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DISSERTATION

Multiple Gene Editing of T Cell Inhibitory Receptors via CRISPR/Cas9 in EL4 cells / Mehrfache genetische Editierung von inhibitorischen T-Zell Rezeptoren mittels CRISPR/Cas9 auf EL4 Zellen

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Vorwort

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Index

Index of Tables and Figures

List of Abbreviations

ICB	Immune checkpoint blockade
irAEs	Immune related adverse events
TIR	T cell inhibitory receptor
CD	Cluster of differentiation
TIL	Tumor infiltrating lymphocyte
NKC	Natural killer cells
INF-γ	Interferon-gamma
IL-12	Interleukin 12
IL-2	Interleukin 2
IL-10	Interleukin 10
TCR	T cell receptor
VEGF	Vascular endothelial growth factor
CCL5	CC-Chemokine-Ligand 5
T_{reg}	Regulatory T cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand
TGF-β	Transforming growth factor beta
IgSF	Immunoglobulin superfamily
TNFSRF	tumor necrosis factor receptor superfamily
DC	Dendritic cell
mAB	Monoclonal antibody
TIM-3	Type I transmembrane/ T cell immunoglobulin mucin-3
Th1	T helper cell
CTL	Cytotoxic T lymphocyte
LAG-3	Lymphocyte activation gene 3
NSCLC	Non-small lung carcinoma
CLL	Chronic lymphocytic leukemia
HL	Hodgkin's lymphoma
MHC	Major histocompatibility complex

CAR	Chimeric antigen receptor
scFV	single-chain variable fragment
CRISPR	Clustered regularly interspaced short palindromic repeat
Cas	CRISPR-associated
sgRNA	Single-guide RNA
PAM	protospacer-adjacent motif
crRNA	CRISPR-RNA
NHEJ	non-homologous end joining
HDR	homology-directed repair
shRNA	short hairpin RNA
MFI	Mean fluorescence intensity
CFD	cutting frequency determination
gRNA	guide RNA
RGENs	RNA-guided endonucleases

Abstract

In cancer, inhibitory signals or specific antigens present on tumor cells induce a state of T cell exhaustion. Targeting these immune inhibitory mechanisms and reactivating the anticancer immune reaction has been the idea of immune checkpoint blockade (ICB). Despite the initial success and remarkable long-term responses emerging from monoclonal antibody-based ICB, checkpoint inhibition entails immune-related adverse events (irAEs). In order to avoid immunotherapy-associated toxicity while improving therapeutic efficacy, we investigated the feasibility of the genetic editing of tumor inhibitory receptors (TIRs) to be major players in the inhibition of T cell activity. We successfully generated triple edited EL4 cells via CRISPR/Cas9-mediated disruption of PD-1, LAG-3 and TIM-3. Transfected EL4 cells showed reduction in immune checkpoint molecule expression, especially for PD-1 signals. Furthermore, we selected triple edited clones with a reduction of protein expression of all three targeted molecules and performed Sanger sequencing on their DNA. The sequencing analysis showed a mixture of different sequence profiles. This genetic chimerism often occurs when mutating a gene of interest using the CRISPR/Cas9 method. Taken together, we were able to create EL4 lymphoma cell clones with genetic disruption of all three TIRs: PD-1, LAG-3 and TIM-3. We have developed a temporary concept of CRISPR/Cas9-mediated triple gene editing of TIRs on EL4 lymphoma cells which needs to be transferred to T cells and further explored for a convertible option for T cell therapy in a clinical setting.

Abstrakt

Bei Tumorerkrankungen führen inhibierende Signale oder spezifische Antigene, die auf den Tumorzellen präsentiert werden, zu einem Erschöpfungszustand der T Zellen. Das Grundprinzip der Immuncheckpoint Blockade (ICB) ist es, in diese immuninhibierenden Mechanismen einzugreifen und die körpereigene Immunreaktion auf Tumorzellen wiederherzustellen. Obwohl die Immuncheckpoint Blockade, basierend auf monoklonalen Antikörpern, zunächst großen Erfolg und ein bemerkenswertes, langfristiges Therapieansprechen zeigte, resultieren daraus andererseits auch immunvermittelte Nebenwirkungen, sogenannte „immune related adverse events“ (irAEs). Mit dem Ziel einer geringeren Immuntherapie assoziierten Toxizität bei gleichzeitig verbesserter therapeutischer Effizienz, prüften wir die Durchführbarkeit einer genetischen Editierung von Tumor inhibierenden Rezeptoren (TIRs), welche eine große Rolle in der Inaktivierung der T Zell Aktivität spielen. Wir generierten dreifach editierte EL4 Zellen, indem wir mittels CRISPR/Cas9 eine Gendisruption für PD-1, LAG-3 und TIM-3 erzielten. Transfizierte EL4 Zellen zeigten eine Reduktion in der Expression der Immuncheckpoint Moleküle. Dies zeigte sich besonders bei PD-1 Signalen. Des Weiteren selektierten wir dreifach editierte Zellklone, die eine verminderte Proteinexpression aller drei Zielmoleküle aufwiesen, und führten eine DNA Sequenzierung nach Sanger durch. Die Sequenzanalyse zeigte eine Mischung verschiedener Sequenz Muster. Dieser genetische Chimärismus tritt häufig bei CRISPR/Cas9 vermittelten Genmutationen auf. Zusammenfassend ist es uns gelungen, EL4 Zellklone mit einer Störung der Gene der Tumor inhibierenden Rezeptoren PD-1, LAG-3 und TIM-3 zu generieren. Wir entwickelten ein vorübergehendes Konzept einer CRISPR/Cas9 vermittelten, dreifachen genetischen Editierung von Tumor inhibierenden Rezeptoren auf EL4 Zellen. Dieses Konzept kann nun auf T Zellen übertragen und weiterentwickelt werden, um als mögliche Anwendung für T Zell Therapie in einem klinischen Setting genutzt zu werden.

1. Introduction

1.1 The crossroads between the immune system and cancer

The classical idea that the immune system is able to recognize and control tumor development to the same extent as it defends the organism from pathogens has prompted a large development of cancer immunotherapies in the last few decades (Murphy et al., 2018). The immune response to tumor cells, now known as *cancer immune editing*, is a versatile and complex process, the first hypothesis for which was formulated in 1909 by Paul Ehrlich (Ehrlich, 1909). Subsequently, in 1957 Thomas and Burnet proposed their theory about cancer immunosurveillance, which represented a milestone in cancer immunotherapy (Burnet, 1957; Dunn et al., 2002). Although the first evidence of an interaction between tumor and immune system came early in the twentieth century, only at the end of it was there the first proof that T cells are able to orchestrate anti-tumor surveillance and responses (Hanson et al., 2000; Mumberg et al., 1999). Since then, further discoveries have also described the ability of cancer cells to escape immune surveillance and the concept of cancer immunoediting was introduced (Dunn et al., 2002; Ikeda et al., 2002). Cancer immunoediting defines the complex relationship that exists between the immune system, the tumor microenvironment and the tumor. The environment in which tumors grow has a crucial effect on tumor development itself. Because of the pressure on tumor cells that results from cancer immunosurveillance, new tumor variants arise, carrying different mutations that could increase the resistance to anti-tumor immune reaction (Dunn et al., 2002). Cancer immunoediting occurs in three sequential phases: elimination, equilibrium, and escape (Figure 1) (Vesely et al., 2011). Given that the concept of immune editing is fundamental to understand how the immune system shapes tumor development and vice versa, each phase will be elucidated in much more detail in the following sections.

1.1.1 Elimination process

The first phase of immunoediting, defined as the elimination phase, is characterized by several steps. Firstly, when tumor cells start expanding, they cause minor disruptions within the neighboring

tissue. This triggers the release of inflammatory signals which, in turn, leads to a recruitment of cells of the innate immune system. These tumor infiltrating lymphocytes (TILs), such as natural killer cells (NK) and subpopulations of T cells, are able to recognize transformed tumor cells, which stimulates the TILs to produce interferon-γ (IFN-γ), a cytokine that functions as a primary activator of macrophages. *In vivo* experiments, on animal models, showed that IFN-γ protects the host against the growth of tumors by recruiting and activating macrophages and other immune effector cells to the tumor site (Dighe et al., 1994). Furthermore, INF-γ induces the production of chemokines, including CXCL10 , CXCL9 and CSCL11 which have angiostatic function, and thereby block the neovascularization of tumor cells (Dunn et al., 2002). Subsequently, a positive feedback loop starts which leads NK cells and macrophages to transactivate one another. Additionally, higher levels of INF-γ, but also interleukin-12 (IL-12) are produced, and cytolytic enzymes like Perforin are released from cytolytic granules of NK cells. In the draining lymph node, newly immigrated dendritic cells activate tumor-specific T helper cells, which are positive for cluster of differentiation 4 (CD4+), which is a glycoprotein that serves as co-receptor for the T cell receptor (TCR). CD4+ T cells express INF-γ, which promotes the development of tumor-specific CD8+ T cells. The CD8 glycoprotein characterizes cytotoxic T cells, a subtype of T cells, which are the main effector of cell immune responses (Murphy et al., 2018). Finally, tumor antigen-specific CD4 and CD8 T cells, primed by the dendric cells, migrate from the lymph node to the tumor site to eliminate the antigen-expressing tumor cells (Vesely et al., 2011). If the elimination process is successful, it allows for the complete clearance of the tumor, otherwise an equilibrium phase is established.

1.1.2 Equilibrium process

The equilibrium phase can be summarized as an immune-mediated tumor dormancy where some of the tumor cells are dormant while others are under immunoselection (Dunn et al., 2002). Tumor cell outgrowth is mainly prevented by the adaptive immune system, more precisely T and B cells, but at the same time the immunogenicity of the tumor cells is shaped. Both the growth inhibitory and cytotoxic reactions of the immune system on the tumor cells provide selection pressure on residual

tumor cells (Schreiber et al., 2011). This selection pressure can lead to the appearance of new variants. These newly created variants are commonly characterized by a lower immunogenicity through downregulation of MHC class I proteins, membrane loss of surface antigen and expression of inhibitory cell-surface molecules like checkpoint molecules (Mittal et al., 2014). Thereby, the immune system promotes outgrowth of tumor cells that have acquired the most immunoevasive mutations. The equilibrium phase can be as long as tumor cells survive, and it is followed by the escape phase as soon as the tumor cells acquire enough mutations and T cells reached a certain state of exhaustion. T cell exhaustion is characterized by a progressive loss of effector function due to prolonged antigen stimulation and thereby favors immune evasion of the tumor cells (Dunn et al., 2002).

1.1.3 Escape process

The last phase of immunoediting is the escape phase. There are different mechanisms by which tumor cells can escape. Firstly, at the tumor cell level, acquired genetic and epigenetic adaptions lead to reduced immune recognition, for instance due to a loss of tumor antigen expression. The result of such Darwinian selection processes determine the appearance of poorly immunogenic tumor cell variants that become unrecognizable to the immune system and able to expand progressively (Dunn et al., 2002). Secondly, tumor cell escape may occur as a result of the establishment of an immunosuppressive state within the tumor microenvironment (Radoja et al., 2000). Tumor cells release various molecules including growth factors, cytokines, angiogenic factors, proteolytic enzymes and chemokines, which not only create an immunosuppressive network within the TME, but also interact with other cells like immune cells or endothelial cells (Saleh and Elkord, 2020). One example is the production of vascular endothelial growth factor (VEGF), which is important for neovascularization of the tumor. Another way is the recruitment of regulatory T cells (T_{reg}) to tumor sites by a variety of chemokines such as CC-Chemokine-Ligand 5 (CCL5). T_{reg} play a crucial role in inhibiting the function of tumor-specific T cells: they produce immunosuppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β), express co-inhibitory immune receptors like cytotoxic T-lymphocyte-associated protein 4(CTLA-4), programmed cell death protein 1

(PD-1) and programmed death ligand 1 (PD-L1), and reduce the level of interleukin-2 (IL-2), which is an important cytokine that sustains the T cell-mediated cytotoxicity (Schreiber et al., 2011). The tumor-mediated immunosuppression is able to support tumor growth and survival, which consequently induces the clinical appearance of the disease.

