Aus dem Institut der pädiatrischen Hämatologie und Onkologie
Institut der Universität München

Vorstand: Prof. Dr. med. Dr. sci. nat. Christoph Klein

CRISPR/Cas9-mediated Generation and Characterization of Glucocorticoid-resistant virus-specific T cells

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Larissa Deisenberger
aus Starnberg

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Dekan:	Prof. Dr. med. Thomas Gudermann
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SUMMARY

Patients after organ transplantation or hematopoietic stem cell transplantation suffer from impaired T-cell function and are at high risk for viral infections, such as cytomegalovirus (CMV) infections. Adoptive T-cell transfer is a way to restore T-cell immunity, by infusing enriched virus-specific donor T cells into the patient. Over the past years it has been shown that this approach can be applied successfully. However, many of these patients need to receive high-dose glucocorticoid therapy, impairing proliferation capacity of adoptively transferred virus-specific T cells by binding to their glucocorticoid receptor (GR).

The most definite way of preventing the adverse effects of glucocorticoids is the genetic knockout of the endogenous GR in virus-specific T cells. To this end, CMV-specific T cells were enriched *via* cytokine capture technique, an IFN-γ-based enrichment method, and the GR was knocked out *via* CRISPR/Cas9. Successful enrichment of CMV-specific T cells, as well as high knock-out rates of the GR in enriched cells were demonstrated. The T-cell product showed a high purity, a stable phenotype eligible for adoptive T-cell therapy and a high cell viability. Further, not only glucocorticoid resistance, but also sensitivity for cyclosporin A were confirmed, providing a rescue treatment concerning safety issues *in vivo*. Glucocorticoid-resistant CMV-specific T cells were functional in terms of IFN-γ secretion upon specific restimulation, proliferation and specific target cell killing *in vitro*. The developed automatable engineering strategy will allow straightforward upgrading to a good manufacturing practice-conform generation of the cell product.

This innovative protocol lays the foundation for a widely applicable T-cell product to treat refractory viral infections in patients needing glucocorticoid therapy.

ZUSAMMENFASSUNG

Bis zur Rekonstitution der T-Zell Immunität birgt eine Organtransplantation oder Stammzelltransplantation das Risiko für virale Infektionen, darunter oftmals Infektionen mit dem Zytomegalie-Virus (CMV). Ein adoptiver T-Zell Transfer ermöglicht, die T-Zell Immunität des Patienten mit Hilfe von virus-spezifischen Spender-Zellen wiederherzustellen. Dieser Therapieansatz wird seit Jahren erfolgreich angewandt. Viele dieser Patienten benötigen jedoch Glukokortikoide in hoher Dosierung, die durch Bindung an den Glukokortikoid-Rezeptor (GR) ebenso die im Rahmen eines adoptiven T-Zelltransfers infundierten, virus-spezifischen T-Zellen in ihrer Proliferation hemmen.

Als eindeutigster Lösungsansatz zur Verhinderung dieser unerwünschten Wirkung ergibt sich der genetische Knock-out des GR in virus-spezifischen T-Zellen. Mittels „Cytokine Capture“ Technik, einer IFN-γ basierten Anreicherungsmethode, und dem „CRISPR/Cas9“-System wurden glukokortikoid-resistente CMV-spezifische T-Zellen hergestellt. CMV-spezifische T-Zellen wurden erfolgreich angereichert und der Knock-out des GR war in den angereicherten Zellen mit hoher Effizienz möglich. Das entwickelte T-Zellprodukt zeichnete sich durch eine hohe Reinheit an T-Zellen, einen stabilen, für den adoptiven T-Zell Transfer geeigneten Phänotyp und eine hohe Lebensfähigkeit der Zellen aus. Nicht nur die Glukokortikoid-Resistenz, sondern auch ein Ansprechen auf Cyclosporin A, das im klinischen Notfall einen Sicherheitsmechanismus zur Hemmung der Zellen darstellt, wurden experimentell bestätigt. Glukokortikoid-resistente CMV-spezifische T-Zellen waren funktionell hinsichtlich ihrer IFN-γ Sekretion nach Restimulation, ihrer Proliferation und ihrer spezifischen zytotoxischen Aktivität gegen Zielzellen *in vitro*. Der entwickelte automatisierbare Ansatz zur Herstellung eines gentechnisch veränderten T-Zellprodukts ermöglicht eine schnelle Umsetzung in die gute Herstellungspraxis-konforme Produktion.

Dieses innovative Protokoll legt den Grundstein für ein breit anwendbares T-Zell Produkt für die Behandlung von Patienten mit refraktären viralen Infektionen und notwendiger Glukokortikoid-Therapie.

TABLE OF FIGURES

ABBREVIATIONS

Abbreviation	Explanation
AdV	Adenovirus
AF-1	Activator function 1
APC	Antigen presentic cell
BCA	Bicinchoninic acid
Cas9	CRISPR associated 9
CD	Cluster of differentiation
CMV	Cytomegalovirus
CRISPR	Clustered regular interspaced short palindromic repeats
crRNA	CRISPR RNA
CC	Cytokine capture technique
CsA	Cyclosporin A
CTV	Cell Trace Violet
Dex.	Dexamethasone
DNA	Desoxyribonucleic acid OR in figure: DNA binding domain
DSB	Double strand break
E:T	Effector to target
EBV	Epstein-Barr virus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoids
GMP	Good manufacturing practice
GR	Glucocorticoid receptor
gRNA	guideRNA
GvHD	Graft versus Host Disease
HD	Hinge domain
HDR	Homology directed repair
HHV-5	Human Herpes virus 5
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HSA	Human serum albumin
HSCT	Hematopoietic stem cell transplantation
ICS	Intracellular cytokine staining
IF	Immunofluorescence microscopy
IFN-γ	Interferon gamma
IL	Interleukin
InDel	Insertions and deletions
KO	Knock out
LBD	Ligand binding domain
MHC	Major histocompatibility complex
Neg.	Negative
NFAT	Nuclear factor of activated T cells
NHEJ	Non homologous end joining
NK	Natural killer
NTD	N-terminal domain

Orig.	Original
PAM	Protospacer adjacent motif
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
Pos.	Positive
RIC	Reduced intensity condition
RNP	Ribonucleoprotein
SCT	Stem Cell Transplantation
SD	Standard deviation
SEB	Staphylococcus enterotoxin B
sgRNA	Single guide RNA
TALEN	Transcription activator-like effector nucleases
Tcm	Central memory T cells
TCR	T-cell receptor
Teff	Effector T cells
Tem	Effector-memory T cells
TIDE	Tracking of InDels by Decomposition
Tn	Naïve T cells
TNF-α	Tumor necrose factor α
tracrRNA	Trans-activating crRNA
Tscm	Stemcell-like T cells
Unstim.	Unstimulated
UT	Untreated
WB	Western Blot
ZFN	Zinc finger nuclease

PUBLICATION

The present work contains already published data, which is marked with the following reference.

Kaeuferle T., **Deisenberger L.**, Jablonowski L., Stief T. A., Blaeschke F., Willier S., and Feuchtinger T. CRISPR-Cas9-Mediated Glucocorticoid Resistance in Virus-Specific T Cells for Adoptive T Cell Therapy Posttransplantation. *Mol Ther* **28**, 1965-1973 (2020).[1]

All required guidelines were followed strictly (to be read at https://www.med.uni-muenchen.de/promotion/downloads/monographie_publ_daten_021220/index.html).

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A. INTRODUCTION

There are various clinical situations in which patients may suffer from refractory viral infections while in need of glucocorticoid therapy, among them solid organ transplantation or graft versus host disease after hematopoietic stem cell transplantation. In the present work hematopoietic stem cell transplantation is introduced as an example.

1. RISKS AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

For nearly half a century hematopoietic stem cell transplantation (HSCT) is a curative treatment for both non-malignant and malignant diseases of the hematopoietic system.[2] The aim of this treatment is to create a recipient-donor chimerism to cure the primary disease, prevent relapses and enable normal hematopoietic and immune function in the patient.[3] To this end, stem cells of either HLA-matched related or unrelated donors or of haploidentical donors are used.[4] To prepare the patients for the HSCT, their T cells are eliminated using various conditioning regimen. Intense schemes include total body irradiation, cytostatic drugs and anti-thymocyte globulin, whereas reduced intensity conditioning (RIC) consists mainly of cytostatic drugs and an anti-CD3 monoclonal antibody.[5] As grafts highly purified CD34+ progenitor cells or CD3/CD19 depleted peripheral blood stem cells are used.[5,6] Immune reconstitution after HSCT takes at least 3 – 6 months. The normalization of CD4:CD8 ratio may even take more than a year.[7,8] This immune deficiency leads to high risk of infections.[9] Infection is the main non-relapse cause of death with 5%- 18% cumulative incidence.[2,8] Among infectious deaths viral infections, which are mainly caused by viral reactivation, play a big role.[2,10] Another important cause of death is graft versus host disease (GvHD). Infection and GvHD contribute highly to the mortality after HSCT.[2]

1.1 ACUTE GRAFT VERSUS HOST DISEASE

Acute GvHD is a systemic inflammatory disease, where donor's T cells attack the tissue of immunodeficient recipients. Patients receiving leukocytes for transplantation are at highest risk for developing this reaction and there is a direct relation between the incidence of GvHD and the degree of HLA mismatch. According to onset, acute GvHD is classified into acute (prior to day 100 *post*-SCT) and late onset acute GvHD (after day 100).[11] The most frequent manifestations are skin, gastrointestinal tract and liver.[12] Acute GvHD (≥°II) occurs in 10% to over 50% of transplanted patients dependent on donor, graft, prophylaxis and various other

factors.[5,6,13] To prevent GvHD cyclosporin A is often used as prophylaxis.[4,13] Lower incidences for GvHD, even without prophylaxis, are reported using purified $CD34^+$ stem cells, but the T-cell reconstitution is delayed in comparison to CD3/CD19 depleted grafts.[5,6] Acute GvHD can be staged in grades I to IV, whereby severe GvHD (grade III and IV) has a very poor prognosis with 25% and 5% long-term survival, respectively.[11] The gold-standard treatment for acute GvHD (except for skin manifestation, grade I) are systemic high-dose steroids. Unfortunately, steroid treatment is reported to be the main risk factor for cytomegalovirus (CMV) infection.[11,13,14] and patients with severe acute GvHD have an over 80% risk of a recurring rising CMV load in the first year after SCT.[15]

1.2 CYTOMEGALOVIRUS (CMV) INFECTION

The human cytomegalovirus is also known as human Herpes Virus type 5 (HHV-5). It has linear double-stranded DNA, a capsid, tegument proteins and an envelope containing different glycoproteins.[16] The immunodominant tegument protein, which is also the most abundant one and the major part of extracellular viral particles, is pp65. It is a useful target for cytotoxic T cells and is therefore used to generate CMV-specific T cells.[17,18] CMV has a high seroprevalence all over the world; in Germany about 50% of adult blood donors are seropositive. In healthy individuals CMV infection remains mainly unnoticed or causes only unspecific mild symptoms, however asymptomatically infected persons can transmit the virus *via* saliva and all other kinds of body fluids. CMV can have a life-long latency in hematopoietic stem cells and monocytes.[19] The virus causes viremia but stays mainly cell-adherent.[16] In the *post*-transplant setting CMV reactivation occurs in one third of patients, when donor and/or recipient are seropositive for CMV, or even more if additional risk factors are present.[10,15] Talking about immunocompromised patients, CMV infection can cause CMV disease in multiple tissues, for example in retina, colon and liver. CMV pneumonia is the manifestation with the highest mortality, which is still about 40%.[15,20] Death due to viral infection occurs in mean during the first three months after HSCT.[14] To prevent CMV infection high-dose acyclovir treatment has shown to be an effective prophylactic treatment and ganciclovir or foscarnet is used for high-risk patients as pre-emptive therapy.[21,22] Unfortunately, even on antiviral drug treatment CMV load increases in nearly 50% of the patients.[14] Though morbidity and mortality due to CMV infection after HSCT has been improved over the last decades there is still the need for developing new therapeutic options.[22]

