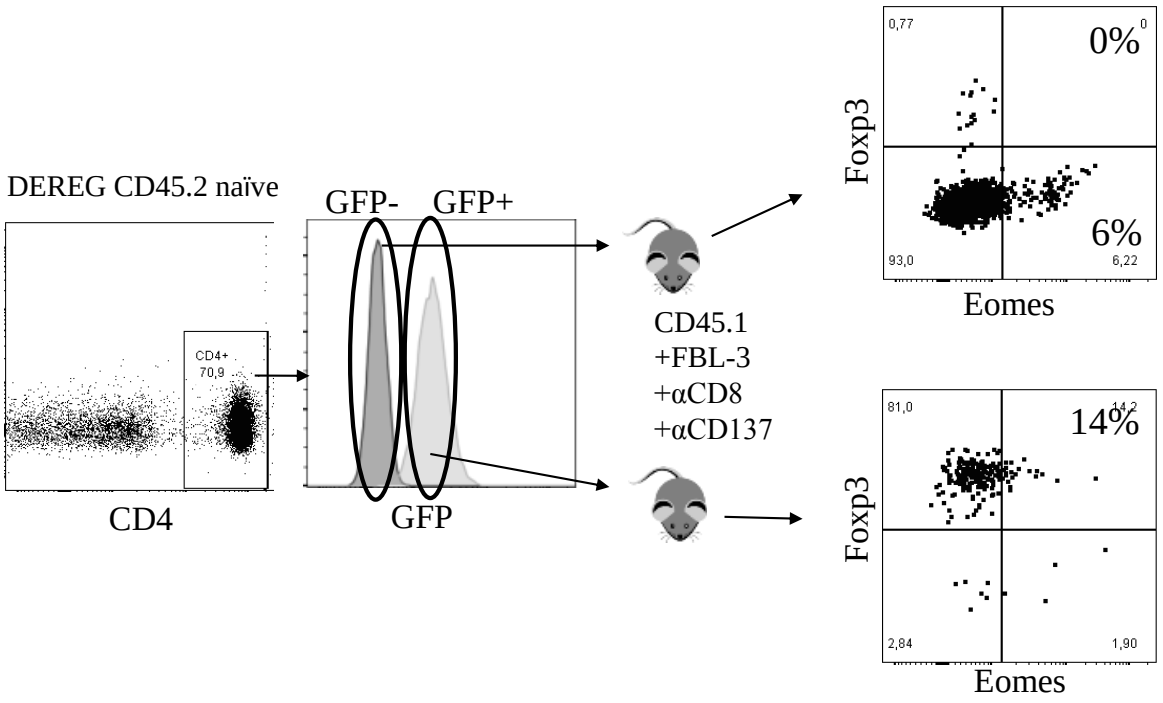

Figure 5.23: Influence of αCD137 treatment on the phenotypic stability of Foxp3$^+$CD4$^+$ T cells

CD45.1 mice were challenged with FBl-3 cells and additionally transferred i.v. with sorted CD4$^+$GFP$^-$ or CD4$^+$GFP$^+$ cells from naïve DEREG mice and treated with αCD8+αCD137 as described in methods. Six days after tumor challenged intracellular expression of Foxp3 and Eomes on transferred CD45.1$^-$CD45.2$^+$ cells was detected. Data are representative for 3 independent experiments.

5.10 Role of CD137 treated Foxp3$^+$ CD4$^+$ T cells in FBL-3 tumor formation

Next, it was of interest to investigate the direct influence of converted Tregs during FBL-3 tumor formation *in vivo*. In order to demonstrate that, we studied tumor regression in mice which either lack Foxp3$^+$ T cells (DT-treated) while treated with αCD8+αCD137 or additionally challenged with Foxp3$^+$ T cells (GFP$^+$-sorted) from αCD137 treated mice. Interestingly, after a selective depletion of Foxp3$^+$ T cells in tumor-bearing DEREG αCD8+αCD137 therapy did not significantly improve tumor elimination. In contrast, αCD8+αCD137 treated mice could eliminate FBL-3 tumor 4 days earlier in comparison to those that additionally were depleted for Foxp3$^+$ T cells (Fig. 5.24). However, these results do not indicate the contribution of converted CD4$^+$ Foxp3$^+$ T cells to tumor elimination, since DT injection depleted both populations of converted CD4$^+$ Foxp3$^+$ T cells and suppressive Tregs. The latter in turn results in effector CD4$^+$ T cell activation and subsequent FBL-3 tumor elimination (2).

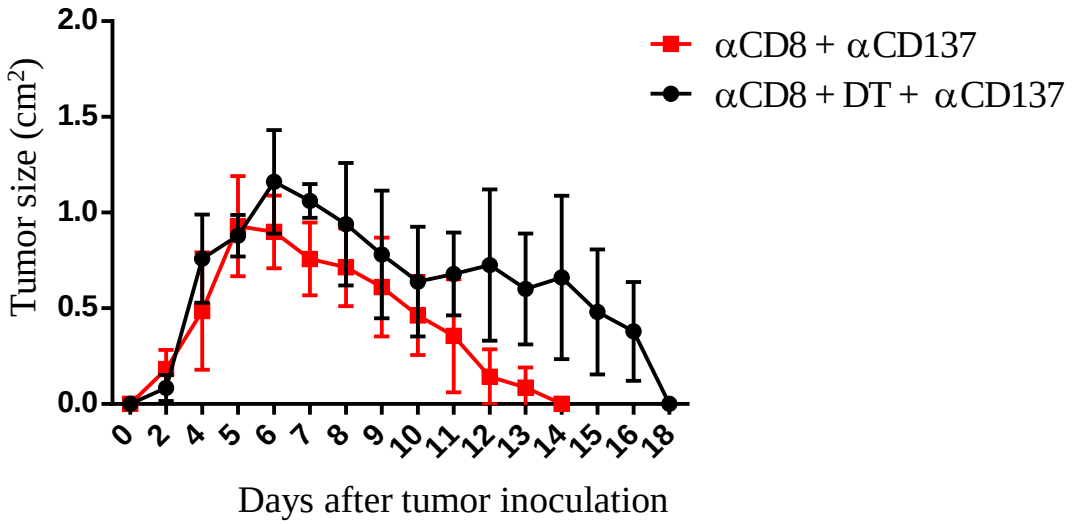

Figure 5.24: Influence of CD8 depletion and αCD137+DT therapy on tumor formation

DEREG mice were injected s.c. with 1×10^7 FBL-3 cells and tumor size was measured. Mice were depleted for $CD8^+$ T cells and treated with αCD137 and DT as described in methods. Lines represent means of tumor progression in 5-7 mice.