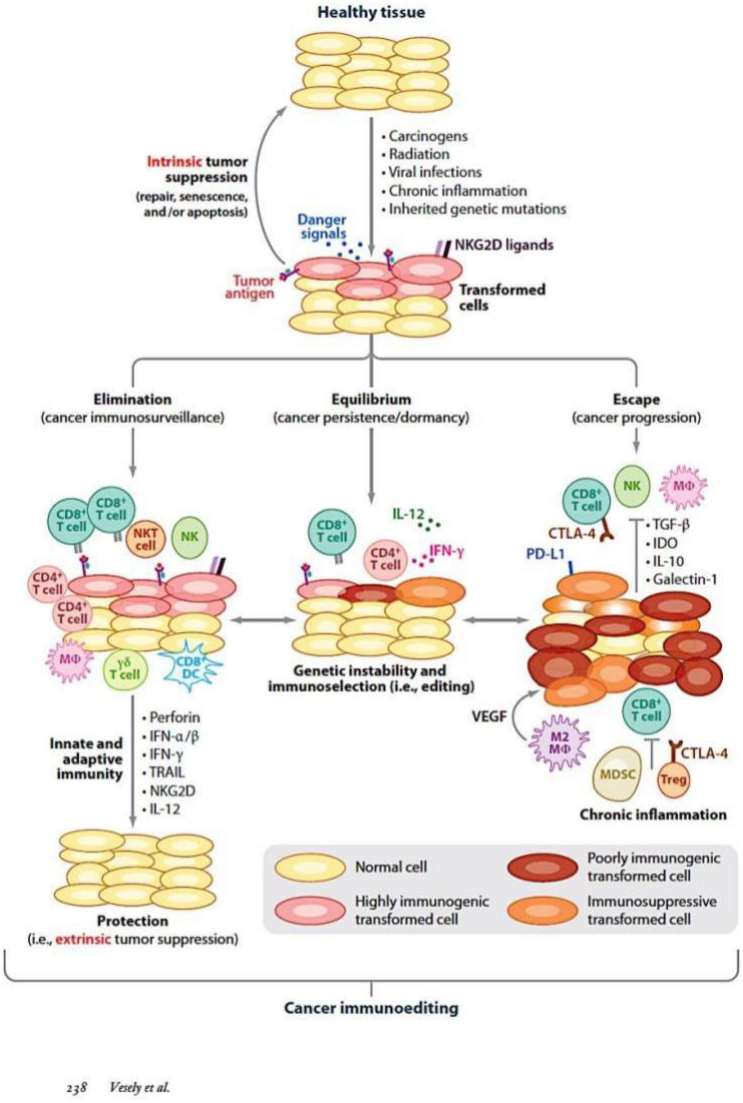

Figure 1. The three different phases of cancer immunoediting: elimination, equilibrium and escape phase. The outcome of these processes controls and shapes cancer. (Vesely M, Natural Innate and Adaptive Immunity to Cancer, 2011[8])

1.2 Immune checkpoint molecules and their role in immunosurveillance

Immune checkpoint molecules and their corresponding signaling pathways are crucial in modulating the immune response. They can be defined as cell-surface molecules with the ability to

transduce co-stimulatory or co-inhibitory signals into T cells, so as to modulate TCR signaling. Once T cells have been activated, the co-stimulatory and co-inhibitory immune checkpoint molecules are translocated to the cell membrane, where they determine the T cell fate by binding specific ligand molecules expressed by hematopoietic and non-hematopoietic cells as well as tumor cells. Co-stimulatory and co-inhibitory receptors are very diverse in their expression and function, which is often context dependent (Chen and Flies, 2013).

A possible classification of immune checkpoint molecules could be based on their molecular structure. There are two superfamilies most immune checkpoint receptors belong to: the immunoglobulin superfamily (IgSF) and the tumor necrosis factor receptor superfamily (TNFSRF). Further subdivisions into specific families are then based on the primary amino acid sequence, protein structure and function. The IgSF consists of diverse families with both co-inhibitory and co-stimulatory receptors (Chen and Flies, 2013). Table 1 shows an overview of all coreceptors with their co-signaling function and corresponding ligands or receptor counterparts.

Receptor subfamily	Molecule name	Extracellular domains	T cell co-signalling	Ligand/counter receptor
CD28	CD28		Stimulatory	B71,B72,B7H2 (human)
	ICOS (CD278)		Stimulatory	B7H2
	CTLA4 (CD152)		Inhibitory	B71,B72,B7H2 (human)
	PD1 (CD279)		Inhibitory	B7H1,B7DC
	PD1H (VISTA)		Inhibitory	Unknown
	BTLA (CD272)		Inhibitory	HVEM, UL144
B7	B71 (CD80)		Inhibitory	B7H1,CD28,CTLA4
	B7H1 (CD274,PDL1)		Inhibitory	PD1, B71
CD226	CD226 (DNAM1)		Stimulatory	CD112, CD155
	CRTAM (CD355)		Stimulatory	NECL2
	TIGIT (VSIG9,VSTM3)		Inhibitory	CD112,CD113,CD155
	CD96 (TACTILE)		Unclear	CD111,CD155
TIM	TIM1 (HAVCR1,KIM1)		Stimulatory	TIM4, TIM1,PS
	TIM2 (TIMD2)		Inhibitory	SEMA4A,H-ferritin
	TIM3 (HAVCR2,KIM3)		Inhibitory	Galectin9,PS
	TIM4 (TIMD4)		Unclear	TIM1,PS
CD2/SLAM	CD2 (LFA2,OX34)		Stimulatory	CD48
	SLAM (CD150,SLAMF1)		Stimulatory	SLAM
	2B4 (CD244,SLAMF4)		Stimulatory/inhibitory	CD48
	Ly108 (NTBA,CD352,SLAMF6)		Stimulatory/inhibitory	Ly108,NTB-A (human)
	CD84 (SLAMF5)		Stimulatory	CD84
	Ly9 (CD229,SLAMF3)		Stimulatory	Ly9
	CRACC (CD319,BLAME)		Stimulatory	CRACC
BTN	BTN1 (BTN1A1)		Unclear	Unknown
	BTN2 (BTN2A1-3)		Unclear	Unknown
	BTN3 (BTN3A1-3)		Unclear	Unknown
LAIR	LAIR1		Inhibitory	Collagens
Orphan	LAG3 (CD223)		Inhibitory	MHCII and unknown
	CD160 (BY55,NK28)		Inhibitory	HVEM

Legend: IgV IgC mucin-like GPI-linked; PS= phosphatidylserine

Table 1. IgSF T cell co-stimulatory and co-inhibitory receptors (Lieping C, Natural Reviews Immunology, 2013 (Chen and Flies, 2013)

The ligand-receptor interaction is not always unique - many of the checkpoint molecules bind to multiple receptors, some of which deliver co-stimulatory signals and other trigger inhibitory signals. Under physiological conditions, co-inhibitory receptors are usually upregulated only after T cell activation to maintain the self-tolerance and modulate immune response. In cancer, this effect is used by tumor cells as an important cancer immune resistance mechanism (Pardoll, 2012). Several inhibitory immunoreceptors have been identified in the past decades, including but not limited to PD-1, CTLA-4, LAG-3 and TIM-3 (He and Xu, 2020).

1.2.1 PD-1

PD-1, also called CD279, is a member of the B7/CD28 family within the IgSF. Both PD-1 and its ligand, PD-L1, are membrane protein receptors with canonical immunoglobulin-like IgV extracellular

domains (Table 1). The PD-1 signaling pathway is actually one of the most characterized mechanisms of tumor immune escape (Cha et al., 2019; Chen et al., 2016; Dermani et al., 2019; Jiang et al., 2019). During persisting exposure to antigens, PD-1 is expressed on the plasma membrane of activated immune cell types such as CD4+ and CD8+ T cells, B cells, NK cells, macrophages and dendritic cells (DCs). Once PD-1 binds to its ligands PD-L1 or PD-L2, immune cell function is suppressed. The two ligands of PD-1 have different functions in the immune regulatory process; PD-L1 inhibits T cell function in peripheral tissues, whereas PD-L2 suppresses immune T cell activation in lymphoid organs. In addition to the downregulation of T cells, PD-1 pathway activation also increases the immunosuppressive regulatory T cell (T_{reg}) function. Early animal studies on PD-1-deficient B6 mice have already shown that PD-1 functions as a negative regulator for the proliferation of antigen-stimulated T cell response (Nishimura et al., 1999). In the tumor environment, the PD-1/PD-L1 axis hampers T cell proliferation, reduces the release of cytokines, and inhibits the cytotoxic function. Therefore, such a system is overactivated by tumors in order to induce T cell exhaustion and apoptosis (Zak et al., 2017). One recent approach to avoid T cell suppression induced by PD-L1 tumor expression was to block the PD-1 signaling pathway through the use of PD-1 blockers, such as specific monoclonal antibodies (Cha et al., 2019). Therefore, the aim of blocking antibodies which target PD-1 signaling is to mainly affect the effector stage of the immune response (Seidel et al., 2018). PD-1/PD-L1 blockade is thought to operate predominantly in the tumor microenvironment, since the PD-L1 ligand is usually overexpressed by tumor cells and myeloid cells within human tumors (Herbst et al., 2014). Recent studies have shown that PD-1 blockades are more effective in tumors that are infiltrated by T cells or that have high mutation rates (Rizvi et al., 2015; Tumeh et al., 2014). There are several clinical reports showing the efficiency of PD-1 checkpoint inhibitors. Fully humanized monoclonal antibodies (mAbs) like Nivolumab or Pembrolizumab were approved by the U.S. Food and Drug Administration (FDA) in 2014 (Sharma and Allison, 2015). The promising therapeutic success of these monoclonal antibodies has been demonstrated in various clinical trials, conducted on patients with melanoma, renal cell carcinoma, Hodgkin's Lymphoma, non-small cell lung cancer and ovarian cancer (Ansell et al., 2015;

Borghaei et al., 2015; Brahmer et al., 2015; Ferris et al., 2016; Larkin et al., 2015; Motzer et al., 2015). Depending on the tumor grade, the overall response rate largely varied among different tumor types and, for example, melanoma showed a 31 – 51% response (Ohaegbulam et al., 2015), while follicular lymphoma showed 66% (Philips and Atkins, 2015). Furthermore, in other studies, significantly better results of overall survival, response rate, and progression-free survival were demonstrated in patients who underwent to mAb treatment in comparison to chemotherapy (Borghaei et al., 2015; Herbst et al., 2016).

Unfortunately, PD-1 blockade also entails several safety issues. PD-1 prevents autoimmunity and limits immune activation under physiological conditions, therefore, an inhibition of PD-1 is often associated with a wide range of side effects that resemble autoimmune reactions. Mild side effects are for instance, diarrhea, fatigue, pruritus, and nausea, whereas severe adverse reactions include severe diarrhea, colitis, inflammation pneumonitis or interstitial nephritis (Abdel-Rahman and Fouad, 2016; Larkin et al., 2015; Robert et al., 2015). In some clinical trials, patients experienced an exacerbation of pre-existing autoimmune conditions such as psoriasis or even developed new ones such as type 1 diabetes mellitus (Kato et al., 2016; Nonomura et al., 2017, 2016).

1.2.2 TIM-3

The type I transmembrane (or T cell) immunoglobulin and mucin (TIM) domain-containing molecules also belong to the IgSF (Barrueto et al., 2020). Their structure contains both an IgV-like domain as well as a mucin-like domain (Table 1). TIM binds to various ligands which are commonly expressed on antigen presenting cells, e.g., Galectin-1, Caecam1, HMGB1 and PtdSer (Chen and Flies, 2013). One of the most characterized members of the TIM family in tumor immunology is TIM-3, whose function is the inhibition of the T helper cell (Th1) responses and the expression of cytokines such as TNF and INF-α (Shayan et al., 2017). The binding of TIM-3 to its ligand galectin-9 in macrophages induces the death of Th1 cells, which means that there is a selective loss of interferon-y producing cells and Th1 autoimmunity is suppressed (Zhu et al., 2005). The role of TIM-3 in T cell exhaustion was largely characterized in the HIV-1 infection, where it exerts inhibition on the human immunodeficiency

virus (HIV-1) cytotoxic T lymphocyte (CTL) specific response. It was shown that blocking TIM-3 in cells from people living with HIV-1 and treated with antiretroviral therapy was associated with the control of viral replication for both *in vitro* and *ex vivo* models (Sanz et al., 2020). These results on HIV-1 models have prompted the use of TIM-3 specific inhibitors in the treatment of cancer. TIM-3 has been recently found to play crucial role in tumor immunity by regulating T cell exhaustion in TILs (Das et al., 2017). It acts as an inhibitory receptor in the setting of chronic activation of T cells. It was shown that an increased level of TIM-3 expression on T cells leads to an inactivation of the CTL (Barrueto et al., 2020). Additionally, the expression of TIM-3 has been found to be significantly upregulated in tumor tissue samples, and such overexpression has been associated with poor prognosis in patients with prostate cancer (Piao et al., 2013), clear cell renal cell carcinoma (ccRCC) (Yuan et al., 2014), colon cancer (Zhou et al., 2015), bladder urothelial carcinoma (Yang et al., 2015), cervical cancer (Cao et al., 2013), and gastric cancer (Jiang et al., 2013). Although the TIM-3-mediated interaction between immune cells and tumor cells has been not completely understood, some evidence suggests that TIM-3 could promote tumor progression through various mechanisms including the direct suppression of CD4+ T cell function and by promoting tumor metastasis (Chiba et al., 2012). There are currently two clinical trials using anti-TIM-3 monoclonal antibodies. The *TSR-022* antibody treatment is currently in phase 1 and is being tested as a monotherapy as well as in combination with anti-PD-1 antibody in patients with advanced solid tumors (NCT02817633). Another clinical trial is using the anti-TIM3 monoclonal antibody *Sym023* in patients with locally advanced, unresectable solid tumors (T03489343).