2. ADOPTIVE T-CELL THERAPY

To address the need for better antiviral therapies in patients after HSCT the possibility of adoptive T-cell transfer was developed. This approach not only treats the infection but restores the viral immunity in immunodeficient patients. Peripheral blood, virus-specific T cells of a seropositive donor are enriched and infused into the patient, where the cells can provide specific reactions against the virus.[23] Adoptive T-cell transfer is a second-line treatment option according to acknowledged guidelines.[24]

Different methods of generating virus-specific T cells have emerged over time. *In vitro* stimulation and expansion of (poly)-clonal ($CD4^+$ and) $CD8^+$ T cells was the first strategy, which provides high cell numbers for infusion. PBMCs are co-cultured with primary antigen-presenting cells (APCs) or cell lines, that present the viral antigens, and hence specific T-cell clones get activated and enriched due to their proliferation.[23,25,26] This procedure takes several weeks, and it was shown for HIV-specific cells that the transferred cells didn't persist *in vivo*, probably due to overstimulation during the *in vitro* expansion.[27] A method which allows the direct and rapid selection of $CD8^+$ specific T-cell clones with high purity is the use of MHC I multimers which are loaded with virus-specific peptide and hence recognize specific TCRs on $CD8^+$ T cells. This method is limited to already known single peptides, HLA-type and to $CD8^+$ T-cell isolation only.[28-30] Cytokine capture technique (CC), which was used in this project, allows the rapid enrichment of virus-specific $CD4^+$ and $CD8^+$ T cells due to their IFN-γ secretion upon stimulation with viral antigens.[31,32] For direct isolation-based methods it is important that the donor has a distinct virus-specific population among PBMCs. A second blood donation or apheresis is needed because of the high required cell amount and for CC purities are lower compared to other techniques. However, CC can be applied without knowing immunogenic peptide sequences and allows the enrichment of $CD4^+$ and $CD8^+$ T cells due to their functionality. Furthermore, it is very fast and compatible with good manufacturing practice (GMP) conditions. The product consists of a relatively small cell number and the T cells are expanding in the patient after infusion. **Figure 1** visualizes the different generation methods for virus-specific T cells.

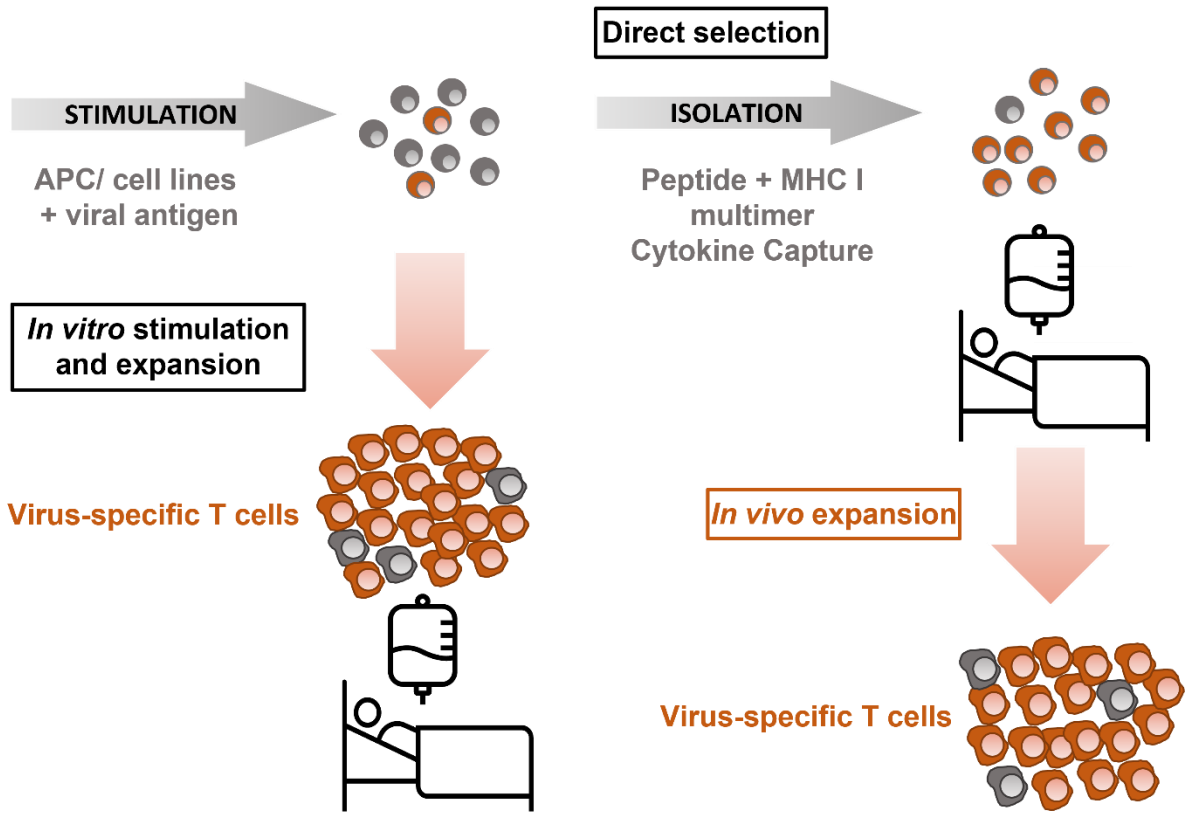

Figure 1 Different methods of virus-specific T-cell generation (based on [33])

Adoptive T-cell transfer was done with CMV-, AdV and EBV-specific T cells using all three methods of generating the cell product.[33] For CMV, *Riddell* et al. were the first to transfer specific T cells for CMV prophylaxis to human patients using *in vitro* stimulation in 1992.[23] *Cobbold* et al. followed 2005 using MHC I multimer- isolated T cells.[34] Using cytokine capture technique, *Feuchtinger* et al. treated heavily pretreated patients suffering from refractory CMV infection with pp65-specific T cells. Multiple other clinical studies followed in this field using all three enrichment methods for different viruses. **Figure 2** gives an overview of the studies done with CMV-specific T-cells.

Reference	Method	Patients	Results
In vitro stimulation and expansion			
Riddell et al. (1992)	Allogeneic CMV-spec. $CD8^+$ clones	3	3/3 Prevention of viremia and pneumonia
Einsele et al. (2002)	Allogeneic CMV-spec. polyclonal $CD8^+$ and $CD4^+$ T cells	8	5/7 evaluable patients eliminated infection
Meji et al. (2012)	CMV-specific polyclonal $CD8^+$ and $CD4^+$ T cells Phase I/II	6	6/6 patients eliminated infection
Withers et al. (2017)	CMV-specific third party CD8+ and CD4+ T cells Phase I	27	26/27 responded
Direct isolation *via* peptide-HLA multimers			
Cobbold et al. (2005)	Allogeneic CMV-specific $CD8^+$ T cells using MHC-I-Tetramers	9	8/9 patients eliminated infection
Blyth et al. (2013)	Allogeneic CMV-specific polyclonal $CD8^+$ and $CD4^+$ T cells Phase II	50 Prophylaxis	41/50 did not require CMV-directed pharmacotherapy
Neuenhahn et al. (2017)	Allogeneic CMV-specific $CD8^+$ T cells using MHC-I-Streptamers Phase I/IIa	16	Stem cell donor-derived: 7/7 responded Third party transfer: 5/8 responders
Direct isolation *via* cytokine-capture technique			
Feuchtinger et al. (2010)	CMV-specific polyclonal $CD8^+$ and $CD4^+$ T cells	18	15/18 responded
Peggs et al. (2011)	CMV-specific polyclonal $CD8^+$ and $CD4^+$ T cells Phase I/II	18	Prophylaxis: 6/7 virus-free Pre-emptive: 2/11 required no antiviral drug treatment

Figure 2 Clinical efficacy of CMV-specific T cells (based on [33])

In addition to the adoptive transfer of primary human virus-specific T-cells the use of genetically modified virus-specific cell products is a promising therapy, as genetically engineered T-cells already are applied successfully for other indications.

3. GENETIC ENGINEERING AND CRISPR/CAS9

Genetic engineering of cells is done since the discovery of the DNA. **Figure 3** gives an overview of the development of different tools over the last decades. Site-specific manipulation was introduced at first using small molecules or oligonucleotides which paired with certain DNA base sequences. Using for example chemical cleavage, those methods didn't remain state of the art but were a proof that base pairing can be a functional mechanism for site-specific gene engineering.[35] Zinc finger nucleases (ZFN) were then the first effective tool for the induction of specific DNA double-strand breaks (DSB), which led to genomic alterations.[36] The next step in the history of genetic engineering were transcription activator-like effector nucleases (TALENs). TALENs consisted of bacterial virulence factors (TALEs), which bound distinct DNA sequences, and the endonuclease FokI, already used in ZFN.[37,38] Those methods allowed highly specific genetic modifications, but withheld difficulties in design, synthesis and evaluation, which prevented a broad and easier routine application.[35]

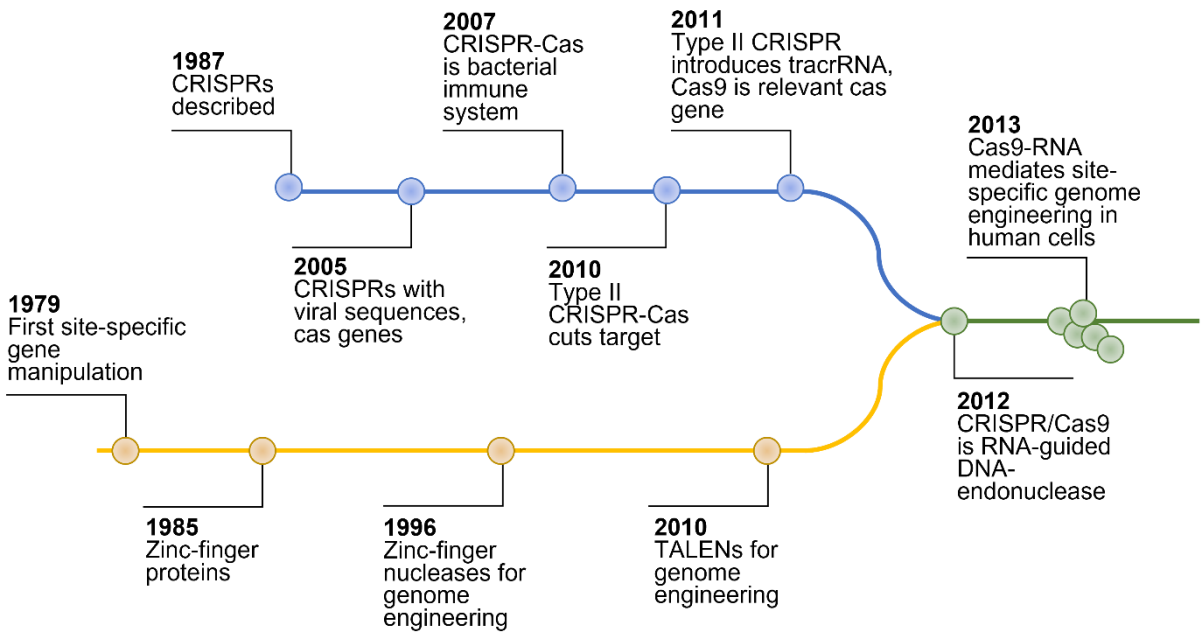

Figure 3 History of genetic engineering and development of CRISPR/Cas9 (based on [35])