To clarify the pure effect of the converted $CD4^+$ $Foxp3^+$ T cells, we used nude (*Foxn1nu)* mice that have deteriorated or absent thymus and therefore no T cells. These mice received GFP^+Foxp3^+ T cells from DEREG tumor-bearing mice either treated or non-treated with αCD8+αCD137 following FBL-3 tumor challenge one day later. None of the groups could control tumor growth (Fig. 5.25). However, an injection of $Foxp3^+$ T cells from αCD8+αCD137 into tumor-challenged nude mice caused the decrease in size of FBL-3 tumor and up for 6 days prolonged the survival of these mice in comparison to control groups (Fig. 5.25). Collectively, these data suggest that following CD137 stimulation $Foxp3^+$ T cells can contribute to effector anti-tumor immune response and delay tumor progression.

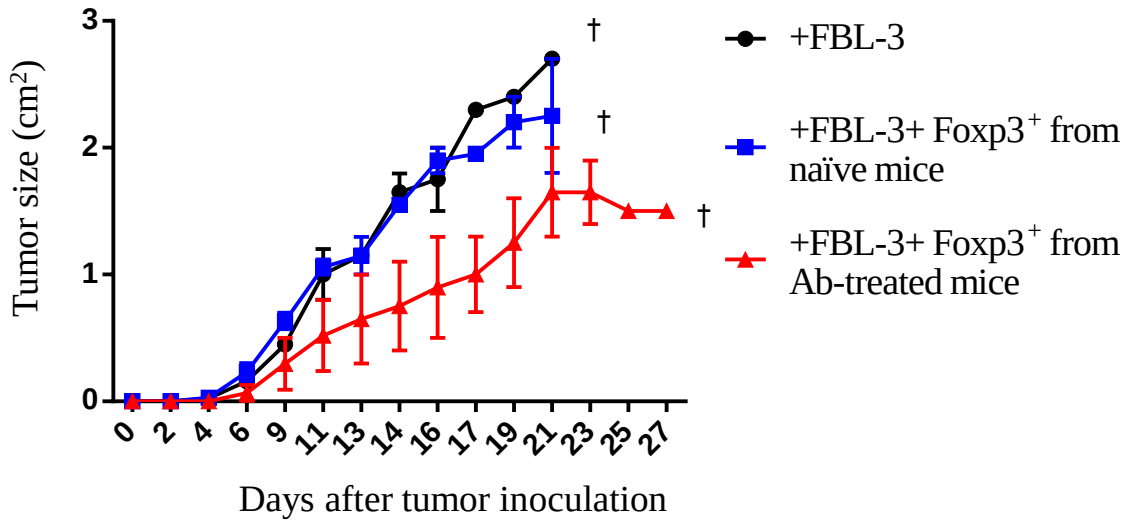

Figure 5.25: Influence of different Treg populations on tumor formation in nude mice

Nude mice (*Foxn1nu*) were injected s.c. with 1×10^6 FBL-3 cells and tumor size was measured. One day before tumor injection some group of mice received GFP^+ $Foxp3^+$ T cells from DEREG mice either treated with αCD137+αCD8 (red line) or not-treated (blue line). The black line represents tumor growth in nude mice challenged with FBL-3 cells only. Lines represent means of tumor progression in 2 mice. Dragger symbol: mice were euthanized due to progressive tumor growth.

5.11 αCD137 treatment also leads to the activation of $Foxp3^+$ effector $CD4^+$ T cells in the presence of $CD8^+$ T cells

It has been shown before that cytotoxic $CD8^+$ T cells are critical in FBL-3 tumor control (64, 133). In cancer patients $CD8^+$ T cells are often exhausted and failed to prevent tumor cell dissemination (159). An administration of CD137 costimulatory antibody into tumor challenge mice with existing $CD8^+$ T cells may not only restore the function of exhausted $CD8^+$ T cells but could also influence effector $CD4^+$ T cells including $Foxp3^+CD4^+$ T cells. However, $CD8^+$ T cells consume large amounts of CD137 ligands, which might prohibit the effect on $CD4^+$ T cells. Therefore, it was of interest to investigate the influence of CD137 agonist antibody on the $Foxp3^+CD4^+$ T cell compartment in the presence of $CD8^+$ T cells. Figure 26 demonstrates that in the presence of $CD8^+$ T cells administration of agonist CD137 antibody into FBL-3 challenged mice nonetheless resulted in a significant expansion of $Foxp3^+$ T cells (Fig. 5.26A) that were highly activated (Fig. 5.26B) and possessed significant

proliferation capacity (Fig. 5.26C) compared to the naïve controls. Remarkably, there was no significant difference in the expansion, activation and proliferation levels of Foxp3$^+$ T cells from CD8-depleted versus non-depleted mice after CD137 agonist treatment (Fig. 5.26B-C).

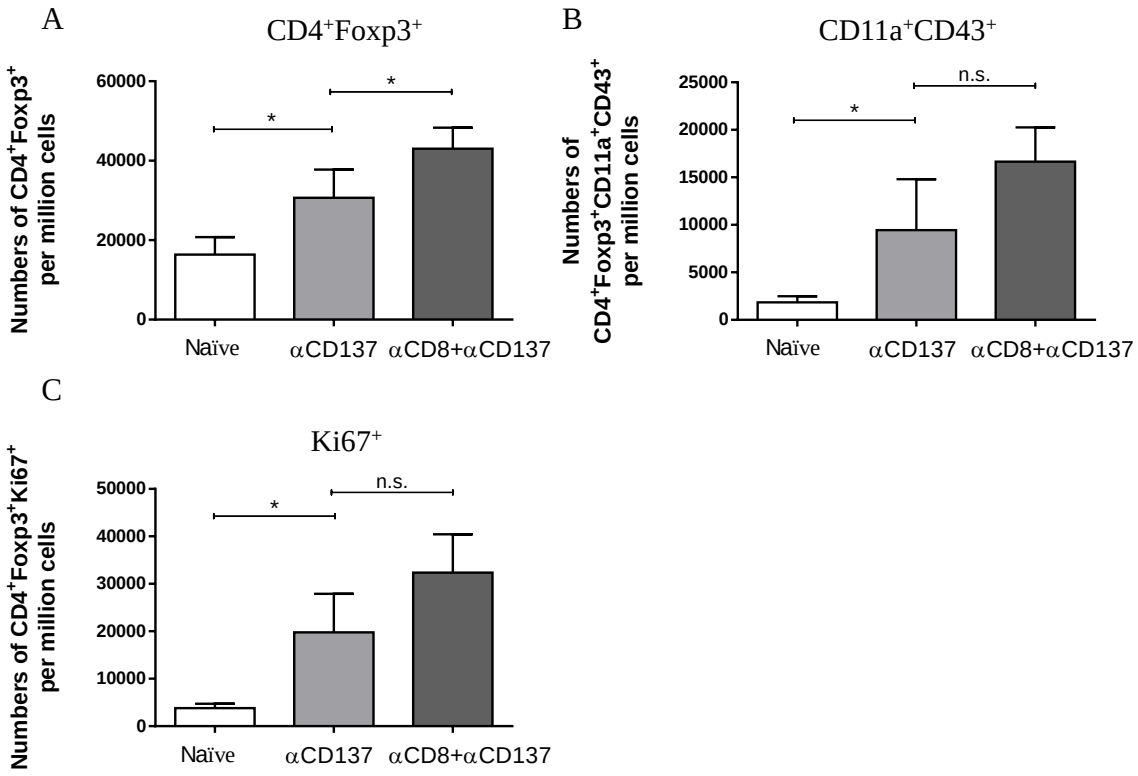

Figure 5.26: αCD137 therapy leads to the activation of Foxp3$^+$ T cells in the presence of CD8$^+$ T cells