1.2.3 LAG-3

The lymphocyte activation gene 3 protein (LAG3 or CD223) shows similar structures and domains to the IgSF members, having an IgV and three IgC domains (Table 1). LAG-3 is expressed on the plasma membrane in close proximity to the CD4 antigen and binds to the major histocompatibility complex II (MHC-II) on antigen-presenting cells (APCs) (Long et al., 2018). LAG-3 is mainly expressed after lymphocyte activation on various immune cells, comprising TILs, activated CD4+ and CD8+ T cells, $\mathrm{T_{reg}}$, NK cells, B cells and DCs. Several data coming from animal studies, also using $\mathrm{LAG-3^{-/-}}$ murine

models, demonstrated that LAG-3 plays an important role in regulating both the expansion of activated primary T cells and the development of the memory T cell pool (Li et al., 2007), (Miyazaki et al., 1996), (Workman et al., 2004). Depending on its binding to MHC-II and signaling through its cytoplasmic domain, LAG-3 may directly control enhanced T cell expansion by secreting certain molecules (Workman and Vignali, 2005). The negative regulatory activity of LAG-3 includes suppression of both CD8+ and CD4+ cells. When LAG-3 binds MHC-II molecules, the TCR-mediated activation in CD4+ cells is impaired, resulting in decreased IL-2 and IFN-γ production, and subsequently CD4+ T cell proliferation is also reduced (Sierro et al., 2011). *In vivo* experiments using mouse tumor models showed that LAG-3 is necessary for galectin-3-mediated suppression of T cell secreted INF-γ and that the depletion of galectin-3 leads to improved tumor-specific CD8+ T cell function. The association between LAG-3 expression and galectin-3 binding might be a mechanism through which LAG-3 can regulate $CD8^{+}$T cells; the exact mechanism is not yet known (Kouo et al., 2015). However, experiments using LAG-3 antibody during antigen-specific T cell stimulation of primary human $CD4^{+}$ and $CD8^{+}$ cells led to enhanced T cell proliferation and function (Maçon-Lemaître and Triebel, 2005).

On the other hand, LAG-3 has been found to be overexpressed on TILs in various human cancers such as melanoma (Hemon et al., 2011), non-small cell lung carcinoma (NSCLC) (He et al., 2017), colorectal cancer (Chen and Chen, 2014), breast cancer (Burugu et al., 2017), chronic lymphocytic leukemia (CLL) (Shapiro et al., 2017), and Hodgkin's lymphoma (HL) (Gandhi et al., 2006), where it has been found to be associated with an aggressive tumor progression and worse prognosis. Blocking LAG-3 by using monoclonal antibodies is a possible therapeutic approach that tries to interfere with the LAG-3/major histocompatibility complex II (MHC-II) pathway in order to potentiate or restore the T cells response to tumor antigens (Sierro et al., 2011). The combination of vaccination and LAG-3 blocking monoclonal antibody showed delayed tumor progression in mice (Prigent et al., 1999). Furthermore, several clinical trials have been executed using *IMP321*, a soluble recombinant LAG-3-Ig fusion protein. It is either used as immunological adjuvant for vaccination against cancer, or used as monotherapy or used in combination with chemotherapy in cancer patients (Sierro et al., 2011). One

clinical trial treating patients with metastatic renal cancer with *IMP321* as monotherapy revealed promising outcomes. In almost all patients, $CD8^+$ T cell activation was sustained and NK cell activation was induced. These patients also exhibited reduced tumor growth. Patients receiving higher doses of *IMP321* had a significantly better progression-free survival. Furthermore, no dose-limiting toxicity and no major adverse events occurred (Brignone et al., 2009). However, most of the clinical trials investigating IMP321 are in Phase I, which means the efficiency of the therapy in the long run remains to be seen.

1.2.4 Adoptive T cell therapy

Adoptive T cell therapy means transferring tumor-specific T cells, or engineered T cells carrying chimeric antigen receptors (CAR), to a recipient specifically targeting tumor antigens and simultaneously activating T cells to produce antitumor effects (Fousek and Ahmed, 2015). Designing CARs and expressing them in immune cells is one kind of the various T cell-based therapies for the treatment of solid tumours. CAR is an artificial T cell receptor, which consists of a single-chain variable fragment (scFV), a transmembrane domain, and an intracellular domain (Kakarla and Gottschalk, 2014). A promising approach to improve the efficacy of ATT was the combination of chimeric antigen receptors CAR-T cells to permanently silence immune checkpoint receptors, such as PD-1 and LAG-3 (Zhang et al., 2017), with the final aim of reducing toxicity. The combination of CAR-T cells with a immune checkpoint blockade through genetic modifications is supposed to boost anticancer activities with a superior safety profile (McGowan et al., 2020). The antitumor activity of PD-1 knockout cytotoxic T cells was firstly demonstrated *in vivo* on a murine xenograft model (Li and Tian, 2019). Pre-clinical studies in various blood and solid tumor mouse models have demonstrated significantly enhanced *in vitro* and *in vivo* activities of PD-1 KO CAR-T cells (Ren et al., 2017). In 2016, the first clinical trial of CRISPR/Cas9-mediated PD-1 KO T cells in patients with lung cancer was started (Cyranoski, 2016). Further clinical trials with PD-1 KO T cells followed for renal cell carcinoma (NCT02867332), prostate cancer (NCT02867345), bladder cancer (NCT02863913) and various other solid tumors (NCT03747965, NCT03545815).

Besides the advantage of combining immune checkpoint inhibitor disruption with T cell therapy, there are also a few problems that the treatment entails. In some studies, it was demonstrated that PD-1 gene edited T cells were susceptible to T cell exhaustion and did not achieve long-term durability (Odorizzi et al., 2015). Furthermore, a study tested the feasibility of disrupting LAG-3 expression in human primary T cells and CAR-T cells using the CRISPR/Cas9 system (Zhang et al., 2017). Interestingly, LAG-3 KO CAR-T cells did not show any enhancement in antitumor potency *in vitro* and *in vivo* compared to the control cells (Zhang et al., 2017). As LAG-3 and PD-1 work in a synergistic manner, blocking only LAG-3 might not be enough to show superior efficacy (Richter et al., 2010).

1.2.5 Combinatorial checkpoint inhibition as a strategy to overcome resistance

With the ongoing use of immune checkpoint blockades in clinical practice, various patients have shown, besides their initial response, progression in their disease. Some patients under immune checkpoint therapy seem to develop resistance against the treatment (Zhou et al., 2021). The underlying mechanisms of this acquired resistance are not completely understood yet, however an upregulation of other immune checkpoints such as TIM-3 and LAG-3 was evidenced (Topalian et al., 2015). To mitigate the tumor cells' development of resistance against ICI therapy due to upregulation of other inhibitory receptors (Huang et al., 2017), it is crucial to understand the interactions between several immune checkpoint inhibitors. There are different mechanisms that induce resistance to immune checkpoint inhibition. On the one hand the appearance of specific mutations makes tumor cells less recognizable to T cell-mediated killing (Zaretsky et al., 2016). On the other hand, tumor cells upregulate other inhibitory receptors as soon as one of them is targeted by antibodies (Tobin et al., 2021). In the following, the synergy between PD-1 and TIM-3 as well as between PD-1 and LAG-3 and possible combinatorial checkpoint inhibition will be elucidated.

There is a close association between the expression of TIM-3 and PD-1 on CD8+ TIL, marking a deeply exhausted T cell population when expressed simultaneously (Barrueto et al., 2020). Several studies showed that TIL expressing both PD-1 and TIM-3 are the most dysfunctional subset of lymphocytes in head and neck squamous cell carcinoma (HNSCC) (Shayan et al., 2017), melanoma

(Fourcade et al., 2010) and leukemia (Kong et al., 2015). Furthermore, it was revealed that there is a connection between TIM-3 upregulation and PD-1 blockade (Shayan et al., 2017). In a study using PD-1 blockers in lung cancer, TIM-3 was significantly upregulated in response (Koyama et al., 2016). It was demonstrated that the TCR-induced TIM-3 expression on T cells can be inhibited by blocking the PI3K pathway (Mujib et al., 2012). In animal models, mice with genetic ablation of PD-1 revealed more exhausted T cell phenotypes marked by the upregulation of TIM-3 (Odorizzi et al., 2015). Little is known about the molecular mechanisms leading to this upregulation, and the whole mechanisms underlying the TIM-3 upregulation in response to PD-1 blockade need to be further investigated (Shayan et al., 2017). However, in several studies, combining anti-TIM-3 and anti-PD-1 antibodies showed better results in tumor regression than a single therapy. For instance, preclinical treatment of murine gliomas with combined ICI PD-1 and TIM-3 resulted in an improved median survival of 100 days, compared to 33 days under anti-PD-1 alone (Kim et al., 2017). Another study treated CT26 tumor-bearing mice with anti-TIM-3 and anti-PD-L1 antibodies, which resulted in a reduction of tumor growth and complete tumor regression in 50% of the mice. The mice exhibiting complete tumor regression even remained tumor free after rechallenge (Sakuishi et al., 2010). Furthermore, $CD8^+$ T cells from advanced gastric cancer patients were investigated after treatment with PD-1 and TIM-3 blockade. It was demonstrated that the dual blockade restores the frequency and effector function of the tumor-specific $CD8^+$ T cells (Lu et al., 2017). In several studies, combining anti-TIM-3 and anti-PD-1 antibodies showed better results in tumor regression than a single therapy. For instance, preclinical treatment of murine gliomas with combined ICI PD-1 and TIM-3 resulted in an improved median survival of 100 days, compared to 33 days under anti-PD-1 alone (Kim et al., 2017). Furthermore, $CD8^+$ T cells from advanced gastric cancer patients were investigated after treatment with PD-1 and TIM-3 blockade. It was demonstrated that the dual blockade restores the frequency and effector function of the tumor-specific $CD8^+$ T cells (Lu et al., 2017).

Furthermore, clinical data has shown that there is a significant synergy between LAG-3 and PD-1. In animal experiments with BALB/c mice deficient for the genes encoding LAG-3 and PD-1, lethal

autoimmune condition was induced. $LAG-3^{-/-}$ single knockout mice did not show autoimmunity (Okazaki et al., 2011). In NY-ESO-1 ovarian cancer samples, for instance, LAG-3 and PD-1 are expressed on CD8+ T cells leading to inhibition of cytokine secretion and enhanced tumor escape capability (Matsuzaki et al., 2010). In a clinical trial, non-small cell lung cancer (NSCLC) patients were treated with PD-1 blockers and five patients from the cohort obtained a upregulation of LAG-3 during treatment (Gettinger et al., 2017). A combination of anti-LAG-3 and anti-PD-1 antibody treatment in murine models showed a complete remission of tumors which have previously been resistant to single antibody treatment (Woo et al., 2012). Within the same study, there were also experiments run on $LAG-3^{-/-}\ PD-1^{-/-}$ knockout mice. The knockout mice showed the prevention of high-dose B16 and MC38 tumor growth as well as ensured survival. Single knockout controls and wild type mice succumbed to illness. (Woo et al., 2012). Recently, bispecific antibodies which are able to engage both LAG-3 and PD-L1 are under investigation in clinical trials. Currently, *FS118* is being tested for patients with advanced malignancies in Phase I/II (NCT03440437).

Nevertheless, the blockade of two checkpoint receptors could potentially lead to immune-related adverse events (irAEs). IrAEs can occur in any organ system, such as the dermatological, gastrointestinal/hepatic, endocrine, pulmonary, and cardiovascular systems (D. Y. Wang et al., 2018). In general, cardiovascular adverse events have the highest mortality risk, followed by neurological toxicities (Reynolds and Guidon, 2019). Dermatological toxicities such as rash, pruritis, mucositis, and dry mouth are the most common irAEs (Belum et al., 2016). There are several studies showing that the occurring adverse events are more severe in cases with combinatorial antibody treatment compared to antibody monotherapy. In a clinical trial, 37 – 42% of patients under anti-PD-1 monotherapy showed dermatological toxicities, whereas it was 58 – 71% of patients on combination therapy (Collins et al., 2017; Sibaud et al., 2016). Colitis occurred in 1 – 5 % of patients treated with anti-PD-1 antibodies and in 20% of patients with combination immune checkpoint therapy (Y. Wang et al., 2018). Hepatitis showed an incidence of 3.8% with anti-PD-1 therapy and up to 17.6% in combination therapy (Spain et al., 2016). Recently, a review on the overall incidence of irAES showed that 20 - 30% of patients

receiving anti-CTLA-4 antibodies reported severe life-threatening irAEs (Spain et al., 2016). In another cohort of patients receiving anti-CTLA-4/PD-1 combination therapy, the overall incidence of irAEs was 55%.

Another approach to improve the outcome of ICI treatment is combining it with chemotherapy. The idea is to use chemotherapy prior to immunotherapy as a preconditioning regimen. In certain cancers, this combination has shown clinical benefits (Barbari et al., 2020). In a clinical trial, patients with NSCLC (non-small cell lung cancer) were treated with both anti-PD-1 antibody pembrolizumab and chemotherapy. The median overall survival of patients who received the combined treatment was 4 months higher compared to the one treated with chemotherapy only (Paz-Ares et al., 2018). Another clinical trial combined *IMP321*, an LAG-3 blocking antibody, with chemotherapy. Breast cancer patients received escalating doses of *IMP321* as well as weekly paclitaxel. The results showed significantly tumor regression, which was also seen during 85 to 170 days of follow up (Brignone et al., 2010). For some tumor entities, this treatment has now become standard (Barbari et al., 2020).