Clustered regular short palindromic repeats (CRISPRs) were first found by *Ishino* et al. in Escherichia coli in 1987.[39] Since 2005 many different groups contributed to the discovery of CRISPR-Cas as a prokaryotic immune system against bacteriophages. When challenged with viruses, bacteria can integrate new genomic sequences, obtained from phages, into their genome. Those so-called spacers are located in between repetitive regions in the CRISPR area of prokaryotic genomes. The following transcription of spacers into crRNA leads to targeted recognition of known foreign DNA sequences and enables the cell to silence them using Cas (CRISPR-associated) proteins guided by the crRNA. By this, CRISPR/Cas renders bacteria resistant against phages and works like an adaptive immune system.[40] There are three different CRISPR/Cas system types with various Cas enzymes. In 2012, Cas9 of *S. pyogenes* in type II system was described as an endonuclease, guided, and activated by two RNAs: the tracrRNA and crRNA, which can also be fused to one single guide RNA (sgRNA). The tracrRNA:crRNA-guided Cas9 endonuclease causes site-specific DSBs in target DNA. The exact location of cleavage is set by base pairing between crRNA and target sequence, as well as the additional DNA sequence "NGG", called protospacer adjacent motif (PAM). DNA cleavage takes place three bases upstream of the PAM (**Figure 4**). As only the crRNA must be replaced to change the target site, the CRISPR/Cas9 system is easily programmable.[41,42]

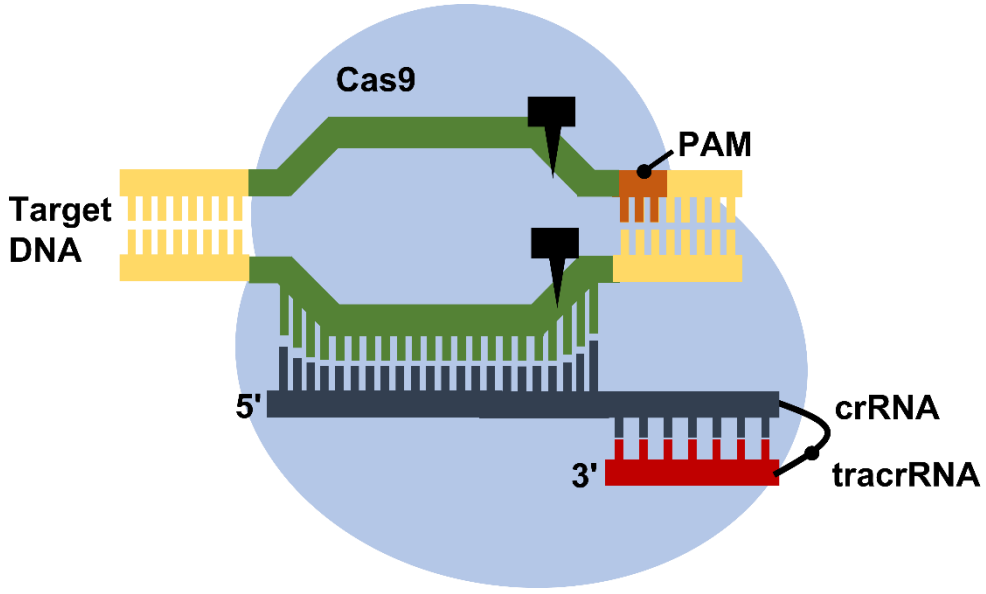

Figure 4 Working mechanism of the CRISPR/Cas9 system (based on [35])

DSBs can be repaired either *via* homology-directed repair (HDR) using a template for insertion of a gene fragment or non-homologous end joining (NHEJ), which is error-prone and therefore induces insertions and deletions (InDels) at the cut site. Those two repair mechanisms can be used for genetic engineering in terms of knock-in or knock-out of gene fragments or whole genes. Therefore, CRISPR/Cas9 is an effective tool for genetic engineering of human cells. **Figure 5** visualizes the use of cell-own repair mechanisms for gene editing.[43-45] The advantages of CRISPR/Cas9 technique are similar or even higher efficacies than with ZFNs or TALENs. For targeting new locations, no protein engineering is required, but only an exchange of the crRNA. The application is easy, low-priced and possible for all cell types and organisms.[35] Though by now it could possibly have more off-target effects than TALEN, the CRISPR/Cas9 system has already overcome other technologies and makes it way steadily towards the clinic.[46]

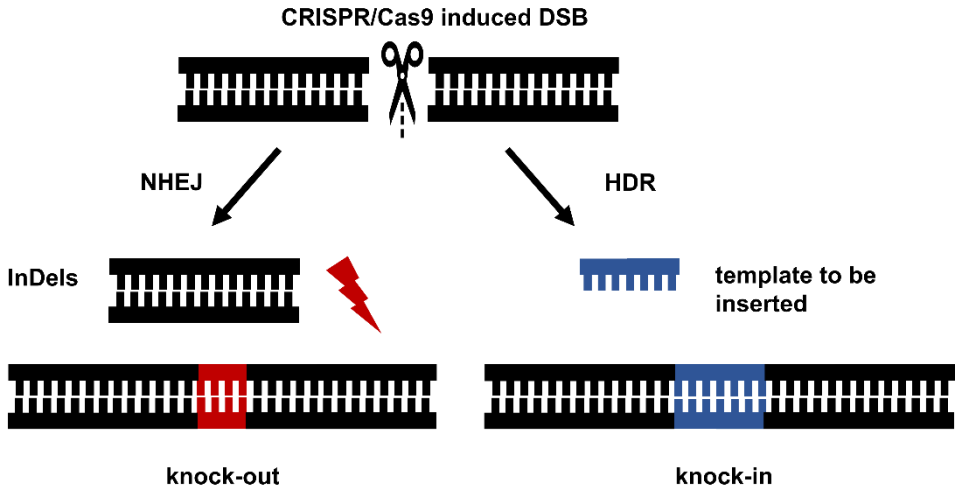

Figure 5 DNA repair mechanisms and their use in gene editing (based on [35])

To use CRISPR/Cas9 successfully on primary human T cells a basic understanding of T-cell subsets and T-cell function is necessary.

4. PRIMARY HUMAN T CELLS

4.1 PHENOTYPIC AND FUNCTIONAL T-CELL SUBSETS

After development out of hematopoietic stem cells in the bone marrow, early T-cell progenitors continue maturation in the thymus, where somatic recombination as well as positive and negative selection takes place. Naïve CD4 and CD8 T cells can get activated by binding their specific antigens presented on either MHC II (CD4 T cells) or MHC I (CD8 T cells) *via* their TCR. Thus, they differentiate to $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells.[47] T cells can be grouped into subsets according to their phenotypic and functional characteristics. There are five T-cell subsets and each of those give rise to the following more differentiated T-cell stages following a linear differentiation model.[48,49] Naïve T cells (Tn) are followed by stem cell like memory T cells (Tscm) and then central memory T cell (Tcm), which give rise to effector memory (Tem) and finally to effector T cells (Teff).[50] **Figure 6** visualizes T-cell differentiation. Early differentiation stages (Tn, Tscm, Tcm) have the ability of homing into lymphoid organs to get activated and therefore express lymphocyte homing receptors such as CD62L.[51] They have high proliferative capacity but low effector functions. Central memory T cells are more sensitive to antigen stimulation and can proliferate and differentiate rapidly into effector stages.[49] Tcm and Tem express the memory marker CD45RO.[52] Tem and Teff carry large amounts of perforine and can secrete cytokines such as IFN-γ and IL-4 rapidly after antigen contact. They lose lymphoid homing molecules and instead express chemokine receptors and other adhesion molecules as surface markers, as they home into inflamed tissues and not into secondary lymphoid organs.[49] With further T-cell differentiation from naïve to effector cell the proliferative capacity decreases which comes along with an increasing effector function.[50]

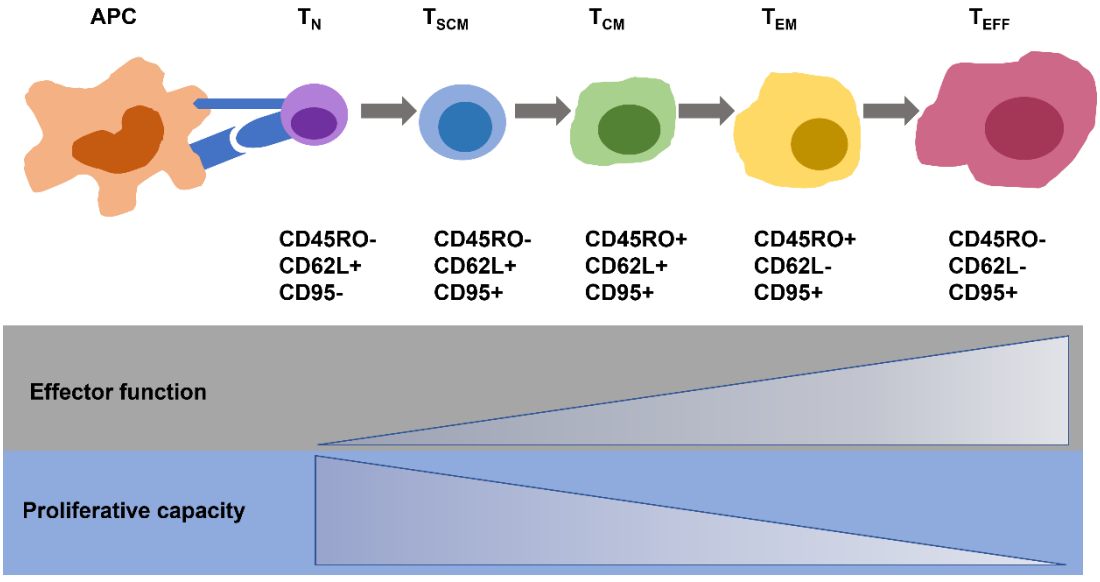

Figure 6 Phenotypic and functional T-cell subsets (based on [50])

4.2 T-CELL ACTIVATION AND FUNCTIONAL CHARACTERISTICS

In vivo, antigen-primed Tcm can traffic through secondary lymphoid organs (homing) where they get activated upon contact with their specific antigen presented by antigen-presenting cells (APCs).[51] Tcm can thereby provide a pool of antigen-specific cells homing through the lymphoid organs, which mediate immune response by stimulating dendritic cells and B cells and differentiating into new effector cells. *In vitro*, stimulation of Tcm leads to proliferation and differentiation into effector stages with pronounced effector functions, such as cytokine secretion and cytotoxic target cell lysis using perforine secretion. IFN-γ and TNF-α is secreted rapidly in high amounts by Tem and Teff.[49] IL-2 and IL-4 are only secreted by Tem. The strongest effector function in terms of cytotoxicity, using perforine and granzyme B, and stimulation of B-cell differentiation is mediated by Teff.[53] IFN-γ is secreted by $CD4^+$ helper T cells type 1 (Th1) and $CD8^+$ T cells and promotes cytotoxic effects by enhancing antigen processing and presentation (through for example up-regulation of MHC I) and strengthening Th1 differentiation and response in a positive feedback loop. In addition, IFN-γ induces antiviral key enzymes and induces IL-12 synthesis in phagocytes.[54] In brief, Tcm have a strong proliferation and differentiation capacity into effector types, whereas Tem and Teff secrete high amounts of IFN-γ, enhancing the immune response, and Teff mediate cytotoxic effects.[53] Several complex T-cell functions are regulated *via* the glucocorticoid receptor, which can also be targeted by therapeutic drugs.