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and treated with αCD137+αCD8 or with αCD137 alone as described in methods. At day 6 ptc draining lymph nodes were analyzed for expression of different molecules by flow cytometry. A, total numbers of Foxp3$^+$CD4$^+$ T cells are shown. B, total numbers of surface expression of CD11a and CD43 (double positive). C, the proliferation of Foxp3$^+$ T cells was measured by the intracellular expression of Ki67. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (*$P<0.05$, n.s. – not significant). The experiment was performed two times.

To study the induction of Foxp3$^+$ CD4$^+$ T cells with helper T cell phenotype after agonistic CD137 therapy we stained for CD154 and TNFa. Interestingly, a significant induction of CD4$^+$ T cell that expressed Foxp3 and at the same time produced TNFα or expressed CD154 after CD137 antibody treatment was observed in the presence

of $CD8^+$ T cells, although the size of this effector $CD4^+$ T cell populations were smaller than in additionally CD8-depleted mice (Fig. 5.27A-B).

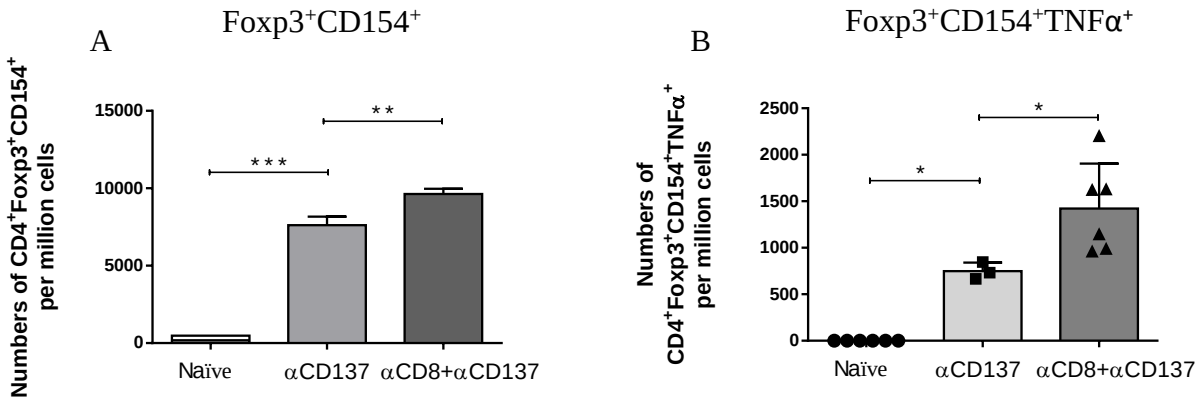

Figure 5.27: Tregs acquire a helper T cell-like phenotypes after αCD137 treatment in the presence of $CD8^+$ T cells

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and treated with αCD137+αCD8 or with αCD137 alone as described in methods. At day 6 ptc $Foxp3^+$ Treg cells from draining lymph nodes were analyzed for different characteristics. A, numbers of $Foxp3^+$ T cells expressing CD154 molecule. C, $CD154^+Foxp3^+$ T cells producing TNF-α. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (**P<0.05, **P<0.005, ***P<0.0005*). The experiment was performed one time (four mice per group).

The cytotoxic characteristics of $Foxp3^+$ T cells following αCD137 therapy in the presence of $CD8^+$ T cells were also analyzed. Again, expression of the transcription factor Eomes and the cytotoxic molecule granzyme B was significantly enhanced in $Foxp3^+$ T cells from tumor-bearing mice treated with αCD137 even in the presence of $CD8^+$ T cells compared to naïve mice (Fig. 5.28A and 5.28B). A representative dot plot in figure 5.28B of con$CD4^+$ and $Foxp3^+$ T cells shows the increased granzyme B production after αCD137 therapy. Only for granzyme B but not for Eomes the population of cells expressing these molecules was significantly bigger after additional depletion of $CD8^+$ T cells. Collectively, these data suggest that even in the presence of $CD8^+$ T cells agonist CD137 costimulatory therapy can convert $Foxp3^+CD4^+$ Tregs into effector cells with a cytotoxic phenotypes.