Taken together, a possible way to overcome resistance in ICI therapy is to simultaneously target several checkpoint molecules, thereby mitigating their upregulation. On the other hand, the toxicity of antibody treatment seems to be enhanced by a combination of antibodies. However, if on the one hand, the combinatorial treatment may induce a more persistent anti-tumor response, on the other it aggravates the serious safety concerns already shown by single checkpoint inhibition (Darnell et al., 2020). These adverse events, namely immune-related adverse events (irAEs), can occur in any organ system such as the dermatological, gastrointestinal/hepatic, endocrine, pulmonary, and cardiovascular systems (D. Y. Wang et al., 2018). In general, cardiovascular adverse events have the highest mortality risk, followed by neurological toxicities (Reynolds and Guidon, 2019). Dermatological toxicities such as rash, pruritis, mucositis, and dry mouth are the most common irAEs (Belum et al., 2016). In a clinical trial, 37 – 42% of patients under anti-PD-1 monotherapy showed dermatological toxicities, whereas it was 58 – 71% of patients on combination therapy (Collins et al., 2017; Sibaud et al., 2016). Colitis occurred in 1 – 5 % of patients treated with anti-PD-1 antibodies and in 20% of patients with

combination immune checkpoint therapy (Y. Wang et al., 2018). Hepatitis showed an incidence of 3.8% with anti-PD-1 therapy and up to 17.6% in combination therapy (Spain et al., 2016). Recently, a review on the overall incidence of irAES showed that 20 - 30% of patients receiving anti-CTLA-4 antibodies reported severe life-threatening irAEs (Spain et al., 2016). In another cohort of patients receiving anti-CTLA-4/PD-1 combination therapy, the overall incidence of irAEs was 55%. Therefore, despite the initial success and the benefits originating from the use of checkpoint inhibition to induce immune-mediated tumor regression, a clinically relevant toxicity profile of the novel immune-checkpoint inhibitors has emerged (Brahmer et al., 2012). Developing and improving alternative immunotherapies such as adoptive T cell therapy might be a path to success.

1.3 Gene editing

1.3.1 CRISPR/Cas9 as a tool for gene editing in immune cells

After the development of the revolutionary gene-editing technology that uses the clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease (Jinek et al., 2012), CRISP/Cas9 gene-editing tools have rapidly spread through laboratories worldwide. CRISPRs are specific repeated DNA sequences within a bacterial genome, which are used in combination with CRISPR-associated (Cas) nucleases to detect and destroy bacteriophages carrying such sequences (Mollanoori et al., 2018). In the CRISPR/Cas9 system, Cas9 is directed by a chimeric single-guide RNA (sgRNA) to a specific genomic locus which is marked by 5'-NGG protospacer-adjacent motifs (PAMs) (Figure 2). The sgRNA is an RNA molecule that functions as guide for the Cas9 and contains a targeting sequence (crRNA) which is homologous to the genomic region of interest, and a Cas9 nuclease recruiting sequence (tracrRNA) which acts as a scaffold linking the crRNA to Cas9. Having recognized the PAM sequence and after the annealing of the crRNA at the genomic target sequence, the Cas9 nuclease cleaves the DNA thus generating a double strand break. Subsequently, the cellular DNA repair machinery takes over by repairing the double break, through a non-homologous end joining (NHEJ) or a homology-directed repair (HDR) pathway. The repair process can then lead to insertions, deletions or mutations at target sites which then might in turn alter the original genomic sequence (Figure 2)

(CRISPR 101 2nd Ed Final May 2018_2.pdf, n.d.). If the CRISPR/Cas9 system targets a specific gene, the alterations introduced can lead to shifts of the open reading frame, thus leading to a de facto knockout of the gene of interest (Campenhout et al., 2019; Qi et al., 2013).

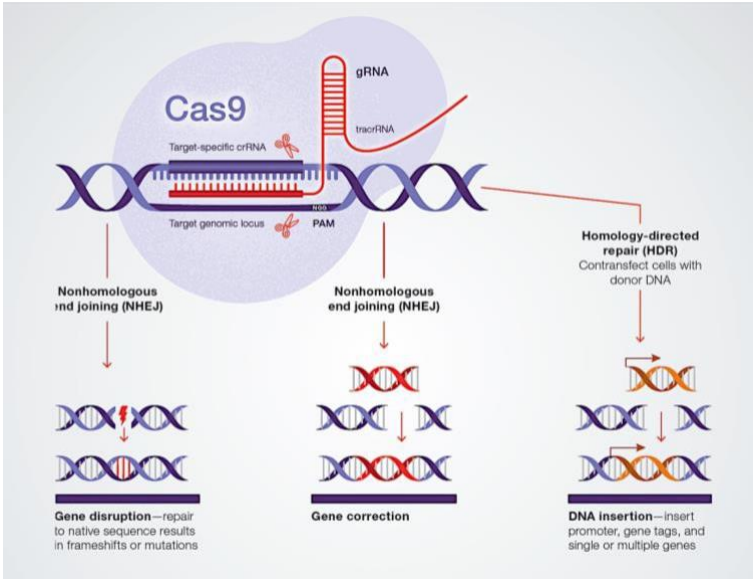

Figure 2. CRISPR/Cas9 system leading to double stranded breaks and possible repair pathways (CRISPR Genome Editing Resource Guide, Thermo Fisher Scientific Inc, 2018, [112])

The use of the CRISPR/Cas9 in T cell gene editing has been already described in the literature (Liu et al., 2017; Ren et al., 2017; Rupp et al., 2017; Zhang et al., 2017), where CRISPR/Cas9-mediated knockout of TIRs such as PD-1, CTLA-4, LAG-3 or TIM-3, in combination with CD19-directed chimeric antigen receptor T cells (CART19) showed feasibility. In addition, studies on single genome editing of PD-1 in T cells using knockout strategies have demonstrated that CRISPR/Cas9 may represent a promising approach for efficient checkpoint inhibitor disruption in T cells (K. Schumann et al., 2015; Su et al., 2016). To establish the CRISPR/Cas9 model system, the EL4 lymphoma cell line was used.

1.3.2 Leukemia EL4 T cells as a system for murine T cells

The EL4 lymphoma cell line was first discovered over 70 years ago isolated from a chemically induced lymphoma (Gorer, 1950). It was established from a lymphoma induced in a C57BL mouse through systemic administration of 9,10-dimethyl-1,2-benzanthracene (Gorer, 1950). Over the years, this cell line has remained very popular in immunological research, due to the ability of the cells in

maintaining a stable phenotype after several cycles of manipulation besides the specifically induced mutation (Logan et al., 2004).

1.4 Aims of the project

Inhibiting immune checkpoint molecules has become an essential component in the treatment of a variety of tumor entities over the last few decades. The application of monoclonal antibody-based immune checkpoint inhibitors has proven to be effective in several malignancies and many patients have benefited from improved clinical outcomes (Garon et al., 2015; Hodi et al., 2010; Robert et al., 2015). However, with the ongoing use of immune checkpoint antibodies in clinical practice, various patients showed, besides their initial response, progression in their disease due to the developing of resistance against the therapy (Saleh and Elkord, 2020), or had to deal with immunotherapy-associated toxicities (Brahmer et al., 2012; Spain et al., 2016). Combinatorial treatments, such as a dual immune checkpoint blockade via using several antibodies, might induce a more persistent anti-tumor response, but also aggravate irAEs which had already occurred in single checkpoint inhibition treatment (Darnell et al., 2020). These adverse events can occur mainly in the dermatological, gastrointestinal/hepatic, endocrine, pulmonary, and cardiovascular systems and significantly affect the morbidity and mortality risk of patients (Reynolds and Guidon, 2019). In order to avoid immunotherapy-associated toxicity while maintaining therapeutic efficacy, an intrinsic blockade of immune checkpoint receptors might be a possible resolution. The approach of genetically editing T cells for a single immune checkpoint inhibitor, such as PD-1 or LAG-3, has already shown relevant results in studies. The T cells, in which only one immune checkpoint inhibitor was genetically knocked out, showed initially enhanced tumor activity, but were prone to exhaustion and did not have a long life span (Rupp et al., 2017; Su et al., 2016; Zhang et al., 2017).

In this scenario, the aim of the project was to investigate the feasibility of combining genetic disruption of PD-1, LAG-3 and TIM-3 with ATT models in order to improve durable anti-tumor activity and at the same time to reduce toxicity. Some studies have already tried to evaluate the possibility of inducing concurrent genetic disruption of PD-1, LAG-3 and TIM-3. In particular, one study tested the

feasibility of a triple short hairpin RNA (shRNA)-mediated silencing of PD-1, LAG-3, and TIM-3 in CAR-T cells, and data showed improved anti-tumor efficiency of the edited CAR-T cells (Zou et al., 2019). However, no enhancement migration of these CAR-T cells was found and the effect of this approach was only transient.

Therefore, in this research project, the possibility of a simultaneous targeting of PD-1, TIM-3 and LAG-3 by using CRISPR/Cas9 strategy in order to induce an efficient triple knockout of PD-1, TIM-3 and LAG-3 in EL4 lymphoma cells was explored, and it was verified that the method was effective in durably altering the expression of PD-1, TIM-3 and LAG-3.

2. Materials and methods

2.1 Materials

2.1.1 Solutions and buffers

All chemicals used in the experiments were purchased from Thermo Fisher, Cell Signaling, Sigma-Aldrich or Carl Roth. Deionized water (Milli-Q Ultrapure Water Purification System, Millipore) was used to make the buffers, solutions and media. The sterilization was performed using an autoclave (25 min, 121°C, 1 bar). All used buffers and solutions are listed in Table 2.

Solution/Buffer	*Ingredients*
PBA	1 x PBS 0.5% BSA
T cell medium (TCM)	10% fetal bovine serum (Life Technologies) 100 U/ml penicillin 100 mg/ml streptomycin 1 mmol/L L-glutamine 1 mmol/L sodium pyruvate 100 mmol/L nonessential amino acids 2 μmol/L mercaptoethanol 500 ml RPMI-1640
RPMI medium	10% fetal bovine serum (Life Technologies) 100 U/ml penicillin 100 mg/ml streptomycin

Taq DNA-Polymerase-PCR-Buffer	200 mM Tris HCl (pH 8.4) 500 mM CKl
1 x TAE buffer	20 ml 50 x TAE 980 ml ddH_2O
1 x Red Blood Cell lysis buffer (1L; pH 7.3)	89.9g NH_4Cl 10.0 g $KHCO_3$ 370.0 mg tetrasodium EDTA
MACS buffer	435 ml ddH_2O 50 ml 10 x PBS 0.5% BSA 5 ml EDTA-stock (0.2 M)
DNA lysis buffer	100 x stock Proteinase K
Tris Buffered Saline (TBS-10X)	137 mM Sodium Chloride 20 mM Tris pH 7.6
1 x Laemmli Buffer	250 mM Tris 2.5 M Glycin 1% SDS
Tris buffered Saline with Tween 20 (TBST)	100 ml 10XTBS stock solution 900 ml distilled water 1 ml Tween 20
Blocking Buffer	1 X TBST with 5% w/v nonfat dry milk
Western Blot Transfer Buffer	20 % Methanol 1 x Laemmli Buffer
Primary Antibody Dilution	1 x TBST with 1% w/v nonfat dry milk ○ LAG-3 (E5S8V) monoclonal Rabbit mAb (#80282 Cell Signaling), 1:1000 ○ PD-1 Rabbit mAb (#86163, Cell Signaling), 1:1000 ○ TIM-3 monoclonal anti-mouse-Antibody (1E5) (#MA5-32841, Thermo Fisher), 1:500
Secondary Antibody Dilution	1 x TBST with 1 % w/v nonfat dry milk

	○ Anti-Rabbit-IgG (R2655, Sigma-Aldrich), 1:80000 ○ Goat Anti-Mouse-IgG (#A32723, Thermo Fisher), 1:500

Table 2. Ingredients of buffers and solutions used in the experiments

2.1.2 Antibodies

The antibodies of interest, labelled to a fluorochrome, were used via flow cytometry or cell sorting. The antibody panel was selected knowing the excitation and emission properties of fluorescent compounds and avoiding overlapping wavelengths of fluorochromes.

Antibody	*Label*	*Titration*	*Isotype*	*Clone*	*Company*
αCD3e	BV-510	1:100			Biolegend
αCD3e	PerCP-Cy5.5	1:200	ArmHamster IgG_1, k	145-2C11	Biolegend
αCD4	BV-510	1:500	Rat IgG_{2a}	RM4-5	Biolegend
αCD8a	APC-Cy7	1:500	Rat IgG_{2a}	53-6.7	Biolegend
αCD25	FITC	1:500	Rat IgG_1, λ	PC61	Biolegend
αCD28	PerCP-Cy5.5	1:200	Syrian Hamster IgG_1	37.51	Biolegend
αCD44	PE-Cy7	1:1000	Rat IgG_{2b}, k	IM7	BD
αCD62L	APC	1:800	Rat IgG_{2b}, k	MEL-14	BD
αCD19	FITC	1:200	Rat IgG_{2a}, k	6D5	Miltenyi
αPD-1 (CD279)	PE	1:200	Rat IgG_{2b}	HA2-7B1	Miltenyi
αLAG-3 (CD223)	BV 421	1:100	Rat IgG_1, k	C9B7W	Biolegend
αTIM-3 (CD366)	PE-Cy7	1:100	Rat IgG_{2a}, k	RMT3-23	eBiosciences

Table 3. Antibodies and corresponding fluorochromes

2.1.3 Primer pairs

Primer	*Sequence 5' → 3'*
PD-1 forward	CCT TTC CGC TAC AGA

PD-1 reverse	GTT CCT CCC CTC CAG
LAG-3 forward	TCT CTC TCC CTT TGT CCG GC
LAG-3 reverse	TGC ATC TTC TTC GTG GCC TTA T
TIM-3 forward	CTA TCT ACA CCT GGG GCA CTT G
TIM-3 reverse	GGA AGT CAG ATG TGA GCA TCC TC

Table 4. List of primer pairs used for the PCR amplification of PD-1, LAG-3 and TIM-3

2.2 Cell biology methods

2.2.1 Cultivation and activation of EL4 cells

The leukemia cell line EL4 (strain C57BL/5N) was cultured in T75 flasks with RPMI containing 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 mg/mL), and in an incubator at 37°C with 5% CO_2. Every 2-3 days, the medium was changed. Therefore, cells were centrifuged (400x g, 5 min at 4°C), old medium was carefully removed and the remaining cell pellet was resuspended with fresh medium. For the activation of the EL4 cells, each well of a 6-well plate was coated with 1 mL of anti-CD3/CD28 antibodies (3 $\mu g/mL$ and 5 μg/mL), dissolved in 1 x PBS overnight at 4°C. On the next day, the coating solution was removed and wells were washed with PBS. 1 x 10^6 cells were seeded in each well in the presence of RPMI and incubated for at least 3 days.