4.3 THE GLUCOCORTICOID RECEPTOR (GR)

Glucocorticoids (GCs) can bind to the Glucocorticoid Receptor (GR; gene name NR3C1). It is a highly conserved intracellular receptor, which acts as a ligand-dependent transcription factor. The GR gene consists of nine exons, which code for the N-terminal domain (NTD), the DNA-binding domain, the hinge domain and the ligand-binding domain (LBD) of the protein.[55] The NTD includes the powerful transcription-activating function AF-1.[56] The GR shuttles between the cytoplasm and, when bound by a ligand, the nucleus, where it can induce transactivation and transrepression of many genes.[55,57] In addition, glucocorticoids also have rapid nongenomic effects *via* interactions with cytosolic and membrane-bound GRs and non-specific membrane interactions.[58] In the classical way, glucocorticoids can rapidly diffuse through the cell membrane and bind to the LBD of the GR, which homodimerizes and shuttles into the nucleus. There it can bind DNA *via* glucocorticoid-responsive elements (GRE) in the promotor region of regulated genes or induce and repress gene expression indirectly through interaction with other transcription factors.[59] An example for transrepression is the inhibition of pro-

inflammatory cytokine expression, an example for transactivation is inducing gluconeogenic liver enzymes.[60] Physiologically, glucocorticoids are steroid hormones, which are essential for survival and responsible for the homeostasis and coordination of response to stressors, as well as for maintaining circadian rhythms. The GR is expressed in most cell types and mediates physiological effects in all kinds of tissues.[61,62] The structures of modern synthetic glucocorticoids are chemically adapted from the physiological steroid hormone cortisol. Widely used glucocorticoids are for example dexamethasone, prednisolone or budesonide, which have a higher affinity for the GR and better pharmacokinetics.[63]

4.4 T CELLS UNDER IMMUNOSUPPRESSIVE TREATMENT

Glucocorticoids are small lipophilic molecules and have strong anti-inflammatory effects by inhibiting the synthesis of many cytokines (for example IFN-γ, IL-2, IL-4 and TNF-α) and influencing T-cell behaviour.[64,65] Activated T cells respond with apoptosis to glucocorticoid treatment,[66] whereas resting mature T cells only redistribute in other compartments, which causes lymphocytopenia *in vivo*.[67] Apoptosis is mediated through the mitochondrial pathway and genes needed for this programmed cell death are transactivated by GR signalling.[65] This may explain the strong immunosuppressive and anti-inflammatory effects of glucocorticoids when used in therapeutic doses.[66]

Cyclosporin A (CsA) is a cyclic peptide[68], which acts GR independent, and is a commonly used immunosuppressant in the therapy of autoimmune disorders as well as in the HSCT-setting[69]. It forms complexes with immunophilins and thereby inhibits calcineurin phosphatase activity, which physiologically induces the nuclear translocation of NFAT (nuclear factor of activated T cells) and leads to cytokine synthesis and T-cell activation.[68,70] CsA increases immunosuppressing effects on regulatory T cells[71], inhibits T-cell proliferation and decreases cytokine secretion of $CD4^+$ and $CD8^+$ T cells.[72]

5. AIMS OF THE STUDY

To provide a treatment option for patients suffering from chemo-refractory viral infection under ongoing glucocorticoid therapy, this study aims to generate and characterize glucocorticoid-resistant virus-specific T cells for adoptive T-cell therapy. The T-cell product will be generated by using the cytokine capture technique for enrichment of virus-specific T cells and the CRISPR/Cas9 technique for GR KO. Furthermore, the product will be extensively characterized in terms of purity, phenotype, functionality, and drug sensitivity *in vitro*. This study aims to establish a GMP-compatible protocol, which is reasonably priced and efficacious, and strives for a novel treatment option for patients under glucocorticoid therapy suffering from viral infections.

B. MATERIALS

1. EQUIPMENT AND SOFTWARE

Description	Product	Manufacturer
Electroporator	Nucleofector® II	Amaxa Biosystems GmbH, Cologne, Germany
Film Processor	CP1000	Agfa-Gevaert N.V., Mortsel, Belgium
Flow Cytometer	MACSQuant Analyzer 10	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	BD FACS Canto™ II Flow Cytometer	BD, Franklin Lakes, New Jersey, USA
Freezing Container	Nalgene Mr. Frosty	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
Gel Imager	Gel iX20 Imager	INTAS Science Imaging Instruments GmbH, Göttingen, Germany
Magnetic cell separator	MACS MultiStand	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	MidiMACS Separator	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	MiniMACS Separator	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	DynaMAG™ -2	Thermo Fisher Scientific Corp.; Waltham, Massachusetts, USA
Microplate reader	FLUOstar® Omega	BMG LABTECH GmbH, Ortenberg, Germany
Microscope	Leica DM IL	Leica Microsysteme, Wetzlar, Germany
	Axiovert 200M	Carl Zeiss AG, Oberkochen, Germany
Software	BD FACSDiva Version 6	BD Biosciences, San Jose, California, USA
	FlowJo 10.0.7r2	FlowJo LLC, Ashland, Oregon, USA
	Gel iX20 Imager Windows Version	INTAS Science Imaging Instruments GmbH, Göttingen, Germany
	GraphPad Prism 5.0	GraphPad Software Inc., San Diego, California, USA
	Image J	Wayne Rasband, NIH, Bethesda, Maryland, USA
	Microsoft Office 2010	Microsoft Corp., Redmond, Washington, USA
	TIDE	Bas van Steensel lab and Desktop Genetics Ltd., London, UK
Spectrophotometer	Nanodrop ND-1000 spectrophotometer	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
Thermocycler	peqSTAR 96 Universal Gradient	Isogen Life Science B. V., De Meern, Netherlands
Western Blot components	Mini-PROTEAN® Tetra System	Bio-Rad Laboratories, Inc., Hercules, California, USA

2. CONSUMABLES

Description	Product	Order Number	Manufacturer
Cell Scraper	Corning® Small Cell Scraper	3010	Corning Inc., Corning, New York, USA
Film	CL-XPosure™ Film	34090	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
Filter Paper	Western Blotting Filter Paper	88600	
Freezing tubes	Cryo Pure Gefäß 1.8 ml	72.379	Sarstedt AG & Co. KG, Nümbrecht, Germany

Magnetic separation columns	MS columns, LS columns, LD columns	130-042-201 130-042-401 130-042-901	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Nitrocellulose Membrane	Nitrocelluclose Membrane, 0.2µm	162-0146	Bio-Rad Laboratories, Inc., Hercules, California, USA

3. CHEMICALS AND REAGENTS

Description	Product	Order Number	Manufacturer
Acrylamid/Bisacrylamid	Rotiphorese® Gel 30 (37.5:1)	3029.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Agarose	Seakem Le Agarose	50004	DMA, Rockland, Maine, USA
Brefeldin A	Brefeldin A	5936	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
Cas9 Nuclease	Alt-R® S. p. Cas9 Nuclease V3	1081059	Integrated DNA Technologies, Inc. , Skokie, Illinois, USA
Cell stimulants	T Cell TransAct, human	130-111-160	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	Dynabeads™ Human T-Activator CD3/CD28	11131D	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
	Cytostim	130-092-172	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	Stapyhlococcal enterotoxin B	4881	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
Cyclosporin A	Sandimmun® 50 mg/ml Ciclosporin	-	Novartis AG, Holzkirchen, Germany
CMV pp65	PepTivator CMV pp65 - premium grade, human	130-093-435	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
DAPI	ProLong™ Gold Antifade Mountant with DAPI	P36935	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
Developer	Roenteroll 25	104700	Tetenal Europe GmbH, Norderstedt, Germany
Dexamethasone	Dexamethasone ≥98% (HLPC), powder	D1756	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
DNA ladder	100 bp DNA Ladder Ready to Load	01-11-00050	Solis BioDyne, Tartu, Estonia
DNA Loading Dye	DNA Gel Loading Dye (6X)	R0611	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
DMSO	Dimethylsulfoxid	4720.4	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
ECL	Clarity™ Western ECL Substrat	170-5060	Bio-Rad Laboratories, Inc., Hercules, California, USA
Electroporation Enhancer	Alt-R® Cas9 Electroporation Enhancer	1075916	Integrated DNA Technologies, Inc., Skokie, Illinois, USA
Ethidium bromide	Ethidium bromide	2218.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ficoll	Biocoll separating solution	L6115	Biochrom GmbH, Berlin, Germany
Fixer	Superfix 25	104701	Tetenal Europe GmbH, Norderstedt, Germany
Interleukins	IL-7 and IL-15 human, premium grade IL-2 IS, premium grade	130-095-363, 130-095-764 130-097-745	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
	rh IL-4	1403-050	CellGenix GmbH, Freiburg, Germany

Magnetic beads	CD3 MicroBeads CD56 MicroBeads	130-050-101 130-050-401	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PFA	Paraformaldehyd	P6148	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
PHA	Phytohemagglutinin-L (PHA-L) Solution (500X)	00-4977-93	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
PI	Propidium Iodide solution	130-093-233	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Poly-L-lysine	Poly-L-lysine solution 0.1%	P8920	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
Proteinase Inhibitor	FAST Proteinase Inhibitor Tablet	S8820	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
Protein ladder	Spectra™ Multicolor Broad Range Protein Ladder	26634	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
Streptamers	MHC I-Strep HLA-A*0201: CMV pp65; NLVPMVATV	6-7001-001	IBA GmbH, Göttingen, Germany
	MHC I-Strep HLA-A*0201: NY-ESO-1; SLLMWITQV	6-7013-001	IBA GmbH, Göttingen, Germany
TEMED	TEMED 99% p. A.	2367.3	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
tracrRNA	Alt-R® CRISPR-Cas9 tracrRNA	1073191	Integrated DNA Technologies, Inc., Skokie, Illinois, USA
Triton™ X-100	Triton™ X-100	T-9284	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
Trypan blue	Trypan Blue Solution, 0.4	15250061	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA