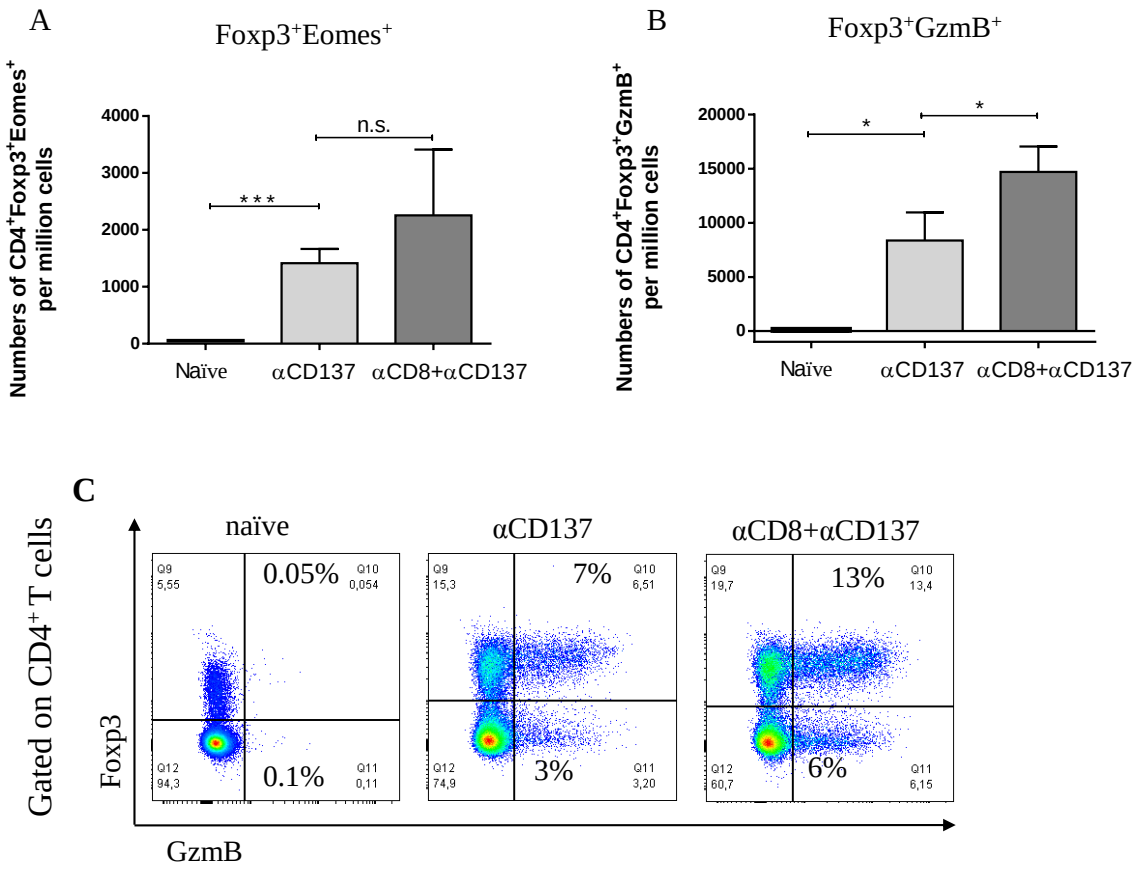

Figure 5.28: Tregs acquire effector-like phenotype following αCD137 therapy in the presence of CD8$^+$ T cells

B6 mice were inoculated s.c. with 1×10^7 FBL-3 cells and treated as described in methods. At day 6 ptc Foxp3$^+$ Tregs from draining lymph nodes were analyzed for transcription factor Eomes (A) and production of GzmB (B). C, representative dot plots of Eomes$^+$Foxp3$^+$ Tregs in different treatment of mice are shown. Differences between the two groups were analyzed by using one-way ANOVA test. Statistically significant differences between the groups are given in the figures (**P<0.05, ***P<0.0005,* n.s. – not significant). The experiment was performed one time (four mice per group).

6 DISCUSSION

Cell-mediated immunity plays an important role in immune responses against cancer. The $CD4^+$ T cell immune response represents a critical part of a functional immune system and is well characterized in many tumor diseases but its role in immunity against oncogenic viruses remains unclear. Over the last years the idea that Tregs, a subset of $CD4^+$ T cells, can undergo functional plasticity has received growing attention. In the current study, we have used the highly immunogenic FV-induced FBL-3 tumor cell line as a model to study the mechanisms of immunological tumor control by $CD4^+$ T cells and Tregs. In a previous study, we reported that in this FBL-3 tumor model Tregs suppress effective anti-tumor $CD4^+$ T cell immune responses and thereby contributed towards tumor progression (2). Cytotoxic $CD4^+$ T cells could fully compensate for the anti-tumor effect of $CD8^+$ T cells, when their suppression by Tregs was abrogated due to Treg depletion. In the current work we show that administration of an agonist antibody against CD137, an activation induced costimulatory molecule, into mice lacking $CD8^+$ T cells promoted effective cytotoxic anti-tumor $CD4^+$ T cell immune responses and led to FBL-3 tumor elimination in the presence of Tregs. Interestingly, αCD137 therapy converted a subpopulation of $Foxp3^+$ Tregs into tumor cell killing effector $CD4^+$ T cells. Our results reveal a unique population of $Foxp3^+CD4^+$ T cells adopting the anti-tumor effector functions of conventional $CD4^+$ T cells upon CD137 signaling.

The TNFR family members which have been found to play major roles as co-stimulatory receptors for $CD4^+$ and $CD8^+$ T cells are CD134, CD137, CD27, CD357, CD30, CD270, DR3, CD267, and CD120b. Targeting these receptors expressed by immune cells is a promising approach for treating patients with cancer. When these receptors are ligated either by their cognate or by agonist antibody, a wide variety of cellular outcomes have been reported ranging from cell differentiation, proliferation, apoptosis and survival to increased production of cytokines and chemokines (36, 40, 42, 45, 98). The first of the TNFR family members to be identified as a possible immunotherapy target was CD137 (92). The efficacy of its ligation was shown in different tumor studies as well as viral infections, demonstrating that treatment of tumor-bearing mice with CD137 agonist therapy promoted effective anti-tumor and anti-viral immune responses. Another costimulatory member of the TNFR family is CD134 that has stimulatory effects on all T helper cell lineages (151), including

increased cytokine production. Interestingly, a combination of CD137 and CD134 treatment drives cytotoxic $CD4^+$ T cell differentiation which in turn results in effective tumoricidal immune functions in a mouse melanoma model (109). In the study presented here, the usage of CD137 agonist therapy alone was sufficient to control FBL-3 tumor formation even in the absence of $CD8^+$ T cells.

Since the use of αCD137 agonist antibody as an anticancer immunotherapy was first proposed, it has been assayed in a number of different tumor model systems (84). CD137 expression is more prominent on $CD8^+$ T cells than on $CD4^+$ T cells and therefore most tumor immunotherapy studies have been focused on the impact of αCD137 on $CD8^+$ T cells (122). In the murine tumor model of sarcoma and mastocytoma, administration of immune co-stimulatory anti-CD137 has been found to significantly decrease the tumor size (92). This effect was highly dependent on $CD8^+$ as well as on $CD4^+$ T cells, since agonist CD137 therapy failed to suppress tumor growth in mice deficient for $CD4^+$ and/or $CD8^+$ T cells (72, 146). In a murine model of breast cancer, treatment with αCD137 resulted in the $CD8^+$ T cell-mediated regression of pre-established tumors and a survival rate of 87% (88). In our tumor model both $CD4^+$ and $CD8^+$ T cell alone could control the growth of FBL-3 cells when stimulated with αCD137. However, depletion of all T cells ($CD8^+$ and $CD4^+$ T cells) during αCD137 treatment resulted in progressive tumor growth (Fig. 5.1D). The results clearly demonstrate the anti-tumor effector function of $CD4^+$ T cells when stimulated with CD137 antibody (Fig. 5.1D). In many tumor entities and tumor models tumor specific $CD8^+$ T cells are functionally impaired or dysfunctional, which likely contributes to the often observed limited efficacy of immunotherapy (159). Previously, we showed that tumor specific $CD4^+$ T cells apart from being helper cells can substitute for the function of $CD8^+$ T cells and efficiently control tumor growth (2). A targeted activation of tumor-specific $CD4^+$ T cells by co-stimulatory CD137 antibody might therefore substantially contribute to anti-tumor immunity and immune control of tumor progression.