2.2.2 Isolation of murine T cells

For the harvesting of murine T cells, mice were sacrificed by cervical dislocation. The spleen was collected and placed into a 6-well plate containing a few milliliters of 1X PBS 2%FBS. Then, the spleen was smoothly mashed through a 40 μm cell strainer and washed with 1 x PBS. After centrifugation (400x g, 5 min at 4°C) and removal of the supernatant, the pellet was resuspended in 500 μL/spleen of Red Blood Cell Lysis Buffer to remove erythrocytes. After incubation at room temperature for 5 min, the reaction was stopped by adding PBA (PBS with 0.5% BSA). Cells were counted via a hemocytometer (Sigma Aldrich), centrifuged (400x g, 5 min at 4°C), and resuspended in MACS Buffer at a concentration of 2.5 x 10^9 cell/mL. Subsequently, $CD8^+$ T cells were purified using a

$CD8^{a+}$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany, #130-104-075). After purification, the cells were activated overnight by seeding them on a 6-well plate (up to 5 x 10^6 per well), previously coated with murine anti-CD3 (3 $\mu g/mL$) and anti-CD28 (5 μg/mL) antibodies. During activation, T cells were incubated in TCM medium (RPMI, 10% FBS, 1 mM sodium pyruvate, 100 mM MEM nonessential amino acid, 5 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin). T cells were subsequently transferred into fresh 6-well plates and cultured in TCM with added interleukin-15 (IL-15, 50 ng/mL, Peptrotech, Cranbury, NJ, USA, #210-15) and interleukin-7 (IL-7, 10 ng/mL, Peprotech, Cranbury, NJ, USA, #217-17).

2.2.3 Single cell cloning of EL4 cells

Transfected EL4 cells were counted via a hemocytometer. T cells were then resuspended in growth medium at a concentration of 0.5 cells/mL and seeded in a 96-well plate (round bottom) in order to have 0.5 cell/well. Single cell clones were left to expand and a certain amount of cells was lysed for DNA extraction by resuspending them in DNA lysis buffer. The lysate was transferred to tubes and put in a PCR cycler, starting the following program: 57°C for 2 h – 95°C for 10 min – 8°C for 10 min. Genomic DNA was purified by adding 100 μl isopropanol to the lysate. Tubes were gently mixed for 10 min and centrifuged at 14000 rpm for 5 min. After discarding the supernatant, the pellet was washed with 200 μl of Ethanol (70%). Then, samples were centrifuged again at 14000 rpm for 5 min. Finally, the pellet was dried and resuspended in 50 μl bidistilled sterile water.

2.2.4 Transfection via CRISPR/Cas9

Specific crRNAs (0.1 mmol/L each; PD1_crRNA1, TIM3_crRNA2, LAG3_crRNA3; Integrated DNA Technologies, IDT) and universal transactivating crRNA (tracrRNA-ATTO™550, 0.1 mmol/L; Integrated DNA Technologies, IDT) were mixed at equimolar concentrations and heated at 95°C for 5 minutes in a ThermoMixer® (Eppendorf). The mixture was then cooled down until room temperature (RT) was reached. Precomplexing of Cas9 (30 pmol/μl, TrueCut™ Cas9 Protein v2, Thermo Fisher, #A36498) endonuclease with all crRNA/tracrRNA complexes (80 pmol/μl of PD1_crRNA1/tracrRNA,

TIM3_crRNA2/tracrRNA and LAG3_crRNA3 /tracrRNA) was done by mixing and incubating 3.75 µl of each crRNA/tracrRNA complex with 11.25 µl Cas9 protein for 10 minutes at room temperature. The crRNA/tracrRNA/Cas9 mixture was electroporated into a $2.5x10^5$ EL4 lymphoma cell line using a 4D-Nucleofector® X Unit (Lonza). EL4 cells positive for ATTO™550 were sorted and EL4 positive cells were cultured in RPMI 10% FBS.

2.2.5 Flow cytometry

For phenotypic characterization, 1.5 x 10^5 transfected and not transfected EL4 cells were centrifuged and resuspended in 20 µl of Master Mix containing antibodies against cell surface markers (Table 3). Samples were incubated in the fridge for 10 minutes in the dark. EL4 cells were then centrifuged at 400 g for 5 min at 4°C and resuspended in 100 µl of PBA and centrifuged (400 x g for 5 min at 4 °C). The supernatant was removed and the cell pellet was resuspended in 20 µl PBA. For acquirement of flow data *Fortessa* was used and data analysis was performed with FlowJo software.

2.2.6 Protein quantification

For the quantification of the protein concentration of transfected cells, Bradford's reagent (Bradfords's Quick Start Kit 2, Bio Rad #5000202) was used. Protein standards were prepared according to a scheme of different dilutions. Samples were transferred into a 96-well plate and 100 µl Bradford reagent was added. After an incubation for 10 min at room temperature, samples were measured by a spectrophotometer (Infinite 200Pro) with absorbance at 595 nm. A standard curve was created by plotting the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). The unknown sample concentration was determined using the standard curve.

2.3 Molecular biological methods

2.3.1 Amplification of LAG-3, PD-1 and TIM-3 genome by quantitative polymerase chain reaction (qPCR)

Genomic DNA from EL4 cells was extracted by using DNA extraction buffer containing Proteinkinase K. Samples were put into the ThermoMixer® (Eppendorf) (1000 rpm for 5 min at 57°C).

Then, a washing step with Isopropanol (1mL) and Ethanol (2mL) followed, both with centrifugation of the cells after applying the solution (400 x g for 5 min at 57°C). Next, 1 ml of distilled water was added to each sample. The concentration of DNA was determined by measuring the optical density. The extracted DNA was used to amplify PDCD1, HAVCR2 and LAG-3 by gene specific primers and PCR. Table 5 lists components for amplification. To improve PCR amplification specificity, the touchdown PCR technique was performed. With this method, the initial annealing temperature was higher than the optimal Tm of the primers and was gradually reduced from 95°C to 65°C over 11 cycles. Subsequently, the PCR products were purified by using the QIAquick PCR Purification Kit (ID: 28104, Qiagen).

Component	*Amount per reaction*
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
Taq DNA-Polymerase-PCR-Buffer	5 µl
cDNA	Variable
dNTPs	2 µl
Taq Polymerase	0.8 µl
H_2O	To 50 µl

Table 5. Components and concentrations of the PCR mix for amplification of PDCD1, HAVCR2 and LAG-3

2.3.2 Western Blot

Samples of lysed protein were prepared aiming to have 10 – 30 µg protein/lane. 2 x Laemmli buffer (950 µl + 50 µl Mercaptoethanol) was added to each sample and the tubes were shortly spun. 7.5 µl protein molecular weight marker (Rainbow Marker, VWR, #RPN800EP) and the samples were applied on SDS-PAGE (Mini Protean® TXG Gels, Biorad). Then, the gel was run at 140 mA. Proteins were transferred from the gel to a nitrocellulose membrane using a Trans-Blot-TurboTM Transfer Pack (Biorad, #170-4156). The reaction was blocked by incubating the membranes for 30 min in 5% milk powder PBST. Subsequently, primary antibodies were applied using 1 % milk powder in TBST (see Table

2). Membranes were incubated overnight at 4°C and then washed with TBST (3 x 15 min). Next, secondary Antibody, anti-Rabbit-IgG (1:40000) was applied using 1 % milk powder in TBST. After 1 h of incubation another washing of the membranes followed (3 x 15 min). The membranes were then developed.

2.3.3 T7 Endonuclease Assay

For validation of genome editing, the T7 endonuclease I (T7EI) assay is a quick method. The assay is based on a family of mismatch-specific enzymes, the endonucleases, which recognize base substitutions, insertions and deletions. The method can be divided into three main steps: Amplification of the gene of interest by PCR, heteroduplex formation and T7EI digestion. For the heteroduplex formation, the components listed in Table 6 were mixed and incubated in a PCR thermocycler. Initially, the mixture was heated to 95°C to denature double strands and then slowly ramped to room temperature for rehybridization. Thereby, three possible products could be obtained: A wild-type-mutant heteroduplex product, a wild-type-wild-type homoduplex product and a mutant-mutant homoduplex product. Heteroduplex products are recognized and cleaved by T7 endonuclease I (T7EI, NEB). Therefore, 1 µl T7EI was added (final volume of 20 µl) to the mixture and incubated for 1 hour at 37°C to allow cleavage of mismatching DNA. Then, probes were loaded with loading dye (6x) and separated by agarose gel electrophoresis. The parenteral band of the positive control (600 bp) should have provided fragments of approximately 200 and 400 bp. By PCR reaction with specific LAG-3 primers (Table 4), the T7EI digestion of the transfected amplicon should have yielded fragments of approximately 160 bp in addition to the parental band (330 bp).

Component	*Stock concentration*	*Final concentration*
Buffer 2 (NEB)	10x	1x
PCR amplicon	Variable	200ng
H_2O	-	To 19 µl

Table 6. Components and concentrations used for T7E assay

2.3.4 Agarose gel electrophoresis

For the separation of PCR products by size, 1.5 % (w/v) agarose gels were used. Therefore, agarose was mixed with 1x TAE buffer and dissolved by a microwave. Then, ethidium bromide was added to the dissolved solution (1µg/ml) and transferred into a gel tray. A comb was used to create wells. After 30 minutes at room temperature, the gel was ready to use and the comb was removed. The gel tray was transferred into an electrophoresis apparatus and filled with 1x TAE buffer. Subsequently, the samples were mixed with loading dye (6x) and loaded into the wells. As a reference, a 1kb ladder (GeneRuler 1kp plus DNA ladder) was used. All gels were run at 100-120V for 30-60 minutes. Afterwards, the gel bands were visualized by UV light.

2.3.5 Sanger sequencing

The Sanger method was used for DNA sequencing via Eurofins Genomics. The samples were transported to their facilities with the aid of their proprietary PCR Plate “MixSeq2” service. They were premixed with the addition of their respective primer. For the sequencing reaction normal deoxynucleoside triphosphates (dNTPs) and modified dideoxynucleoside triphosphates (ddNTPs) were used for strand elongation. The modified and fluorescently labelled ddNTPs caused the DNA polymerase to stop the reaction whenever a ddNTP is incorporated. The resulting DNA fragments were separated by size through a capillary and the fluorescence of each molecule was detected. The emitted fluorescence signal from each excited fluorescent dye determines the identity of the nucleotide in the original DNA template.

3. Results

3.1 Phenotype of EL4 lymphoma cells and murine splenic T cells

The aim of the project was to investigate the possibility of a triple gene editing of PD-1, TIM-3 and LAG-3 via CRISPR/Cas9. Parts of the project have already been published (Ciraolo et al., 2022). To establish the model system, the EL4 lymphoma cell line was used. Initially, EL4 cells were analyzed for the expression of the immune checkpoint molecules, i.e., LAG-3, PD-1 and TIM-3. The aim was to verify

that such a cell line in some terms maintains some similarities to the murine splenic T cells in the phenotypic expression of cell surface markers (Ahmed and Smith, 1983; Gays et al., 2000). The antigen expression profiling was performed by comparing EL4 lymphoma cells and murine splenic T cells. Splenic T cells isolated from wild type mice were subjected to magnetic separation in order to obtain CD3+ T cells. EL4 and murine splenic T cells were therefore activated for 24 hours on anti-CD3/anti-CD28 coated plates, stained with specific fluorescent antibody against CD3 and CD44 antigen, and analyzed by flow cytometry. CD44 is overexpressed in several malignancies and seems to have an effect on the development of cancer (He et al., 2018; Ponta et al., 2003). In Figure 3A, representative dot plots of CD3 versus CD44 are shown. The percentage of $CD3+$ CD44+ cells was high in both populations, representing 99.2% of the murine splenic T cell population and 99.7% of the EL4 lymphoma cell population.

Subsequently, the expression level on the plasma membrane of LAG-3, PD-1 and TIM-3 was defined on EL4 and murine splenic T cells subjected to specific CD3/CD28 activation for 72 hours. EL4 and murine splenic T cells were then stained with anti CD3, LAG-3, PD-1 and TIM-3 fluorescent antibody, and analyzed through a flow cytometer. Gated on the CD3, the mean fluorescence intensity (MFI) was calculated. Figure 3B shows the distribution of the data. The data appears to be approximately normally distributed, hence we used a t-test to compare the mean difference in MFI between murine splenic T cells and EL4 cells for LAG-3, PD-1 and TIM-3. Flow cytometry analysis showed that after 72 h of stimulation with anti-CD3/anti-CD28 antibodies, both murine splenic T cells and EL4 cells expressed a comparable and significant amount of the LAG-3 and TIM-3 antigen if compared to unstimulated. We observed that EL4 showed a significant higher expression of PD-1 with respect to murine splenic T cells.