4. COMMERCIAL KITS

Product	Order Number	Manufacturer
AccuPrime™ Taq DNA Polymerase, high fidelity	12346086	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	552843	BD Biosciences, San Jose, California, USA
CellTrace Violet Proliferation Kit	C34557	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
DNA Clean & Concentrator™ -5	D4014	Zymo Research Corp., Irvine, California, USA
EasySep™ Human T Cell Enrichment Kit	19051	STEMCELL™ Technologies Inc., Vancouver, Canada
FIX & PERM™ Cell Permeabilization Kit	GAS004	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (PE), human	130-054-201	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
MACS Comp Bead Kit anti-mouse/ anti-REA	130-097-900 130-104-693	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Mix2Seq Kit	-	Eurofins Genomics GmbH, Ebersberg, Germany
QIAamp DNA Mini Kit	51306	QIAGEN GmbH, Hilden, Germany
Pierce™ BCA Protein Assay Kit	23227	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA

5. ANTIBODIES

5.1 FLOW CYTOMETRY

Antigen	Fluorochrome	Clone	Order Number	Manufacturer
CD3	APC-Vio770	REA613	130-113-136	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD3	FITC	REA613	130-109-460	
CD3	PE-Vio770	REA613	130-113-140	
CD4	VioGreen	REA623	130-113-230	
CD4	VioBright FITC	REA623	130-113-791	
CD4	PE/Cy7	SK3	344612	BioLegend, Inc., San Diego, California, USA
CD8	APC/Cy7	SK1	344714	
CD8	APC-Vio770	REA734	130-110-681	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD8	VioBlue	REA734	130-110-683	
CD14	APC	TÜK4	130-091-243	
CD16	PE	REA423	130-106-704	
CD19	PE-Vio770	REA675	130-113-647	
CD20	APC	2H7	302310	BioLegend, Inc., San Diego, California, USA
CD45	VioBlue	5B1	130-092-880	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD45RO	PE	UCHL1	304206	BioLegend, Inc., San Diego, California, USA
CD45RO	PE-Vio770	REA611	130-113-560	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD56	APC	NCAM16.2	341027	BD BioSciences, San Jose, California, USA
CD56	PE	REA196	130-100-653	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
CD56	PE-Vio615	REA196	130-114-550	
CD62L	APC	145/15	130-091-755	
CD62L	VioBlue	145/15	130-098-699	
CD95	APC	DX2	130-092-417	
IFN-γ	PE	25723.11	340452	BD BioSciences, San Jose, California, USA
Streptactin	PE	-	6-5000-001	IBA GmbH, Göttingen, Germany
TCR α/β	FITC	IP26	306705	BioLegend, Inc., San Diego, California, USA
Viability	7-AAD	-	420404	
Viability	VioGreen Viobility Dye	-	130-110-206	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

5.2 WESTERN BLOT AND IF

Antibody	Order Number	Manufacturer
Alexa Fluor® 488 Goat anti-mouse IgG	405319	BioLegend, Inc., San Diego, California, USA
GAPDH (0411)	Sc-47724	Santa Cruz Biotechnology, Inc., Dallas, Texas, USA
Goat anti-mouse IgG-HRP	Sc-2005	
GR (G-5)	Sc-393232	

6. OLIGONUCLEOTIDES

Description	Sequence	Manufacturer
crRNA GR1	AACCAAAAGUCUUCGCUGCU	Integrated DNA Technologies, Inc., Skokie, Illinois, USA
crRNA GR2	CUUUAAGUCUGUUUCCCCCG	
Primer fwd GR	TCCTGGTAGAGAAGAAAACCCC	metabion international AG, Planegg, Germany
Primer rev GR	CCTGCAAAATGTCAAAGGTGC	

7. BUFFERS, MEDIA AND SUPPLEMENTS

7.1 COMMERCIALS

Description	Product	Order Number	Manufacturer
FCS	FCS (Fetal Bovine Serum)	F0804	Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany
HSA 5%	Albiomin 5 % infusion solution human albumin (HSA)	623 050	Biotest AG, Dreieich, Germany
Human AB serum	Human AB serum was kindly provided by Prof. R. Lotfi, University Hospital Ulm, Institute for Transfusion Medicine and German Red Cross Blood Services Baden-Württemberg—Hessen, Institute for Clinical Transfusion Medicine and Immunogenetics, both from Ulm, Germany		
IMDM	IMDM Iscove's Modified Dulbecco's Medium	12440061	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
L-Glutamin	L-Glutamin (200 MM)	K0283	Biochrom, Merck KGaA, Darmstadt, Germany
MACSQuant Buffers	Running Buffer Storage Solution Washing Solution,	130-092-747 130-092-748 130-092-749	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PBS	PBS Dulbecco's phosphate-buffered saline	14190144	Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA
RPMI	VLE-RPMI 1640	F1415	Biochrom, Merck KGaA, Darmstadt, Germany
TAE Buffer	TAE Buffer (50x)	A4686	Applichem GmbH, Darmstadt, Germany
TM	TexMACS Medium	130-097-196	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
TM GMP	TexMACS GMP Medium	170-076-307	

7.2 COMPOSITION

Description	Ingredient	End Concentration
Activating T-cell medium	Human AB serum IL-2 IL-7 IL-15 TexMACS Medium	5% 30 U/ml 10 ng/ml 10 ng/ml Ad 50 ml
Basic feeder cell medium	Human AB serum IMDM	2% Ad 50 ml
Basic T-cell medium	Human AB serum TexMACS Medium	2.5% Ad 50 ml
Blast medium	L-Glutamin	1%

	FCS	10%
	RPMI	Ad 50 ml
Cell culture buffer	Human AB serum	2%
	PBS	Ad 50 ml
Electroporation Buffer M	KCl	5 mM
	$MgCl_2$	15 mM
	Na_2HPO_4/ NaH_2PO_4	120 mM
	Mannitol	50 mM
Feeder cell medium	Human AB serum	2%
	IL-4	12.5 ng/ml
	IMDM	Ad 50 ml
Freezing medium	DMSO	10%
	HSA 5%	4.5% HSA
Laemmli Buffer 6x pH 6.8	SDS	0.5 M
	DTT	0.6 M
	Tris	0.4 M
	Bromphenol Blue	0.9 mM
	Glycerol	9 M
	ddH_2O	Ad 50 ml
RIPA Buffer	Tris-HCl pH8	50 mM
	NaCl	150 mM
	Glycerin	10%
	Triton X-100	1%
	EDTA	1 mM
	dH_2O	Ad 100 ml
Running Gel	TRIS-HCl	0.58 M
	SDS	0.1%
	Acrylamid/Bisacrylamid	9%/ 0.24%
	Ammonium persulfate	0.2%
	TEMED p. A.	0.05%
	ddH_2O	Ad 10.9 ml
Stacking Gel	TRIS-HCl	0.38 M
	SDS	0.1%
	Acrylamid/Bisacrylamid	3.9%/ 0.1%
	Ammonium persulfate	0.1%
	TEMED	0.1%
	ddH_2O	Ad 5 ml
Staining buffer	FCS	1%
	PBS	Ad 50 ml
TBS-T	TRIS	20 mM
	NaCl	140 mM
	Tween20	0.1%
	dH_2O	Ad 1 L
T-cell medium	Human AB serum	2.5%
	IL-7	10 ng/ml
	IL-15	10 ng/ml
	TexMACS Medium	Ad 50 ml
Western Blot Running Buffer 1x	TRIS	25 mM
	Glycine	0.2 M
	SDS	3.5 mM
	dH_2O	Ad 1 L
Western Blot Transfer Buffer 1x	TRIS	25 mM
	Glycine	0.2 M
	SDS	3.5 mM
	Methanol p. A.	20%
	dH_2O	Ad 1 L

C. METHODS

1. GENERATION OF T-CELL PRODUCT

1.1 PBMC ISOLATION

About 90 ml peripheral venous blood was taken from healthy donors using 9 ml EDTA precoated vials. Informed consent was always given written before blood withdrawal.[1] Ethics approval was obtained from the local Ethics Committee and the study was performed in accordance with the Declaration of Helsinki.[1] Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation. The blood was diluted 1:2 with PBS before it was layered on Biocoll separating solution. After centrifuging for 30 minutes at 800 g without brake the PBMC layer was carefully removed and washed twice with PBS. Cell count and viability was determined microscopically after Trypan blue staining.

1.2 CYTOKINE CAPTURE TECHNIQUE (CC)

PBMCs were seeded at a concentration of $1x10^7$ cells/ml in 6 well plates (4 ml/well) in basic T-cell medium. PBMCs were stimulated and incubated at 37°C and 5% CO_2 for four hours either with Staphylococcus enterotoxin B (SEB) or with cytomegalovirus pp65 Peptivator.[1] Using the IFN-γ Secretion assay kit the stimulated cells were first coated with a bispecific antibody, which binds the leukocyte surface marker CD45 and captures the secreted IFN-γ of each cell on its surface.[1] Then the cell-bound IFN-γ was labelled with a secondary PE IFN-γ detection antibody and at last a third antibody labelled with magnetic beads binds PE, thereby marking all cells which secreted IFN-γ after stimulation with SEB or pp65.[1] All labelling steps were done according to manufacturer`s instructions.[1] Enrichment was performed by applying all cells on a LS or MS column in a MidiMACS or MiniMACS separator depending on expected cell counts. Basic T-cell medium was used to flush out the positive cell fraction. Positive and negative cell fraction were centrifuged at 300 g for 10 min and resuspended at $1x10^6$ cells/ml in activating T-cell medium and Dynabeads (in a 1:2 bead to cell ratio); at least 1 ml medium was used. Stimulation beads were washed beforehand according to manufacturer´s instructions and removed using the DynaMAG™ -2 magnet two or three days after application. Samples for flow cytometric analysis were collected at various steps of the procedure. Enrichment was evaluated *via* flow cytometry. In case of SEB stimulation, cells were gated on

lymphocytes, single cells and IFN-γ$^+$ cells; for CMV pp65 stimulation cells were gated on lymphocytes, single cells, CD3$^+$ CD56$^-$ T cells and IFN-γ$^+$ cells.

1.3 PREPARATION OF FEEDER CELLS

Autologous PBMCs were used to generate feeder cells for the ICS. Therefore, PBMCs were CD3 and CD56 depleted according to manufacturer´s instructions.[1] In brief, cells were simultaneously labelled with anti-CD3 and anti-CD56 microbeads for 15 min, washed once and applied onto a LD column in the MidiMACS separator. The negative unbound fraction was rested overnight in feeder cell medium in a 24 well plate at a concentration of 1,5x10^6 cells/ml (2 ml/well). The next day feeder cells were either used directly or frozen until needed. For cryopreservation cells were frozen at -80°C in freezing medium using a freezing container. One day later the freezing tubes were transferred into a -180°C nitrogen tank and stored until needed. For thawing, cells were pre-warmed in a 37°C water bath and subsequently transferred into pre-warmed medium and centrifuged at 450 g for 5 min. Cells were resuspended in basic T-cell medium at a concentration of 2x10^6/ml for ICS.