It has already been shown that there are many effector mechanisms by which $CD4^+$ T cells can combat tumor cells: cytotoxins (granzymes and the pore forming protein perforin), cytokines (IFN-γ, TNF-α, IL-2) and membrane-associated death proteins (FasL signaling) (49, 107, 111, 117, 123). In the current study, we investigated whether $CD4^+$ T cells in drLN expressed pro-inflammatory cytokines (IFN-γ, TNF-α,

and IL-2), as well as granzyme B in response to FBL-3 challenge following CD137 co-stimulatory treatment.

$CD4^+$ T cells are characterized by their ability to produce cytokines, and play a critical role in the anti-tumor immunity (71). For example, the effective immune response to HPV which controls the development of benign lesions, is mediated by $CD4^+$ T cells producing cytokines (126). In the FV mouse model IFN-γ production by $CD4^+$ T cells is an important component in the infection control (100). One major form of T cell help comes from the transient expression of CD154 (CD40L) on activated $CD4^+$ T cells, which interacts with CD40 on the surface of dendritic cells (DCs), enhancing their expression of both MHC and co-stimulatory molecules, as well as by stimulating the production of cytokines (e.g. IL-2) (116, 132). In both anti-cancer and anti-viral therapies, agonist CD137 therapy expands effector cells that can produce high levels of cytokines, such as IFN-γ, TNF-α and IL-2 (93, 99, 155). Studies using cytokine and cytokine receptor knockout mice have also provided additional information. For instance, mice that were deficient in their ability to produce IFN-γ failed to develop effector anti-tumor immune responses even after administration of αCD137 antibody. Here we show that CD137 agonist therapy resulted in significantly increased numbers of $CD4^+$ T cells producing the pro-inflammatory cytokines, IFN-γ, TNF-α and IL-2 (Fig. 5.6). Not unexpectedly, the cytokine producing $CD4^+$ T cells in tumor drLNs were CD154 positive cells. This observation indicates that these $CD4^+$ T cells were activated by tumor antigen and became tumor specific cells that contributed to FBL-3 tumor control. Interestingly, studies performed by using a blocking CD154-specific antibody inhibited the formation of effective anti-tumor immune responses that was not overcome by CD137-mediated signals (93). In contrast, blocking CD137 expression was associated with downregulation of IFN-γ and TNF-α in $CD8^+$ T cells and NK cells in chronic obstructive pulmonary disease (57).

Here, we show that the anti-tumor $CD4^+$ T response in the absence of $CD8^+$ T cells was altered by αCD137 immunotherapy. This alteration resulted in an increased expression of activation molecules, such as CD11a, CD43 and a higher proliferation capacity. Moreover, KLRG-1, a cadherin receptor that is associated with terminal differentiation and senescence in T cells (56), was significantly increased on $CD4^+$ T cells after CD137 ligation (Fig. 5.3). Recently, it has been demonstrated that in B16 melanoma KLRG-1^+ tumor-infiltrating $CD4^+$ T cells play an important role in response to immunotherapy with a Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) blockade and

CD137 agonist antibody (27). The authors showed that CD137 agonist antibody treatment induced a widespread expression of KLRG-1 on both $CD8^+$ and $CD4^+$ effector T cells that correlated with positive therapeutic outcome. We show here that expansion of highly activated and differentiated $CD4^+$ T cells corresponded with the tumor-specific $CD4^+$ T cells expansion and tumor control after CD137 agonist antibody therapy (Fig. 5.4).

Tumor-specific $CD4^+$ T cells can mediate killing of established tumors as demonstrated by the tumor-specific $CD4^+$ T cells used in the B16 melanoma model (108). The dominant cytotoxic mechanism utilized by effector $CD4^+$ T cells is the induction of apoptosis by the direct release of cytotoxic granules into a target cell. Contained within these granules are granzymes that gain intracellular access into target cells with the help of the protein perforin, which polymerizes to form channel pores through the plasma membrane (described in Introduction). Granzyme/perforin-mediated cytolytic activity has been suggested for virus-specific $CD4^+$ T cells that recognized peptides derived from EBV latent membrane proteins (50), HIV-1 gag protein (103) or poliovirus (142). Perforin-deficient mice are more susceptible to spontaneous lymphomas (124), suggesting that the perforin-granzyme pathway plays a role the in immune surveillance of several types of cancers. It has become apparent that the cytotoxic potential of tumor-specific $CD4^+$ killer T cells depends on the expression of the transcription factor Eomes (28). Additionally, it was demonstrated that Eomes plays an important role in initiating granzyme production in T cells (109). In agreement with this study, we show here that an increased level of granzyme B following αCD137 therapy correlates with levels of Eomes expression in the $CD4^+$ T cell compartment (Fig. 5.7). The study by Qui et.al. also described in vitro differentiation of naïve $CD4^+$ T cells into cytotoxic T-helper cells, in which Eomes, similar to $CD8^+$ T cells, initiates the cytotoxic function (109). However, in this study immunotherapy with two agonists of co-stimulatory molecules, αCD137 plus αCD134, was used. In the current study αCD137 therapy alone was able to initiate cytotoxic $CD4^+$ T cell function and expansion of $CD4^+$ T cells expressing Eomes (Fig. 5.7). Thus, CD137 signaling can obviously initiate a transcriptional program in $CD4^+$ T cells that results in potent cytotoxic activity of these cells.

In order to demonstrate that the increased expression of granzyme B by effector $CD4^+$ T cells resulted in tumor specific lysis of target cells after CD137 co-stimulatory therapy, we performed an *in vivo* CTL assay. $CD4^+$ T cells showed potent killing

activity of peptide-loaded targets in the drLN of FBL-3-bearing mice (Fig. 5.9). The acquisition of cytotoxic activity by tumor-reactive $CD4^+$ T cells emerged when the co-stimulatory treatment was performed. Cytotoxic $CD4^+$ T cells targeting viral antigens (54, 55, 104) and alloantigens (58, 108, 125) have been described previously, but in these models the effector $CD4^+$ T cell activity was not regulated by co-stimulation and the pathogen was eliminated either by adoptive transfer of effector $CD4^+$ T cells (62, 108) or by improved $CD4^+$ T cell recognition (54). Quezada et al. (108) showed effector $CD4^+$ T cell dependent tumor rejection when radiotherapy and adoptive transfer of tumor-reactive $CD4^+$ T cells were combined with the blockade of CTLA-4, a protein receptor that is involved in downregulating the immune system (114). We now report that targeting a costimulatory pathway alone resulted in maintaining immune control over the FBL-3 tumor via direct killing by effector $CD4^+$ T cells.