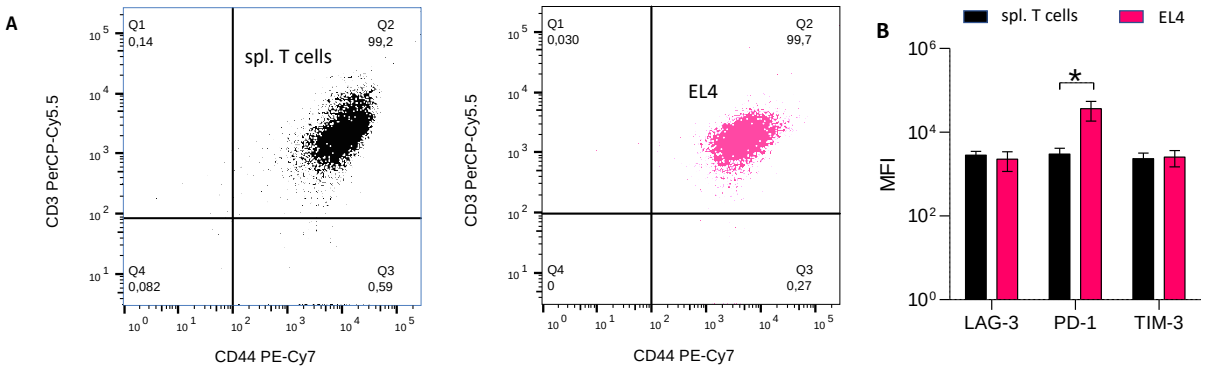

Figure 3. Comparative expression of LAG-3, PD-1 and TIM-3 on murine splenic T cells (spl. T cells) and EL4 cells. (A) The expression of CD3 against CD44 coreceptor was analyzed on murine splenic T cells (black) and EL4 cells (pink) to set up a gating strategy. The expression of the antigens was measured after 24 h stimulation with anti-CD3/anti-CD28 antibodies. Fluorochrome conjugates from the antibodies are shown in Table 3. (B) Mean Fluorescence Intensity (MFI) of LAG-3, PD-1 and TIM-3 in splenic T cells and EL4 cells after 72 h stimulation with anti-CD3/anti-CD28 antibodies. Bars are representative of three independent experiments with murine splenic T cells derived from three different animals. Statistical analysis: t-test. *$p < 0.05$

3.2 CRISPR/Cas9 gene editing of LAG-3, TIM-3 and PD-1

We took advantage of the CRISPR/Cas9 system to accomplish our principal aim of inducing a permanent disruption of LAG-3, PD-1 and TIM-3 expression in EL4 lymphoma cells. Therefore, a duplex preparation of crRNA and tracrRNA was performed, followed by precomplexing with the Cas9 endonuclease. Particular crRNA sequences were designed in order to bind a specific exon of the target gene. To mitigate the problem of accidental, nonspecific modifications due to the mismatch binding of crRNAs, poor efficiency for off-target binding and the cutting frequency determination (CFD) score were calculated, and only crRNA with less off-targets were selected for further experiments. Furthermore, PD1_cRNA was designed to bind the first exon, TIM3_crRNA the second and LAG3_crRNA the third exon (Table 7).

Gene	cRNA Name	RNA Sequence	PAM	Binding	CDF	Off-Targets
Pdcd1	PD1_crRNA1	ACAGCCCAAGTGAATGACCA	GGG	Exon 1	87	0-0-3-18-140
Lag-3	LAG3_crRNA3	ACCCGCACCCGGTCGCTACA	CGG	Exon 3	98	0-0-0-1-16
Havcr2	TIM3_crRNA2	ATGTGACTCTGGATGACCAT	GGG	Exon 2	80	0-0-1-14-133
-	trRNA	AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU	-	-	-	-

Table 7. List of crRNA used for CRISPR/Cas9 gene editing. PAM, protospacer adjacent motif, DNA region targeted for cleavage by the CRISPR system. CFD, cutting frequency determination score. It summarizes all off-targets into a number from 0 to 100, where a higher number corresponds to fewer off-targets, and vice versa. Off-target numbers represent the number of off-targets with 0, 1, 2, 3, 4, mismatches, respectively.

Once designed, the gene editing efficiency of each specific crRNA was tested experimentally. Therefore, the preassembled PD-1, LAG-3 and TIM-3 crRNA with fluorescent-labeled tracrRNA (tracrRNA-ATTO™550) were mixed together with Cas9 protein in order to form a crRNA/tracrRNA/Cas9 complex. All complexes were then transferred into EL4 lymphoma cells by electroporation. After transfection, EL4 cells were positively selected for ATTO through cell sorting in order to isolate the cells that had received the crRNA/tracrRNA/Cas9 complex. After sorting, EL4 cells were expanded for 48 hours to let them recover, and single cell clones were isolated by using limiting dilution in a 96-well plate. Every clone was in part frozen and in part lysed to extract the genomic DNA and proteins. Protein expression was analyzed through Western blot analysis and T7 endonuclease I assay. The presence of PD-1, LAG-3 and TIM-3 in EL4 protein lysates was measured via quantitative analysis related to the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Figure 4A). Positive clones for the genetic modification were selected only if the residual expression of each protein was lower than 30%. In order to verify the Western blot analysis, clones were analyzed via Sanger sequencing. Each sequence was compared with that relative to non-transfected clones. The analysis of the sequences showed that all clones with less than 30% of PD-1, LAG-3 and TIM-3 expression had DNA alteration in the region close to the PAM sequence (Figure 4B-D). In total, 15 clones with a reduction in the protein expression higher than 30% were identified. The Western blot analysis and

the Sanger sequencing were repeated several times on the selected clones. The results showed very good reproducibility.

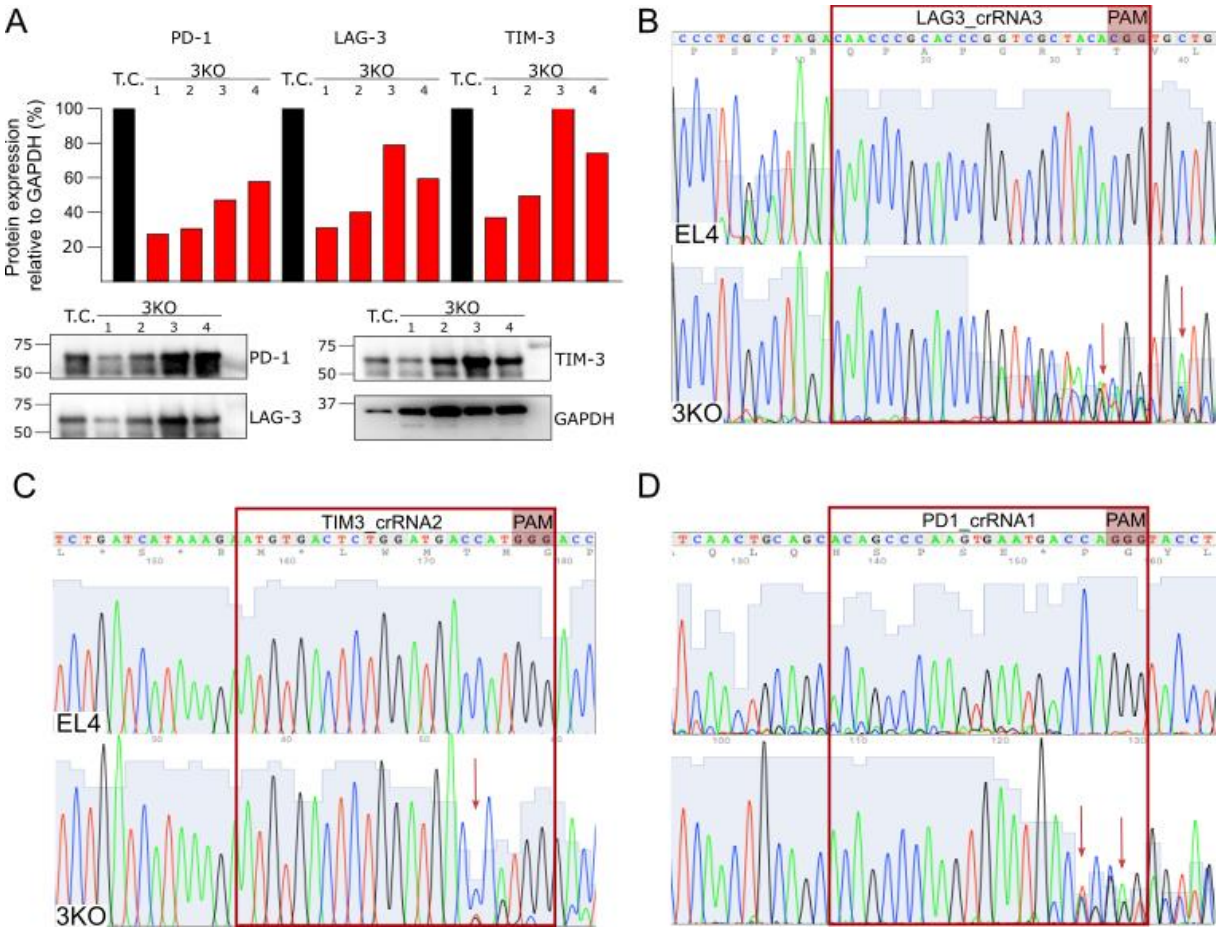

Figure 4. **Reduction in protein expression in clones positive for the CRISPR/Cas9-mediated gene modification of LAG-3, TIM-3 and PD-1**
(A) Western blot representing the expression of transfection control EL4 (T.C.) and EL4 single cell clones transfected with all three crRNA (3KO, clones 1-4) (B), (C) and (D). DNA sequence of LAG-3, TIM-3, and PD-1 on the annealing region of the crRNA of wild type (up) and transfected (low) EL4 cells. (Data shown here were also published in a peer-reviewed journal and the author of the present thesis is co-author of the published article (35328630).)

As mentioned above, the second approach, used to verify the efficiency of the CRISPR/Cas9 genome editing of LAG-3, was the T7 Endonuclease I (T7EI) assay. Regarding the LAG-3 genes, after positive digestion of the T7 Endonuclease, a fragment of 160 bp instead of 330 bp was expected in those clones positive selected for gene editing with Western blot analysis (Figure 5). However, no digestion product was identified in the clones 3KO 1 and 3KO 2 (Figure 5), as well as in the positive control provided with

the T7 Endonuclease I assay, which clarifies why this approach was not further pursued in following experiments.

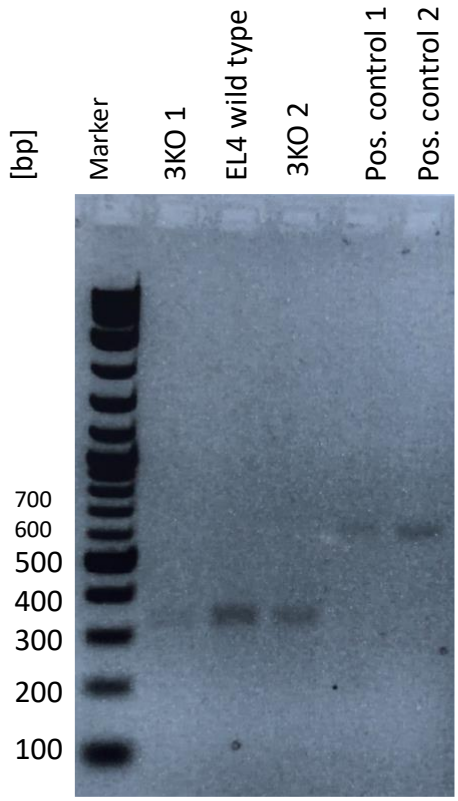

Figure 5. T7 Endonuclease I digestion
T7 endonuclease I digestion of PCR fragments from triple edited single cell clones and wild type EL4 representative of 15 independent experiments. Line 1: molecular weight marker; lane 2: tripled edited (3KO) EL4 cells, clone 1; lane 3: control wild-type EL4 cells; lane 4: 3KO EL4 cells, clone 2; lane 5 and lane 6: positive controls of the T7 endonuclease I digestion assay.

3.3 Effect of CRISPR/Cas9 genetic editing on LAG-3, PD-1 and TIM-3 expression in EL4 cells

We further analyzed the effect that the triple CRISPR/Cas9 editing had on the EL4 cell membrane expression profile of LAG-3, PD-1 and TIM-3. After activation for 72 hours on plate coated with anti-CD3/CD28 antibodies, the membrane expression of LAG-3, PD-1 and TIM-3 was assessed by flow cytometry analysis (Figure 6A). Transfection control (T.C.) and not transfected (N.T.) EL4 cells did not show difference in the plasma membrane expression of LAG-3, PD-1 and TIM-3. Conversely, 3KO EL4 cells showed only a comparable expression for LAG-3 and TIM-3, while they displayed a 30% reduction for PD-1. Subsequently, we wanted to analyze the effect of non-specific activation with anti-CD3/CD28 antibodies on the TIR expression of EL4 cells in comparison to the samples without

stimulation. Figure 6B shows the distribution of the data, which appears to be approximately normally distributed. We used ANOVA to compare the mean difference between not transfected EL4 cells (NT), transfection control EL4 cells (TC) and triple edited EL4 cells (3KO). In N.T. EL4 cells, stimulation with CD3/CD28 induced a slightly increased expression of LAG-3 and TIM-3, while no difference in PD-1 expression was identified after activation (Figure 6B). On the other hand, in 3KO EL4 cells TIR expression was not increased after activation of the CD3/CD28 antigen. However, the editing of PD-1 within the triple editing induced a strong reduction of the PD-1 signals if compared with the correspondent N.T. controls.