1.4 GLUCOCORTICOID RECEPTOR KNOCK-OUT (GR KO)

Depending on cell numbers knock-out of the glucocorticoid receptor was performed two to eight days after CC using CRISPR/Cas9 system.[1] Up to 1x10^6 cells were resuspended in 100 µl of electroporation buffer M and mixed with pre-incubated RNP complex containing a guideRNA, consisting of crRNA and tracrRNA heated for 5 min at 95°C, Cas9 enzyme and Enhancer.[1] For MOCK control cells were electroporated in electroporation buffer M without any further reagents.[1] Untreated control cells were resuspended in electroporation buffer M and directly transferred to a 24 well plate without electroporation. After electroporation using Amaxa nucleofector MOCK and GR KO cells were subsequently seeded in pre-warmed basic T-cell medium in a 24 well plate at a concentration of 1x10^6 cells/ml and were splitted every two to five days according to density and change in media colour.[1] During the following 18 days of culture the cells were characterized on genetic, phenotypic and functional level. **Figure 7** gives a schematic overview of the workflow.

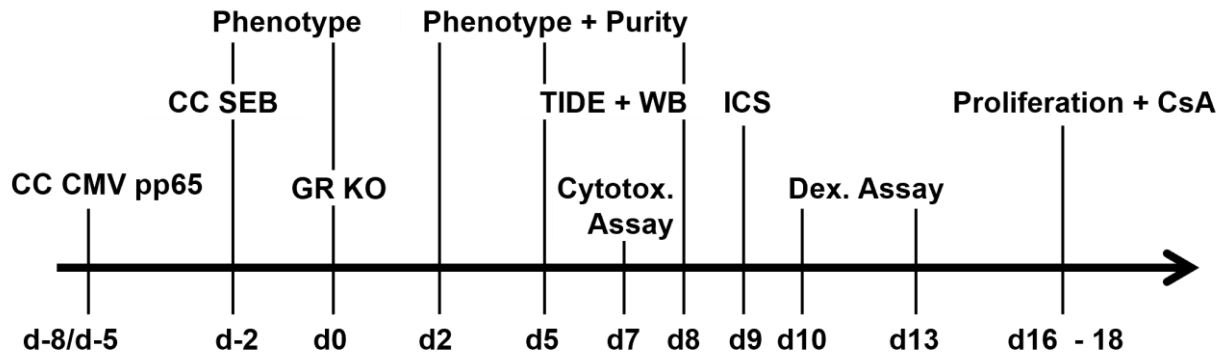

Figure 7 Workflow of the generation of virus-specific GR KO T cells

PBMCs were stimulated either with SEB or with CMV pp65 and (virus-)specific cells were enriched *via* CC (=positive fraction). The GR KO *via* CRISPR/Cas9 was performed on d0 in the positive, as well as the negative fraction. Afterwards cells were expanded. Analysis and assays were performed at the respective days.

2. Evaluation of GR KO

2.1 DNA Isolation

$5x10^5$ cells were centrifuged, resuspended in 200 µl PBS and stored at -20°C until further use. DNA isolation was performed according to manufacturer`s instructions using the QIAamp DNA Mini Kit.[1] DNA concentrations were measured with the spectrophotometer and isolated DNA was stored at -20°C until PCR was performed.

2.2 PCR and Sequencing

50 ng of genomic DNA was added to a mastermix containing nuclease free water, HSA, buffer, both forward and reverse primers, covering the CRISPR cut site in the glucocorticoid receptor locus exon 2, and a high-fidelity polymerase.[1] The final PCR volume was 25 µl. A non-template control was always included.

Initial denaturation	95°C	2 min	
Denaturation	95°C	40 sec	
Annealing	55°C	40 sec	45 cycles
Elongation	68°C	1 min	
Final Elongation	68°C	10 min	

Subsequently 5 µl of PCR product was mixed with 6x loading dye and applied onto a 1,5% agarose gel. After applying 120 V for 45 minutes on the gel chamber the bands were checked for size and specificity. The PCR products were purified using DNA Clean and Concentrator Kit. After measurement of DNA concentration, 75 ng of DNA were mixed with either forward or reverse primer, diluted with nuclease free water and sent for sequencing. Knock-out rate on genetic level was then determined by using TIDE.[73] In brief, raw data files of Sanger sequencing and a crRNA sequence were uploaded, and a test sample was aligned to a control sample. By comparing the sequences after the expected cut site of the crRNA the tool gave information about deletions and insertions (InDels) around the cut site. The sum of all InDel frequencies was determined as GR KO rate on DNA level.

2.3 PROTEIN LYSATE GENERATION

To generate protein lysates 1,5 – 2x10^6 cells were washed once with PBS and resuspended in 100 µl cold RIPA buffer. After 30 minutes of incubation on ice the mixture was centrifuged at 4°C with 15,000 g for 20 minutes. Subsequently the supernatant was stored as complete protein lysate at -20°C until Western Blot was performed.

2.4 BCA ASSAY

Protein concentrations of lysates were measured at FLUOstar® Omega using Pierce™ BCA Protein Assay Kit following manufacturer`s instructions. In brief, lysates, RIPA buffer and protein standards were thawed on ice. Standards and 1:2 with RIPA diluted lysates were pipetted in duplicates into a 96 well flat bottom plate. BCA solution was prepared and 200 µl BCA solution was added to each well while working fast and avoiding bubbles. The plate was incubated for 30 min at 37°C and subsequently absorbance was measured at the microplate reader. According to the standard curve and dilution factors, the lysates' protein concentrations were calculated.

2.5 WESTERN BLOT

Western Blots were performed following standardly used protocols. In brief, running and stacking gels were poured between two glass panels and a 10 well comb was added. After polymerization the gels were fixed into the chamber and running buffer was added. At least 20 µg of protein (in about 10 µl) were mixed with 6x Laemmli, heated to 95°C for 5 min and loaded onto the gel.[1] The gel was run at 90 V for 20 min and subsequently at 120 V for additional 70 min. Blotting onto a nitrocellulose membrane was performed using transfer buffer and applying 90 V for 70 min to the chamber.[1] This was followed by blocking the membrane in 5% milk/TBS-T for one hour at room temperature on a shaker and then incubating with primary GR antibody 1:500 (or 1:1,000) in 5% milk/TBS-T overnight.[1] After washing the membrane three times for 10 min with TBS-T on the shaker, primary GAPDH antibody staining followed (1:10,000 for one hour).[1] After another washing step, the secondary HRP antibody was added 1:5,000 in 5%milk TBS-T.[1] For detection the membrane was incubated with 1 ml of ECL solution for 1 min and developed after 5 – 30 min exposure time. Blots were analysed *via* Image J.[1] To this end, each GR band was normalized on the housekeeper band of the same sample. The normalized values were then used to calculate the GR KO rate by dividing GR KO samples by MOCK, or MOCK by UT.

2.6 IMMUNOFLUORESCENCE MICROSCOPY (IF)

Immunofluorescence microscopy was performed by Tanja Weißer. GR KO cells were fixed with 1% PFA and permeabilized with 0.1% TritonX-100.[1] Subsequently cells were labelled with purified GR (G-5) antibody 1:200 and stained with an AlexaFluor® 488 secondary antibody 1:100.[1] Stained cells were applied onto a Poly-L-lysine coated slide and closed under a cover slip with DAPI solution.[1]

3. FUNCTIONALITY ASSAYS

3.1 DEXAMETHASONE TREATMENT

Cells, which were SEB-stimulated und enriched due to IFN-γ-secretion, were used on day 8 after GR KO for functional testing of dexamethasone resistance. $0.5x10^6$ cells of each condition were seeded in 500 µl T-cell medium in a 48 well plate. Dexamethasone was added at 200 µM to the cells.[1] After 2 and 5 days of dexamethasone treatment, cells were resuspended and 150 µl each were washed and stained with 7-AAD (staining procedure described in C.4).[1] Cells were measured at MACSQuant flow cytometer.[1] In addition, $2x10^6$ cells were seeded in a 12 well plate. Dexamethasone was added in a concentration of 200 µM and cells were harvested after five days of incubation. Protein lysates were generated, and Western Blot was performed as described in C.2.3 and C.2.5.

3.2 CYCLOSPORIN A TREATMENT

Virus-specific GR KO and control cells were treated with 400 ng/ml CsA or with the same volume of ethanol without CsA for 5 days.[1] Subsequently cells were labelled with 1 µM CTV for 5 min at 37°C using the CellTrace Violet Proliferation Kit.[1] After removal of unbound dye and washing according to manufacturer´s instructions $2x10^5$ cells/well were seeded in a 96 well round bottom plate and re-stimulated with the CD3/CD28 stimulant TransAct.[1] 24 h, 48 h and 72 h after re-stimulation cells were stained for Viability, CD4 and CD8 and CTV intensity was measured *via* flow cytometry.[1] For evaluation of T-cell proliferation, cells with reduced CTV intensity compared to the 24h measurement were determined as proliferating cells, as the CTV amount/cell reduces with each mitosis.[1] Proliferation of T cells was calculated out of $CD4^+$ proliferating and $CD8^+$ proliferating cells taking the $CD4^+$/ $CD8^+$ proportion into account.

3.3 INTRACELLULAR CYTOKINE STAINING (ICS)

Virus-specific GR KO and control cells were harvested for ICS on d8. Cells were centrifuged at 450 g for 5 min, resuspended at $2x10^6$/ml in basic T-cell medium and incubated in a 24 well plate for 24 hours. Feeder cells were thawed or generated freshly and, in both cases, rested overnight in basic feeder cell medium. On day 9 feeder cells were counted, centrifuged at 450 g for 5 min and resuspended at $2x10^6$/ml in basic T-cell medium. $2x10^5$ of either virus-specific GR KO or control cells were co-cultured with feeder cells in a 1:1 ratio in a 96 well plate.[1]

Except for unstimulated control cells, cells were stimulated with CMV pp65 Peptivator (0,6 nmol per peptide/ml) or Cytostim (10µl/ml; positive control).[1] All wells were mixed gently by resuspending once with a multichannel pipet. After 2 hours of incubation at 37°C and 5% CO_2 10µg/ml Brefeldin A was added.[1] 6 hours after re-stimulation IFN-γ secretion was stopped by washing the cells with cold PBS.[1] Cells were stained extracellularly for Viability, CD3, CD4, CD8, CD56, CD45RO and CD62L to identify T-cell subsets.[1] Afterwards cells, except FMO control, were stained intracellularly for IFN-γ using FIX & PERM™ Cell Permeabilization Kit according to manufacturer´s instructions and were measured *via* flow cytometry.[1]

3.4 CYTOTOXICITY ASSAY

Autologous PBMCs were thawed and seeded at a concentration of $1x10^6$/ml in a 12 well plate (3 ml/well).[1] Cells were incubated in blast medium supplemented with 1 µg/ml PHA for three days to induce blast transformation.[1] Subsequently cells were washed and cultured in fresh blast medium containing 200 U/ml IL-2 for three days.[1] Cells were washed again and cultured in fresh blast medium containing 100 U/ml IL-2 for one day. Blasts were then resuspended in fresh blast medium and divided into 15 ml tubes. One condition remained unpulsed, whereas the other was pulsed with 20 µl/ml CMV pp65 Peptivator.[1] Blasts were incubated for 20 h, labelled with CTV (as described in 3.2) and resuspended in TexMACS GMP medium at $2x10^5$/ml to serve as target cells.[1] Virus-specific GR KO cells were harvested on day 7 and the T cell Enrichment Kit was used according to manufacturer's instructions to get rid of contaminating cells.[1] To serve as effectors, cells were then resuspended in TexMACS GMP.[1] Targets and effectors were co-cultured in a 96 well round-bottom plate at different effector-to-target (E:T) ratios for 24 h.[1] Unpulsed targets without effector cells served as control. PI was added 1:100 to the wells directly before flow cytometric measurement.[1] To calculate cytotoxic capacity, living target cell count in co-cultured wells was divided by averaged living target cell count in control wells.[1]

4. FLOW CYTOMETRY

Compensation was performed using compensation beads for antigen stainings and dead cells for viability stainings. Extracellular staining was performed in 50 µl staining buffer, adding the prepared antibody mastermix and staining the cells for 10 min on ice in the dark. Cells were washed once before and after staining. In case of low cell numbers, cells were not washed but diluted with staining buffer before acquisition.