It is of significance to the current discussion, that it has been recognized that in addition to being expressed on activating effector T cells, many TNFR family members, including CD137 are constitutively expressed on $CD4^+Foxp3^+$ Tregs (20, 91) (Fig. 5.11). Until now it has been unclear whether agonist CD137 antibody treatment exerts pro- or anti-suppressive effect on Tregs (20, 33, 73, 156). The engagement of CD137 on Tregs can increase their number (33, 156), and the stimulation of Tregs with a CD137-Fc fusion protein expands Tregs without changing their function (156). In contrast, another study showed that CD137 ligation on Tregs with an agonist antibody suppressed their function *in vitro* without inducing proliferation (20). The differences in these studies, although unsolved, might reflect alterations in the signal strength or in other aspects of the stimulation conditions. In the current work mice lacking $CD8^+$ T cells and challenged with tumor showed a significant expansion of $Foxp3^+$ Tregs comprising up to 30% of all $CD4^+$ T cells in the tumor-drLNs after αCD137 treatment (Fig. 5.12A). Additionally, the Tregs found following αCD137 therapy *in vivo* were highly activated, differentiated, and had high proliferation capacity. (Fig. 5.13 and 5.14). This clearly demonstrates that αCD137 indeed induces signaling in Tregs.

Tregs can be divided into the two groups of natural occurring thymus-derived Tregs (nTreg), and peripherally induced Tregs (iTreg) that can develop from conventional T cells (26). It has recently been reported that nTregs express Neuropilin-1 while iTregs do not (152). The analysis of the expanded Treg subset showed that the vast majority of Tregs expanding after αCD137 treatment in FBL-3-bearing mice

expressed Neuropilin-1 (Fig. 5.15), suggesting that those cells were mostly thymic-derived nTregs.

It is well known that Tregs suppress beneficial immune responses against pathogens and limiting antitumor immunity (139). Dietze et al. showed that exhausted $CD8^+$ T cells were reactivated and reduced viral loads after depletion of Tregs during chronic FV infection (29). During acute FV infection Treg depletion in DEREG mice augments cytotoxicity of virus-specific $CD4^+$ T cells (86). Iwashiro et al. demonstrated that mice persistently infected with FV approximately doubled the normal percentage of splenic $CD4^+CD25^+$ Tregs and lose their ability to reject the implantation of FBL-3 cells (64). We have previously demonstrated that direct anti-tumor effects of $CD4^+$ T cells in FBL-3 tumor model were limited by Tregs (2). When $CD8^+$ T cells were absent, Tregs suppressed effector $CD4^+$ T cell responses and promoted tumor growth (Fig. 5.10) (2). Therefore, under most circumstances the role of Tregs is clearly immunosuppressive. In regards to our study, the αCD137 therapy overruled this Treg suppression and enabled effector $CD4^+$ T cell control of FBL-3 tumor growth, suggesting that CD137 signaling may influence Treg functions. One possible pathway is that Foxp3 expressing Tregs retain a certain degree of plasticity and under specific conditions they may adopt a pro-inflammatory phenotype (77). This phenotype includes a “helper-like” role and has been observed in different models, including vaccination (137), tumors (1) and graft rejection (140). In particular, Addey et al. (1) showed that in the murine urothelial carcinoma model Tregs can upregulate genes characteristic for a T helper cell phenotype, such as pro-inflammatory cytokines. Remarkably, in the current work CD137 agonist therapy induced expression of markers characteristic for T helper cells (CD154) or cytotoxic T cells (Eomes) in $CD4^+$ $Foxp3^+$ cells. We show here that upon αCD137 therapy CD154 was expressed on a subset of $Foxp3^+CD4^+$ T cells. Moreover, the $CD154^+Foxp3^+$ $CD4^+$ T cell subset expressed the pro-inflammatory cytokine TNF-α, supporting the functional plasticity of the $Foxp3^+CD4^+$ T cells. It has been shown that in drLNs of vaccinated mice Tregs can undergo reprogramming and upregulate CD154 (118), which goes in line with our studies. Interestingly, Sharma et al. (118) advanced that Tregs reprogrammed in response to inflammatory stimuli are specifically needed to provide help in the early cross-priming of $CD8^+$ T cells, leading to effective tumor immunity. Of note, in this work, the function of reprogrammed Tregs depended on the loss of Foxp3 expression, which contradicts the studies suggesting that once established

Foxp3 expression is highly stable in Tregs (95). In contrast, we report that functionally reprogrammed Tregs upon CD137 agonist therapy maintained Foxp3 expression. In a recent study by Sharma et al. (119) it has also been demonstrated that in response to specific inflammatory signals $Foxp3^+CD4^+$ T cells transformed into biologically important helper cells, without loss of Foxp3 expression. Interestingly, *in vitro* restimulation in the presence of transforming growth factor β (TGF- β) induced a major subset of Tregs to reacquire Foxp3 expression, whereas Tregs without TGF- β retained an effector phenotype and produced pro-inflammatory cytokines (74), indicating that mechanisms controlling Foxp3 stability are reversible and may allow adaption to changing microenvironments.

Importantly, Tregs plasticity can contribute to several human diseases. IPEX patients have an increased frequency of IL-17-producing cells that is thought to be sustained by inflammatory driven conversion of Tregs that develop in the thymus (106). Similarly, $Foxp3^+IL\text{-}17^+$ cells were also recognized in patients with allergic rhinitis and polyposis (81) and in the intestinal mucosa of Crohn's disease patients (59). Another study by Kryczek et al. identified a population of $Foxp3^+IL\text{-}17^+$ cells that accumulate in the patients with colon carcinoma as well as in ulcerative colitis (76). These cells originated from memory T cells or Tregs and displayed both an inflammatory and regulatory phenotype. There is also sufficient evidence to suggest that Tregs can be reprogrammed towards a Th1 phenotype as $Foxp3^+IFN\text{-}\gamma^+$ cells were found to be increased in type 1 diabetes patients compared to healthy controls (89). However, it has not been addressed so far whether $Foxp3^+CD4^+$ T cells can undergo conversion that will change their functional capability and turn them into cytotoxic $CD4^+$ T cells. In the current study we describe for the first time a subpopulation of $CD4^+Foxp3^+$ T cells that expresses the cytotoxic transcription factor Eomes while maintaining $Foxp3^+$ Treg identity after αCD137 treatment. These cells constituted up to 15% of all $Foxp3^+$ T cells (Fig. 5.18). The conversion of Treg cells into cytotoxic $Eomes^+CD4^+$ T cells was accompanied by granzyme B production (Fig. 5.19). Moreover, these reprogrammed $Foxp3^+Eomes^+$ $CD4^+$ T cells contributed to FBL-3 tumor clearance (Fig. 5.21). It has been recently shown that granzyme B producing Tregs can kill tumor cell targets (19). However, these cells were redirected by bispecific antibodies, and classical TCR involvement in specific tumor cell recognition was absent. Although we presume that the granzyme pathway is used in the FBL-3 model by $Foxp3^+CD4^+$ T cells to kill tumor cells, there are still unanswered questions regarding

this pathway in Foxp3$^+$-mediated killing. Because granzyme B appears to be an induced molecule in Tregs, it is also important to determine what signaling factors, apart from improved co-stimulatory conditions, are required to activate this pathway.