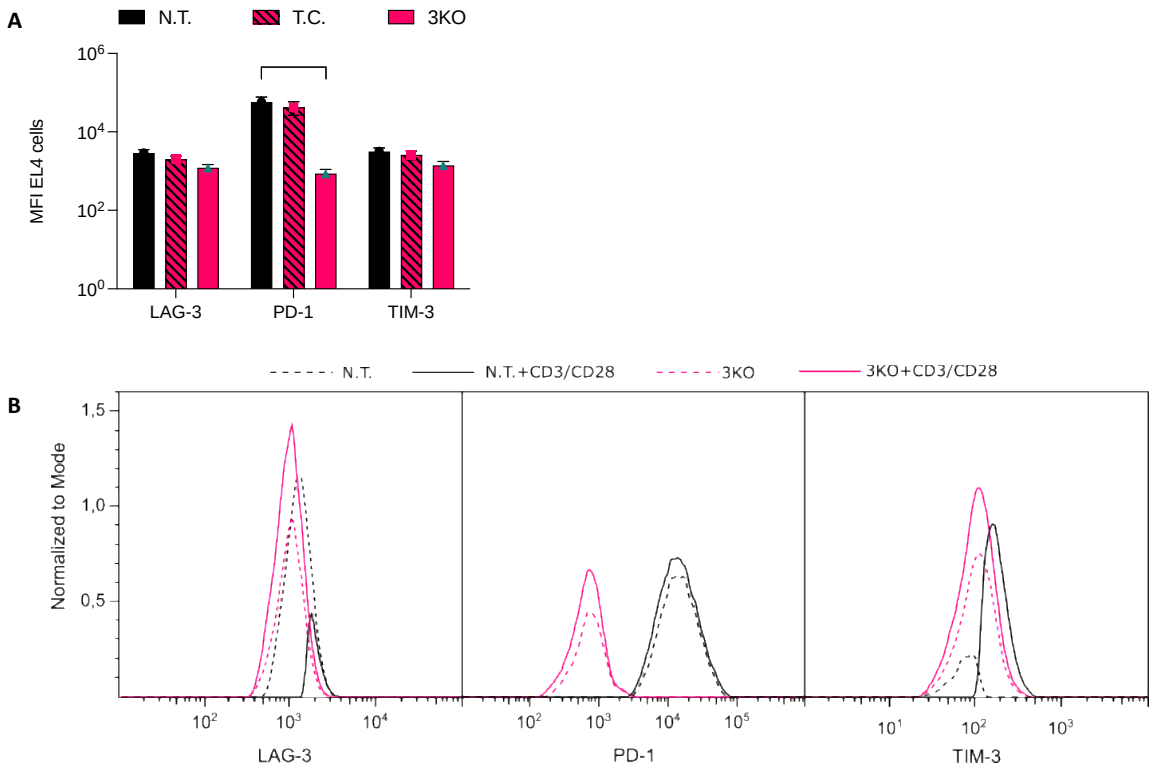

Figure 6. TIR expression on stimulated EL4 cells after CRISPR/Cas9-mediated triple-knockout of LAG-3, PD-1 and TIM-3.
(A) Mean Fluorescence Intensity (MFI) of LAG-3, PD-1 and TIM-3 in EL4 cells after 72 h stimulation with anti-CD3/CD28 antibodies. (B) Representative dot plot curves for LAG-3, PD-1 and TIM-3 intensity on 3 KO EL4 cells (pink) and N.T. EL4 cells (black) after 72 h stimulation with anti-CD3/CD28 antibodies, and on 3 KO EL4 cells (dotted pink) and N.T. EL4 cells (dotted black) with no stimulation. Bars are representative of two independent experiments. N.T., not transfected; T.C., transfection control; 3KO, Triple Knockout for PD-1, LAG-3 and TIM-3 genes; N.S., not stimulated. Statistical analysis: ANOVA * $p < 0.05$.

4. Discussion

4.1 EL4 lymphoma cell line constitutively expresses high levels of PD-1

Immune checkpoint receptors are cell-surface molecules which are capable of modulating the TCR signaling. After TCRs are triggered by specific antigen peptides presented on the surface of cells in a MHC context, immune checkpoint molecules synergize with TCR signaling and determine the T cells' fate. There are co-stimulatory and co-inhibitory molecules, and their exact function is often context dependent (Chen and Flies, 2013). In chronic infection or cancer, with prolonged exposure to antigen and inflammatory signals, the inhibitory signals induce a state of exhaustion, often characterized by an altered transcriptional status and plasma membrane expression of multiple inhibitory receptors. In cancer, tumor cells likely act as antigen presenting cells by interacting with the T cells and triggering T cell exhaustion. Such an immune inhibitory mechanism has represented a promising target for the final aim of reactivating the immunosuppressed anticancer immune reaction (Wang et al., 2019). Over the last few decades, monoclonal antibody (mAb)-based immune checkpoint blockade (ICB) has proven to be effective in malignancies (Wang et al., 2019). Despite the success and benefits emerging from ICB, the main problem of this approach is the immunotherapy-associated toxicity (Brahmer et al., 2012; Spain et al., 2016). In order to mitigate immunotherapy-associated toxicity while maintaining therapeutic efficacy, we investigated the feasibility of disrupting the expression of immune checkpoint molecules by genetic modifications in combination with ATT. The focus of most preclinical studies has only been on single knockout of TIRs. In some studies, it was shown that T cells which were only edited for one immune checkpoint inhibitor were prone to T cell exhaustion and did not have a long life span (Odorizzi et al., 2015). Therefore, we performed a CRISPR/Cas9-mediated knockout of PD-1, LAG-3 and TIM-3 to investigate the feasibility of the genetic editing of TIRs as major players in the inhibition of T cell activity (Davoodzadeh Gholami et al., 2017). Due to the complexity of transfection and gene editing of primary murine T cells, we were looking for a comparable cell line with similar phenotypical characteristics. We tested our CRISPR/Cas9 approach on the T cell lymphoma cell line EL4. This cell line has remained very popular in immunological research, due to the ability of the cells to maintain a

stable phenotype after several cycles of manipulation (Logan et al., 2004). The direct comparison of CD3/CD44 expression on murine splenic T cells versus EL4 lymphoma cells showed a similar expression pattern. CD3 is part of the TCR and it is involved in activating both $CD4^+$ and $CD8^+$ T cells (Murphy et al., 2018), while CD44 is responsible for enhancing T cell receptor signaling and functions as co-receptor in the T cell activation (J. Schumann et al., 2015). Our flowcytometric analysis demonstrated that EL4 lymphoma cells show positive membrane staining of CD3, CD44, PD-1, LAG-3 and TIM-3, thus suggesting their ability to be activated through the anti-CD3/CD28 antibodies. In previous studies, it was already shown that EL4 cells express CD4, a molecule which functions as coreceptor with the T cell receptor (Chen et al., 1994; Logan et al., 2004). Regarding immune checkpoint inhibitors, studies have demonstrated the expression of CTLA4 on EL4 lymphoma cells (Chen et al., 1994). Furthermore, a study on the anti-tumor efficacy of PD-1/PD-L1 checkpoint blockade was carried out on EL4 cells (Grauers Wiktorin et al., 2019), (Rong et al., 2021), (Taylor and Rudd, 2017). Another study demonstrated the sensitivity of EL4 lymphoma cells towards galectin-9 or anti-TIM-3 blocking antibodies (Nakajima et al., 2019). The results from our flow cytometry analysis and gene sequencing confirmed the expression of PD-1 and TIM-3 on EL4 lymphoma cells and additionally showed the expression of LAG-3. It has already been reported in the literature that PD-1 is constitutively expressed at high levels on EL4 lymphoma cells and is upregulated on CD8 T cells after stimulation (Oestreich et al., 2008). In our analysis, EL4 showed a significant higher expression of PD-1 with respect to splenic murine T cells in resting condition. Resting T cells did not show expression of PD-1 (Jin et al., 2010), whereas EL4 lymphoma cells constitutively expressed PD-1 on the plasma membrane. Furthermore, in a study where the expression of PD-L1 in three natural killer/T cell lymphoma (NKTL) cell lines was evaluated, it was found that the PD-L1 protein was highly expressed in these lines in comparison to Raji cells. They also supposed a correlation between the expression of PD-L1 and NKTL prognosis (Xue et al., 2019). Since EL4 cells belong also to the lymphoma cell line, the high expression of PD-1 might be characteristic for the aggressivity and self-defence of lymphoma cell lines. Other studies showed that PD-1 Is

constitutively expressed in T cells which exhibit an exhausted phenotype, such as HIV-specific CD8 T cells (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006).

4.2 Simultaneous targeting of multiple immune checkpoint inhibitors is essential for the efficacy of the gene editing

It was shown that the number of antigens which are expressed by tumor cells correlate with the severity of T cell exhaustion (Wherry and Kurachi, 2015) and at the same time numerous evidence suggests that it might be not enough to target a single co-inhibitory receptor (Huang et al., 2017; Riaz et al., 2017). Animal models have already shown that a dual genetic knockout of LAG-3 and PD-1 is more effective in preventing tumor progression and improving prognosis (Woo et al., 2012). In this study, we therefore aimed for a triple knockout of PD-1, TIM-3 and LAG-3 through the CRISPR-Cas9 strategy. The use of the CRISPR/Cas9 in T cell gene editing has been already described in the literature (Liu et al., 2017; Ren et al., 2017; Rupp et al., 2017; Zhang et al., 2017), where CRISPR/Cas9-mediated knockout of T cell inhibitory molecules (TIRs) such as PD-1, CTLA-4, LAG-3 or TIM-3, in combination with CD19-directed CAR T cells (CART19) showed feasibility. In addition, other studies investigated the efficacy of CRIPR/Cas9-mediated knockout of PD-1 in T cells. CRISPR-Cas9 has recently been used to genetically edit primary human T cells by reducing PD-1 expression via CRISPR-mediated indel mutations (K. Schumann et al., 2015). In another study by Su et al., it was demonstrated that precise knockout of the PD-1 gene via electroporation of plasmid-encoded sgRNA and Cas9 into human T cells led to significantly decreased PD-1 expression (Su et al., 2016). Thus, T cell immune responses to cancer cells and their antitumor activity was improved and it was demonstrated that CRISPR/Cas9 may represent a promising approach for efficient checkpoint inhibitor disruption in T cells. CRISPR/Cas9-mediated genome editing technology has attracted the attention of many other researchers in the treatment of cancer as it has shown great promise and has many advantages (Chen et al., 2019). Firstly, compared to the extensively investigated antibody-based immune checkpoint blockade, which showed adverse events resulting from unintended effects of the activation of the immune system, mediated by the immune checkpoint inhibitors (D. Y. Wang et al., 2018), genome editing might be able to

mitigate these autoimmune toxicities. Furthermore, compared to first generation gene disruption techniques using zinc-finger nuclease (ZFNs) and transcription activator like effector nucleases (TALENs), CRISPR/Cas9-mediated genome editing shows more efficiency and flexibility due to its easier and more precise Watson-Crick base pairing between sgRNA and target DNA (Hsu et al., 2014). In addition, the simultaneous expression of different sgRNAs within the CRISPR-Cas9 system opens up the possibility of multiple double-strand breaks (DSBs), and due to simple alterations of the sgRNA sequence, new DNA sequences can be easily retargeted by the CRISPR-Cas9 system (Cong et al., 2013). Another advantage of CRISPR/Cas9 genome editing is the ability to inhibit or activate the target gene transcription by specifically targeting transcriptional repressors (CRISPRi) and activators (CRISPRa) (Gilbert et al., 2014). This enables the modulation of gene expression and improves the gene modification in T cell-based cancer therapies. Moreover, we decided to use electroporation, a non-viral mediated CRISPR-Cas9 genome editing method, for the disruption of PD-1, TIM-3 and LAG-3, as the delivery of genetic agents using for example lentivirus is labor-intensive and might thereby limit their use in the clinic. Furthermore, the nucleofection method has shown higher transfection efficacy and better cell viability (Chicaybam et al., 2013), which plays a crucial role in successful genome editing and can be a limiting factor of CRISPR/Cas9 genome editing (Su et al., 2016).

4.3 Triple edited clones showed decreased protein expression of targeted immune checkpoint molecules

Western blot analysis has represented a valuable tool to select and isolate positive clones for a successful genetic editing. A 30% residual expression of the protein was set as threshold. Less than 30% leads to a not functional phenotype and it indicates genetic editing in both allele of the gene. However, any EL4 clone with no expression was identified, suggesting an ineffective cloning strategy or the presence of signal background due to the primary antibody. Surprisingly, the percentage of triple edited clones was quite low. Only around 4% of the final selection of clones showed a simultaneous reduction in PD-1, LAG-3 and TIM-3. This emphasizes the challenge of targeting multiple inhibitory receptors at once. It is one of the main aims of gene therapy to achieve a simultaneous knockout of

multiple inhibitory checkpoint molecules, which is difficult using immune checkpoint blockade treatment due to the upregulation of the molecules among themselves (Topalian et al., 2015) (Riaz et al., 2017) (Huang et al., 2017). Our low percentage of triple edited clones might be due to the fact that we did not indicate a genetic editing in both allele of all three genes and therefore did not achieve a null allele in all of the three genes. Furthermore, the more sgRNAs are present in the cell at a given time, the more challenging it is to predict the exact editing outcome. It is also harder to account for all possible fragment deletions or translocations that could result from triple cutting by any given three guide RNAs (gRNAs). An approach to improve the problem of multiplex targeting of genes may be performing sequential editing. Another technique for improving our results could be to perform one edit at a time, select the edited clones and perform several transfection rounds (CRISPR 101 2nd Ed Final May 2018_2.pdf, n.d.). Thus, the efficacy of simultaneous gene editing might be improved. This will depend highly on the cell line used as well as transfection associated cytotoxicity.