Streptamer staining was done according to manufacturer´s protocol. In brief, 0.2 µg Streptamer, either CMV p65 NLVPMVATV or NY-ESO-1 SSLMWITQV as a control, was labelled with a Streptactin PE backbone.[1] The next day cells were adjusted to $1x10^7$/ml and stained with the labelled Streptamer for 45 min at 4°C. After washing, cells were additionally stained for CD3, CD4, CD8, CD56 and Viability.[1] Streptamer panel was measured at BD FACS Canto™ II Flow Cytometer, whereas all other measurements were performed at MACSQuant Analyzer 10.

5. STATISTICS

Statistics were performed with GraphPad Prism 5. If not indicated otherwise, paired t-test was used. Significances are indicated as follows: * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

D. RESULTS

1. GENERATION OF THE CELL PRODUCT

1.1 ENRICHMENT OF VIRUS-SPECIFIC T CELLS

To generate antigen-specific T cells and assess the efficacy of enrichment, the cytokine capture technique (CC) was used.[1] Therefore, freshly isolated PBMCs were stimulated with SEB or CMV pp65 for four hours and IFN-γ secreting cells were enriched *via* CC.[1] SEB is a superantigen, which stimulates a high percentage of T cells. Upon SEB stimulation, 6.5% of lymphocytes secreted IFN-γ. After the sort, the superantigen-responding cells were highly significantly (p=0.002) enriched to 59.6% in the positive fraction, whereas the negative fraction contained 2.5% IFN-γ^+ cells (**Figure 8**A).

CMV pp65, a peptide-pool, which covers the immune-dominant protein of CMV, showed similar results, though the initial frequency of pp65-specific T cells was much lower and highly donor-dependent.[1] There were 0.3% specific T cells in the original fraction, 48.3% in the positive fraction and 0.1% remaining cells in the negative fraction. The enrichment was highly significant (p=0.0047; **Figure 8**B).[1] To investigate enrichment *via* an IFN-γ-independent detection method, the original fraction as well as the positive and the negative fraction were stained using streptamer method. Therefore, a pp65 MHC I streptamer against the single peptide epitope NLVPMVATV was used.[1] The respective peptide-specific cells were enriched from 0.04% in the original fraction up to 1.97% in the positive fraction thereby confirming IFN-γ staining results (**Figure 8**C).[1]

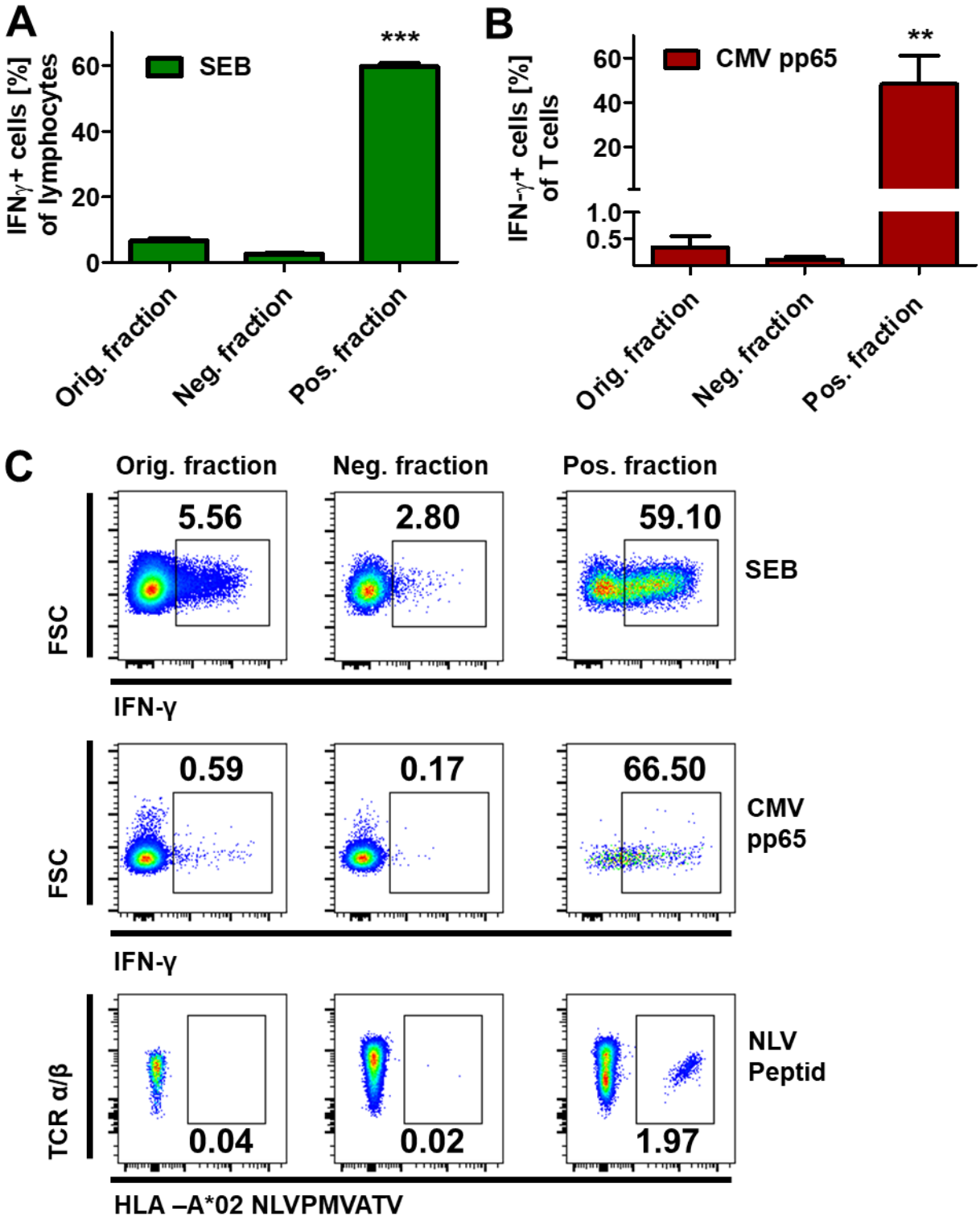

Figure 8 Enrichment of virus-specific T cells (A+B: based on [1]; C[1])

Enrichment of IFN-γ-secreting cells *via* CC after SEB-stimulation (A) or CMV pp65-stimulation (B) and detection of enriched cell population. Mean and SD of n=3 (A) or n=4 (B). Paired t-test; ** = p<0.01; *** = p<0.001. (C) Exemplary flow cytometry plots of cells before (orig. = original fraction) and after (pos. = positive and neg. = negative fraction) enrichment *via* CC. SEB and CMV pp65: Detection of extracellularly stained IFN-γ directly after CC, NLV Peptide: Detection of one CMV-specific T-cell receptor *via* HLA-A*02 NLVPMVATV Streptamer staining before and two weeks after CC. Pre-gated on CD8+ T cells.

1.2 CONFIRMATION OF THE GR KO

To evaluate the knock-out of the glucocorticoid receptor, cells were harvested at two time points after CC and CRISPR/Cas9-mediated GR KO. To assess the KO on DNA level, InDel frequencies were determined using TIDE and summed up to the GR KO rate (**Figure 9**A).[1] Independent of enrichment the KO rate remained stable, showing about 60% on day 5 (59.3 ± 6.8% in the positive fraction and 62.7 ± 9.9% in the negative fraction) and about 60% on day 8 (59.5 ± 5.0% in the positive fraction and 65.8 ± 3.4% in the negative fraction).[1] **Figure 9**C shows that a donor-independent InDel pattern could be observed.[1] The KO rate mainly consisted of 1-base insertions (27.1% frequency), 3-base deletions (11.9% frequency) and 1- and 8-base deletions (6.2% and 5.2%, respectively). With lower frequencies (2.5% and 1.8%) 13- and 15-base deletions were observed.

Similar results could be shown regarding the KO on protein level. To this end Western Blot pictures were analysed *via* Image J.[1] Independent on enrichment, the KO rate was stable around 90% on day 5 (84 ± 17% in the positive fraction and 91 ± 16% in the negative fraction), as well as on day 8 (79 ± 18% in the positive fraction and 90 ± 8% in the negative fraction; **Figure 9**B).[1] KO rate on protein level was higher than on DNA level (84% in the positive fraction on day 5 on protein level, compared to 60% on DNA level). A representative Western Blot is shown in **Figure 9**D, where a clear reduction of the GR band is visible in the GR KO lanes. A highly significant correlation between KO on DNA level and KO on protein level was observed ($p<0.0001$; Pearson $r= 0.9469$), as shown in **Figure 9**E. To visualize the KO, immunofluorescence microscopy pictures were generated. In the GR KO sample most of the cells show a reduction in GR staining intensity (**Figure 9**F).