It was of interest to establish the origin of Foxp3$^+$ CD4$^+$ T cells coexpressing the cytotoxic transcription factor Eomes, and thus provide formal proof of the plasticity of those cells. To this end, an adoptive transfer experiment was performed with DEREG mice, expressing GFP under the Foxp3 promoter to measure conversion of Foxp3$^-$ to Foxp3$^+$ cells following αCD137 therapy. Our results showed that no conversion of conventional CD4$^+$ T cells to Foxp3$^+$ cell was observed but already Foxp3$^+$ T cells started to co-express Eomes after αCD137 treatment (Fig. 5.23). However, it is still unclear, whether cells that coexpress Foxp3 and Eomes represent an intermediate, transitional stage of reprogrammed Tregs that retain the potential to engage in reprogramming or revert to their maternal origin. Moreover, it is also unclear if Foxp3$^+$ cells are cytolytic and suppressive at the same time. This is very important as it might influence the course of disease and choices of immunotherapy.

Additional studies will be required to clarify the antigen specificity of the reprogrammed Foxp3$^+$ CD4$^+$ T cells. In general, the Foxp3$^+$CD4$^+$ T cell population is thought to include many TCRs that recognize self peptides (38). It has been hypothesized that this recognition allows reprogrammed Foxp3$^+$ CD4$^+$ T cells to interact spontaneously with APCs without cognate specificity for the new antigen (118).

In conclusion, our studies have established critical role for effector CD4$^+$ T cells and effector Foxp3$^+$ T cells in the control of oncoviral diseases. When CD8$^+$ T cells were dysfunctional and unable to control FBL-3 tumor development, effector CD4$^+$ T cells were able to attack and abolish the tumor following CD137 agonist therapy, suggesting a direct anti-tumor capacity for those cells. In the current work we provide evidence that CD137 stimulation can induce granzyme B production in Tregs and converts these cells into Foxp3$^+$ cytotoxic killer cells that contribute towards antigen-specific tumor rejection *in vivo*. The findings presented here may have implications for vaccination against tumors that escape immunosurveillance to establish cancer. We suggest that a cytotoxic CD4$^+$Foxp3$^+$ T cell response may be induced by co-stimulatory signals to enhance resistance against virus-associated tumors. Finally, our results should encourage researchers to develop new reagents to manipulate

Tregs and $CD4^+$ T cells *in vivo* that may be used for immunotherapy of malignancies caused by oncoviruses.

7 SUMMARY

Recent successes in immune therapeutic strategies aimed to improve control over tumor growth have sparked the hope that long-lived control of cancer through stimulation of the immune system can be possible. However, the underlying immunological mechanisms that are induced by immunotherapeutic strategies are not well understood. Here, we used the highly immunogenic FV-induced FBL-3 tumor as a model to study the mechanisms of immunological tumor control by $CD4^+$ T cells in the course of CD137 (4-1BB) agonist immunotherapy and in the absence of a visible CD8 T cell response. CD137 is a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily, which is involved in T cell activation and function, including expansion, survival, and cytokine production of effector T cells. We demonstrate that treatment with a CD137 agonist resulted in complete FBL-3 tumor regression in $CD8^+$ T cell deficient mice. CD137 signaling enhanced the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-2, and cytotoxic molecule granzyme B in tumor-specific $CD4^+$ T cells. Over the last years the idea that Tregs, a suppressive subset of $CD4^+$ T cells, can undergo functional plasticity has received growing attention. Interestingly, in addition to being expressed on activating effector T cells the CD137 molecule is constitutively expressed on $CD4^+Foxp3^+$ Tregs. In the current study, we showed that the engagement of CD137 on Tregs resulted in expansion of their numbers and that they were mostly thymic-derived nTregs. Additionally, the Tregs found following αCD137 therapy *in vivo* were highly activated, differentiated, and had high proliferation capacity. Moreover, a subset of $CD4^+Foxp3^+$ Treg cells was reprogrammed to eliminate virus-induced tumor cells in response to CD137 agonist treatment. These converted Tregs expressed a marker characteristic for T helper cells (CD154) and produced the cytokine TNF-α or the cytotoxic transcriptional factor Eomesodermin (Eomes) and granzyme B without loss of Foxp3 expression. Foxp3 Eomes double-positive $CD4^+$ cells were capable of eliminating virus-induced tumor cells *in vivo*.

Our results reveal a unique population of $Foxp3^+$ $CD4^+$ T cells adopting the anti-tumor effector functions of conventional $CD4^+$ T cells upon CD137 signaling. Therefore, this study might contribute to the development of new reagents to manipulate Tregs and $CD4^+$ T cells *in vivo* that can be used for immunotherapy of malignancies caused by oncoviruses.

8 ZUSAMMENFASSUNG

Jüngste Erfolge bei der immuntherapeutischen Behandlung von entarteten Krebszellen durch Stimulation bestimmter Immunzellen wecken die Hoffnung, das Tumorwachstums lebenslang kontrollieren zu können. Allerdings sind die immunologischen Mechanismen, die den immuntherapeutischen Strategien zugrundeliegen, bis heute nicht vollständig geklärt.

In dieser Arbeit wurde die Kontrolle des Tumorwachstums durch $CD4^+$ T-Zellen in Anwesenheit des CD137 (4-1BB)-Agonisten untersucht. Als Tumorzelllinie wurden die immunogenen, FV-induzierten FBL-3 Zellen verwendet, die stets in Abwesenheit von $CD8^+$ T-Zellen untersucht wurden. CD137 ist ein Mitglied der Tumornekrosisfaktor-Rezeptor (TNFR) Superfamilie, welches bei der T-Zellaktivierung und T-Zellfunktion, wie z.B. Expansion, Überleben und Zytokinproduktion, mitwirkt. In dieser Arbeit konnten wir zeigen, dass die Behandlung mit dem CD137-Agonisten in $CD8^+$ T-Zell-depletierten Mäusen zu einem vollständigen Rückgang des FBL-3 Tumorwachstums führte. Dabei zeigte sich, dass CD137 die Produktion von proinflammatorischen Zytokinen, wie IFN-γ, TNF-α und IL-2, und des zytotoxischen Moleküls Granzym B in Tumor-spezifischen $CD4^+$ T-Zellen induziert. In den vergangenen Jahren rückten regulatorische T-Zellen (Tregs), welche eine supprimierende Population innerhalb der $CD4^+$ T-Zellen darstellen, in den Fokus der Forschung, da diesen eine funktionelle Plastizität zugesprochen wird. Interessanterweise wird CD137 nicht nur auf aktivierten Effektor T-Zellen, sondern auch konstitutiv auf $CD4^+Foxp3^+$ Tregs exprimiert. Wir konnten zeigen, dass die Bindung von CD137 an Tregs zu einer Expansion dieser bestimmten Tregs führte, welche hauptsächlich dem Thymus entstammen und als natürliche Tregs bezeichnet werden. Nach *in vivo* anti-CD137-Therapie war zu erkennen, dass Tregs einen stark aktivierten und differenzierten Phänotyp aufwiesen und eine vermehrte Proliferation zeigten. Zusätzlich stellte sich heraus, dass eine Subpopulation der $CD4^+$ $Foxp3^+$ Tregs durch Applikation des CD137-Agonisten umprogrammiert werden konnte, sodass diese daraufhin Virus-infizierte Tumorzellen eliminieren konnte. Diese umfunktionierten Tregs exprimierten den für T-Helferzellen charakteristischen Marker (CD154). Zusätzlich produzierten sie das Zytokin TNF-α und waren positiv für Eomesodermin (Eomes) und Granzym B. Interessanterweise führte die Umprogrammierung dieser Tregs nicht zum Verlust der Foxp3-Expression.