4.4 Sequencing analysis of triple edited cells revealed genetic chimerism

In order to have a final proof of the gene editing, the nucleotide sequencing analysis of the CRISPR/Cas9 edited EL4 cells showed a mixture of different sequence profiles. This genetic chimerism may be what usually occurs when mutating a gene of interest using the CRISPR/Cas9 method. It could be explained by a biallelism of the target locus due to the intrinsic activity of the repair machinery, which acts differently on different alleles (Jang et al., 2016). This means that mutations induced might not be the same in the two alleles, which makes it hard to detect a clear mutation pattern. This effect of CRISPR/Cas9-mediated genome editing has already been described in several studies. In animal models, the CRISPR/Cas9 system was used to generate mono- and bi-allelic null mutations in the gene locus for *Tyrosinase*, an enzyme for the production of melanin pigmentation. Besides complete albino mice carrying two different Tyr mutations, mice with pigmentation mosaics and fully pigmented mice also occurred. Deep sequencing demonstrated that the majority of the albinos and the mosaics had more than two new mutant Tyr alleles, thus revealing a somatic mosaicism and allele complexity for CRISPR/Cas9-targeted genes (Yen et al., 2014). Another study using sgRNAs targeting the *Mecp2* gene

in mouse models revealed mosaicism in 17% to 40% of the genetic sequencing of targeted mice (Yang et al., 2013). Usually, the Cas9 nuclease cleaves the DNA, thus generating a double strand break. Subsequently, the cellular DNA repair machinery takes over by repairing the double break, through a non-homologous end joining (NHEJ) or a homology-directed repair (HDR) pathway, which then leads to modifications in the targeted genes (Hille and Charpentier, 2016). However, the CRISPR-Cas nucleases can possibly cleave off-target at non-specific gene loci and therefore produce undesired mutations which reduce the accuracy of CRISPR-Cas9 genome editing (Chen et al., 2019). Recently, two studies reported a high off-target mutation rate in CRISPR/Cas9 transfected human cell lines. Fu et al. showed that off-target mutagenesis can be induced by CRISPR RNA-guided endonucleases (RGENs) in three different human cell types (Fu et al., 2013). Considering this problem, we calculated the CFD and only crRNA with less off-targets were selected for further experiments, though off-target modifications might have occurred leading to sequences with no clear mutation patterns.

4.5 Phenotypic analyses showed strong reduction for PD-1 signals in triple edited EL4 cells

One approach to prove the successful triple editing of the EL4 cells has been the T7 Endonuclease I assay. The assay is based on a family of mismatch-specific enzymes, the endonucleases, which recognize base substitutions, insertions and deletions. The specific CRISPR/Cas9 target region was amplified by PCR and the PCR products were purified and subjected to denaturation and annealing in order to form heteroduplex formation between wild type and mutated amplicons in transfected clones. DNA heteroduplexes were subsequently digested with T7 Endonuclease, which cleaved non-perfectly matched DNA duplexes. The digestion products were subsequently separated and analyzed by electrophoresis on agarose gel. The T7 Endonuclease is able to discriminate between homoduplex and heteroduplex dsDNA and to detect a mutant allele in a DNA mixture with high sensitivity (Vouillot et al., 2015). Furthermore, it is easily practicable and cost effective. Unfortunately, it was not possible to show cleaved fragments of LAG-3 genome. By using T7 Endonuclease I, it is difficult to genotype compound heterozygotes (Kc et al., 2016), which might emerge from biallelism of the target locus. As mentioned above, genetic chimerism may occur when mutating a gene of interest using the

CRISPR/Cas9 method. Due to the intrinsic activity of the repair machinery, which acts differently on different alleles, the mutations induced might not be the same in two alleles, also known as biallelism (Jang et al., 2016). Therefore, it is crucial to consider the extent of polymorphisms on the DNA region amplified by PCR, as it affects the efficacy of the T7 Endonuclease I assay (Vouillot et al., 2015). On the other hand, the use of proofreading polymerases, as suggested in the data sheet of the assay, could generate recombination events during the amplification, which lead to correction or partial correction of the mutation (Judo et al., 1998; Lahr and Katz, 2009; Vouillot et al., 2015).

The efficacy of the multiple CRISPR/Cas9 editing strategy was also tested by phenotypic analysis. The editing of PD-1 in the triple editing approach induced a strong reduction of the PD-1 signals in comparison to the corresponding N.T. controls. The decrease of LAG-3 and TIM-3 signals on the cell surface of triple edited EL4 cells was only limited. Furthermore, we wanted to analyze the effect of non-specific activation with anti-CD3/CD28 antibodies on the TIR expression of EL4 cells in comparison to the samples without stimulation. Surprisingly, there was no significant induction of TIR expression on EL4 cells simulated with CD3/CD28 antigen, either in the not transfected EL4 cells or the triple edited EL4 cells. Therefore, we cannot testify to the sensitivity of the EL4 cells towards activation, which might have provided us insight into the cells' phenotype in a stimulating environment. Intrinsic pathways that modulate the expression of the genes need to be further investigated, so as to completely understand the interaction between the genetic disruption and the expression profile. In a study which investigated the anti-tumor efficacy of LAG-3 edited CAR-T cells, no significant anti-tumor activity *in vitro* and *in vivo* was reported. This shows that genetic disruption of a checkpoint inhibitor does not necessary imply changes in the cells' anti-tumor potency (Zhang et al., 2017).

4.6 Conclusion and Outlook

In conclusion, we investigated the feasibility of genetic editing of the TIRs PD-1, LAG-3 and TIM-3, which play a significant role in the inhibition of T cell activity. After we initially defined the phenotype of our cell model, the T cell lymphoma cell line EL4, we tested our CRISPR/Cas9 approach for the simultaneous editing of PD-1, LAG-3 and TIM-3 on the EL4 cells. Sequence analysis of the clones

identified with reduced TIR expression revealed the expected genetic modifications in the area of the Cas9-specific cleavage site (PAM). The efficacy of the CRISPR/Cas9 triple gene editing strategy was furthermore tested by phenotypic analysis and showed significantly reduced expression of PD-1 on the plasma membrane of EL4 cells. Subsequently, we performed single cell cloning of cells that showed significant reduction in the protein expression. However, we were only able to create a few clones with genetic disruption of all three TIRs: PD-1, LAG-3 and TIM-3. Nevertheless, we have developed a temporary concept of CRISPR/Cas9-mediated triple gene editing of TIRs on EL4 lymphoma cells which can be transferred to patients undergoing T cell therapy.

Clearly, the feasibility of successfully performing triple gene editing on T cells for therapeutical approaches needs to be further explored in the future. The development of immune-oncology drugs has undergone years of improvement. Their development started in 2011 with the anti-CTLA4 monoclonal antibody, followed by the PD-1/PD-L1-blocking antibodies (Hoos, 2016). Eventually, more complex cellular and genetic therapies emerged in the form of CAR-Ts and TCR-transduced T cells (Gill and June, 2015; Restifo et al., 2012). Despite the success of these first- or second-generation treatments, most cancer patients still do not benefit from these treatments (Hoos, 2016). One of the new technologies in immuno-oncology is the engineering of several genes to modulate cell functions (Hoos, 2016). Recently, a study showed that CRISPR-Cas9 genome editing of primary human T cells led to a reduction in PD-1 expression by CRISPR-mediated indel mutations (K. Schumann et al., 2015). Another study tested the feasibility of disrupting LAG-3 expression in human primary T cells and CAR-T cells using the CRISPR/Cas9 system and showed that edited cells kept their functionality (Zhang et al., 2017). Studies like these emphasize the importance of fundamental research on TIRs as potential targets for gene editing, so that they might be able to cure cancer or turn it into a controllable chronic disease one day.

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6. Eidesstattliche Versicherung

„Ich, Miriam Pühl, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: ‚Multiple Gene Editing of T Cell Inhibitory Receptors via CRISPR/Cas9 in EL4 cells' / „Mehrfache genetische Editierung von inhibitorischen T-Zell Rezeptoren mittels CRISPR/Cas9 bei EL4 Zellen" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.
Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.
Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst."

Datum Unterschrift

7. Anteilserklärung an etwaigen erfolgten Publikationen

Pühl Miriam hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Ciraolo, E.; Althoff, S.; Ruß, J.; Rosnev, S.; Butze, M.; Pühl, M.; Frentsch, M.; Bullinger, L.; Na, I-K. Simultaneous Genetic Ablation of PD-1, LAG-3, and TIM-3 in CD8 T Cells Delays Tumor Growth and Improves Survival Outcome. *Int. J. Mol. Sci*. **2022**, 23, 3207. https://doi.org/10.3390/ijms23063207

Beitrag im Einzelnen

Meine Beiträge zur oben genannten Publikation beziehen sich auf den ersten Unterpunkt des Ergebnisteils ‚CRISPR/Cas9 Gene Editing Induces Reduction in PD-1, LAG-3 and TIM-3 Expression'. Ich übernahm die Aufgabe, passende Primer für die Amplifikation des LAG-3 Genoms zu finden. Somit trug ich zur Vervollständigung der Liste aller für das CRISPR/Cas9 Gene Editing benötigten crRNA Sequenzen bei, siehe Tabelle 1. Des weiteren quantifizierte ich die Proteinexpression auf erfolgreich CRISPR/Cas9 editieren Zellen mittels Western Blot Assays. Die statistischen Auswertungen dieser Ergebnisse sind der Publikation als Supplementary Material (S1) angefügt. Außerdem absolvierte ich einen Kurs für Tierversuche mit Mäusen und konnte nach erfolgreichem Abschluss eigenständig murine T Zellen aus der Milz von Mäusen extrahieren und kultivieren, die für die weiterführenden Experimente der Publikation benötigt wurden.

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Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

8. Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

9. Liste der Publikationen

Ciraolo, E.; Althoff, S.; Ruß, J.; Rosnev, S.; Butze, M.; Pühl, M.; Frentsch, M.; Bullinger, L.; Na, I-K. **Simultaneous Genetic Ablation of PD-1, LAG-3, and TIM-3 in CD8 T Cells Delays Tumor Growth and Improves Survival Outcome**. *Int. J. Mol. Sci*. **2022**, 23, 3207. https:/ / doi.org/10.3390/ijms23063207 “.

10. Danksagung

An dieser Stelle möchte ich allen meinen großen Dank aussprechen, die mich bei der Anfertigung meiner Doktorarbeit unterstützt haben.

Mein besonderer Dank gilt zunächst meiner Doktormutter Prof. Il-Kang Na, die mir die Möglichkeit gegeben hat, diese Arbeit unter ihrer Leitung durchzuführen und mich bei der Umsetzung der gesamten Arbeit hervorragend betreut hat.

Für konstruktive Anregungen und vielfältige Unterstützung danke ich ebenso Frau Dr. Elisa Ciraolo, die mir stets für jegliche Fragen und Anliegen zur Seite stand.

Meiner gesamten Arbeitsgruppe danke ich für die freundschaftliche Arbeitsatmosphäre und stete Hilfsbereitschaft. Insbesondere möchte ich Dr. Stefanie Althoff danken, die mich anfangs in die Arbeitsgruppe einführte und mich bis zum Ende hin mit zahlreichen fruchtbaren Gesprächen, Anregungen und mühevollen Korrekturlesen unterstützte.

Mein besonderer Dank gilt schließlich meinen Freunden, die meine Arbeit mit großem Engagement unterstützt haben und mich bei allen Zweifeln und Schwierigkeiten immer wieder ermutigten. Besonders danken möchte ich meinem Freund Christian, der mit emotionalem Support und Fürsorge immer für mich da war und an mich glaubte. Schließlich danke ich meinen Eltern für ihre uneingeschränkte, liebevolle und vielseitige Unterstützung während meines gesamten Studiums, ohne die diese Arbeit so nicht möglich gewesen wäre.

11. Statistische Bescheinigung

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Bescheinigung

Hiermit bescheinige ich, dass Frau Miriam Pühl innerhalb der Service Unit Biometrie des Instituts für Biometrie und klinische Epidemiologie (iBikE) bei mir eine statistische Beratung zu einem Promotionsvorhaben wahrgenommen hat. Folgende Beratungstermine wurden wahrgenommen:

- Termin 1: 08.11.2021

Folgende wesentliche Ratschläge hinsichtlich einer sinnvollen Auswertung und Interpretation der Daten wurden während der Beratung erteilt:

- Darstellung deskriptiver Statistiken
- Beschreibung der gewählten statistischen Verfahren

Diese Bescheinigung garantiert nicht die richtige Umsetzung der in der Beratung gemachten Vorschläge, die korrekte Durchführung der empfohlenen statistischen Verfahren und die richtige Darstellung und Interpretation der Ergebnisse. Die Verantwortung hierfür obliegt allein dem Promovierenden. Das Institut für Biometrie und klinische Epidemiologie übernimmt hierfür keine Haftung.

[redacted]

Unterschrift

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