Es zeigte sich *in vivo*, dass die doppelt positiven $Foxp3^+Eomes^+$ $CD4^+$ T-Zellen virus-induzierte Tumorzellen töten konnten.

Unsere Ergebnisse zeigen, dass eine bestimmte Population der $Foxp3^+$ $CD4^+$ T-Zellen über den CD137-Signalweg zu anti-Tumor Effektorzellen umgewandelt werden konnte. Daher könnte diese Manipulation von Tregs und $CD4^+$ T-Zellen *in vivo* zur Entwicklung von neuen Medikamenten beitragen, welche dann zur immuntherapeutischen Bekämpfung von onkoviralen Krankheiten verwendet werden können.

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10 APPENDIX

10.1 List of Abbreviations

Abbreviations	Full name
°C	Degree Celsius
µl	Microlitre
ADCC	Antibody dependent cell mediated cytotoxicity
AF 700	Alexa Fluor 700
AF488	Alexa flour 488
AF647	Alexa flour 647
AIDS	Acquired Immune Deficiency Syndrome
ALV	Avian leukemia virus
APC	Antigen presenting cells
APC	Allophycocyanin
APC-Cy7	APC-cyanine7
ASV	Avian sarcoma virus
B7RP1	B7-Related protein 1
BCL-6	B cell lymphoma 6
BLV	Bovine leukemia virus
BSA	Bovine Serum Albumin
BV421	Brilliant violet 421
BV605	Brilliant violet 605
CAD	Caspase-activated deoxyribonuclease
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester

CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic T cell
depl	Depletion
DEREG	Depletion of regulatory T cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
drLNs	draining lymph nodes
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid,
eF650	eFlour650
eF780	eflour780
eFluor 450	eFluor 450
Env	Envelope protein
Eomes	Eomesodermin
FACS	Fluorescence Activated Cells Scanner (Flow cytometer)
FASL	FAS ligand
FCS	Fetal Calf Serum
FeLV	Feline leukemia virus
FeSV	Feline sarcoma virus
Fig	Figure
FITC	Fluorescein isothiocyanate
F-MuLV	Friend murine leukemia virus
Foxp3	Forkhead box P3

FSC	Forward scatter
FV	Friend Virus
FVD	Fixable viable dye
g	Gram
Gag	Group specific antigen
GATA-3	Gata binding protein 3
GFI-1	Growth Factor Independent-1
GFP	Green fluorescent protein
Gzm	Granzyme
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HPV	Human papillomavirus
hrs	Hours
HTLV	Human T-cell leukemia virus
HTLV-1	Human T-Cell Leukemia Virus-1
i.p.	Intraperitoneal
i.v.	Intravenous
ICOS	Inducable costimulator
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome
iTregs	Induced Tregs

KLRG-1	Killer cell lectin-like receptor subfamily G member 1
KS	Kaposi’s sarcoma
KSHV	Kaposi sarcoma-associated herpesvirus
L	Liter
LAG-3	Lymphocyte-activation gene 3
LNs	Lymph nodes
LTR	Long Terminal Repeat
Mab	Monoclonal antibody
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
mRNA	messenger RNA
MSV	Murine sarcoma virus
n.s.	Non-significant
NK	Natural Killer cells
Nrp-1	Neuropilin-1
nTregs	Natural Tregs
PAMPs	Pathogen-Associated Molecular Patterns
PBBS	Phosphate Buffered Saline, containing 1.0 g glucose
PBS	Phosphate Buffered Saline
PD-1	programmed cell death 1
PD-L1	programmed cell death ligand 1
PE	Phycoerythrin
PE Cy5	PE-cyanine5
PE Cy7	Phycoerythrin–Cy7
PerCP	Peridinin-chlorophyll-protein complex

PI	Propidium iodide
PMT	Photomultiplier tube
Pol	Polymerase
PRR	Pathogen Recognition Receptors
ptc	post tumor challenge
RNA	Ribonucleic acid
RORγt	Retinoic acid receptor-related orphan receptor-γt
RPMI-1640	Roswell Park Memorial Institute Medium 1640
RSV	Rous sarcoma virus
s.c.	Subcutaneous
SFFV	Spleen focus forming virus
SPF	Specific pathogen-free
SSC	Sideward scatter
STAT	Signal Transducer and Activator of Transcription
Tcon	Conventional $CD4^+$ T cells
TCR	T-cell receptor
Tetr	Tetramer
TetrII	Tetramer Class II
TGF-β	Transforming Growth Factor-beta
Th	T helper cells
TLR	Toll-Like Receptor
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor-alpha
TRAIL	Tumor-necrosis-factor-related apoptosis-inducing ligand
TRAILR	Tumor-necrosis-factor-related apoptosis-inducing ligand receptor
Tregs	Regulatory T cells

10.2 Figure list

10.3 Table list

10.4 List of publications

Akhmetzyanova I et al. Tumor-specific CD4(+) T cells develop cytotoxic activity and eliminate virus-induced tumor cells in the absence of regulatory T cells, Cancer Immunol Immunother. 2013 Feb. 62(2):257-71. doi: 10.1007/s00262-012-1329-y. Epub 2012 Aug 14.

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Akhmetzyanova I, Drabczyk M et al. PD-L1 expression on retrovirus-infected cells mediates immune escape from CD8+ T cell killing, (submitted)

Akhmetzyanova I et al. CD137 agonist therapy can convert regulatory T cells into cytotoxic CD4+ T cells with antitumor activity, (submitted)

Littwitz E, **Akhmetzyanova I** et al, Regulatory T cells suppress effector NK cell responses during an acute retroviral infection by consumption of IL-2, (submitted)

10.5 Acknowledgements

Die Danksagung ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten

10.6 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

10.7 Erklärungen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „*Regulation of the effective anti-tumor CD4$^+$ T cell immunity by agonistic CD137 antibody*“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Ilseyar Akhmetzyanova befürworte.

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