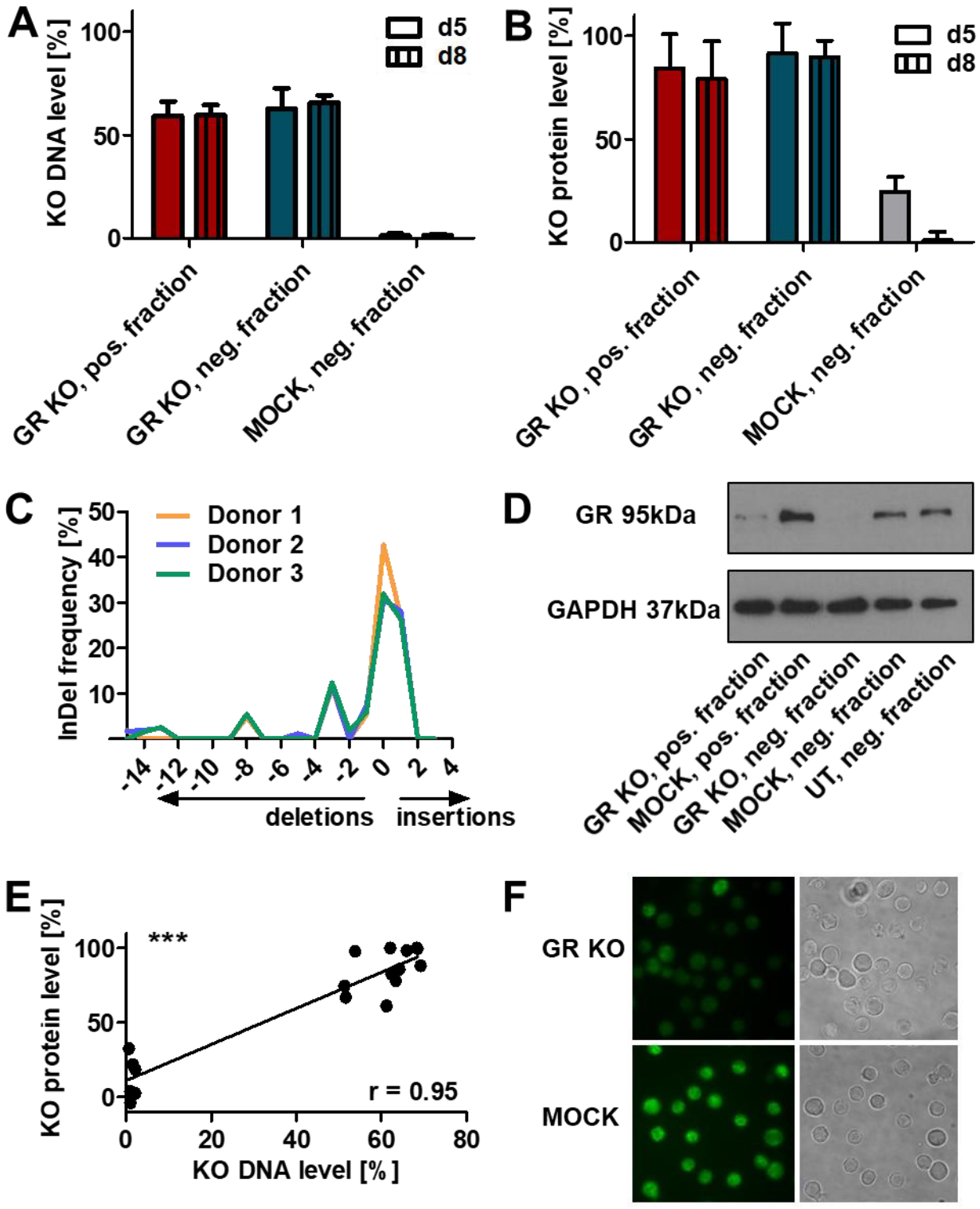

Figure 9 Confirmation of the GR KO *via* CRISPR/Cas9 on genetic and protein level (A-D; F[1])

GR KO cells after CC using SEB-stimulation. Cell harvest on d5 and d8 after GR KO electroporation using the crRNA GR1. (A) Total frequency of insertions and deletions (InDels) at the CRISPR cut site by comparing two PCR-amplified and Sanger-sequenced DNA fragments, using the webtool TIDE. (B) KO rate on protein level *via* Western Blot and Image J analysis. Mean and SD n=3. (C) Characteristic InDel profile of the crRNA GR1, shown with three different donors. InDels with a p-value <0.05 in TIDE are depicted. (D) Representative Western Blot of GR KO and control samples. (E) Correlation between KO evaluation on genetic and protein level, n= 18; Pearson`s r=0.95, *** = p < 0.0001. (F) Microscopy of GR KO and control sample. GR in immunofluorescent staining with GR (G-5) and Alexa Fluor 488 secondary antibody.

To investigate crRNA-specific KO effects, KO rates and InDel profiles of two different crRNAs were compared.[1] Therefore, the above results (crRNA GR1), were compared to the KO rates and InDel pattern of a second crRNA (crRNA GR2).[1] As exon 1 is an untranslated region, both crRNAs are located in the beginning of exon 2 of the GR gene, to avoid expression of a truncated protein version.[1] **Figure 10**A shows the exact DNA sequences of the on-target binding sites of both crRNAs. The binding sites have been chosen based on a PAM motif located directly upstream of the cut site, which is between the third and fourth base of the DNA binding site. Exon 2 codes for the N-terminal domain, which contains the activation function region AF-1.[1] **Figure 10**B visualizes that the CRISPR/Cas9 cut sites are located directly upstream (crRNA GR1) or at the beginning of AF-1 (crRNA GR2). The comparison of the KO on DNA level showed a non-significant tendency of higher rates using crRNA GR2 (crRNA GR1=59.3 ± 6.8% and crRNA GR2 69.7 ± 16.5%; **Figure 10**C). Thus, GR2 was used to generate the virus-specific GR KO T-cell product in the following experiments.

When comparing the InDel profiles of crRNA GR1 (see also **Figure 9**C) to those of crRNA GR2, a different pattern was observed.[1] Whereas crRNA GR1 induced mainly +1 insertions with a frequency of 26.9% and various deletions (from 1-base up to 15-base deletions), using of crRNA GR2 resulted in little +1 insertions (1.3%), but 52.7% 2-base deletions and other smaller deletions (from 1- to 6-base; **Figure 10**D).

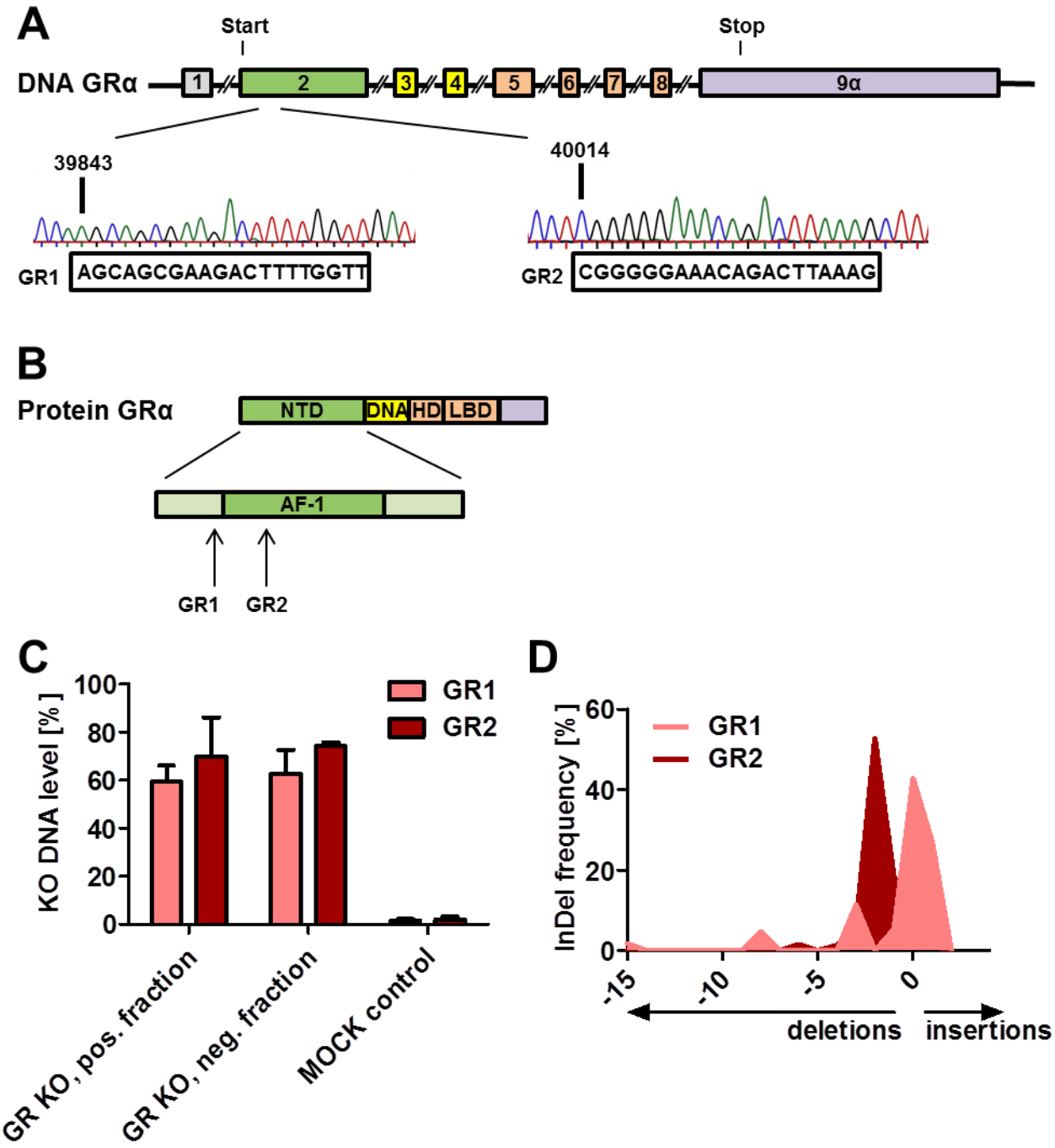

Figure 10 GR gene locus and comparison of different CRISPR/Cas9 cut sites (A[1]+B: based on [55]; D[1])

(A) Genomic DNA of the GRα gene locus with its exons (1 - 9α) and introns, as well as Start and Stop Codon. Region of exon 2, including the crRNAs GR1 and GR2 binding sites. Chromatograms were obtained *via* Sanger Sequencing. (B) Translated and spliced protein components of the GR. NTD = N-terminal domain; DNA = DNA binding domain; HD = Hinge domain; LBD = ligand binding domain. NTD in detail containing AF-1 = activation function-1. (C) Genetic KO efficacy of GR1 and GR2 analysed *via* TIDE. For GR1, cells after CC with SEB-stimulation were harvested on d5 after electroporation. For GR2, cells after CC with pp65-stimulation were harvested on d4 after electroporation. Mean and SD of n=3. (D) InDel profiles of crRNA GR1 and GR2. The DNA of cells after CC with SEB-stimulation on d5 was analysed *via* TIDE, one representative donor is depicted, respectively.

2. CHARACTERIZATION OF THE CELL PRODUCT

2.1 PURITY

To examine the purity of the cell product, cells were harvested at the beginning, on d2 and d8 of the protocol and cellular composition as well as viability was determined *via* flow cytometry.[1] Cells, which were double negative or double positive for CD4 and CD8 or cells, which were negative for all stained surface markers, were declared as “Others”.

Figure 11 shows that the starting material contained 29.8% $CD4^+$ T cells and 13.9% $CD8^+$ T cells and other leukocyte subsets such as monocytes (9.3%), B cells (8.6%) and granulocytes (1.7%).[1] However, the final virus-specific GR KO cell product had a purity of T cells of 87%, showing 1.6% of contaminating NK T cells, 0.4% NK cells, 0.9% B cells and no monocytes and granulocytes.[1] Most T cells were $CD4^+$ T cells at this time point (52.0% $CD4^+$ and 35.0% $CD8^+$ T cells). On day 8, NK T cells and NK cells were observed (7.3% and 0.8%, respectively). The ratio between $CD4^+$ and $CD8^+$ T cells shifted in favour of $CD8^+$ T cells, while the total purity of the product stayed stable at more than 85% (34% $CD4^+$ and 51% $CD8^+$ T cells). Representative flow cytometry plots in **Figure 11**B show the disappearance of $CD14^+$ monocytes and $CD19^+$ B cells (third row), as well as the increase of $CD56^+$ NK T and NK cells in the first and the last row .The disappearance of eosinophil ($CD56^-$ $CD16^-$ SSC high) and neutrophil ($CD16^+$ SSC high) granulocytes is visible in the last row (0.2% eosinophils and 1.5% neutrophils in PBMCs, compared to 0.0% on day 2 and day 8).

The starting material showed a viability of 98%, whereas the virus-specific GR KO cells on day 2 had a slightly decreased viability of 95%, increasing to 97% until day 8 (**Figure 11**A, lower row).[1]

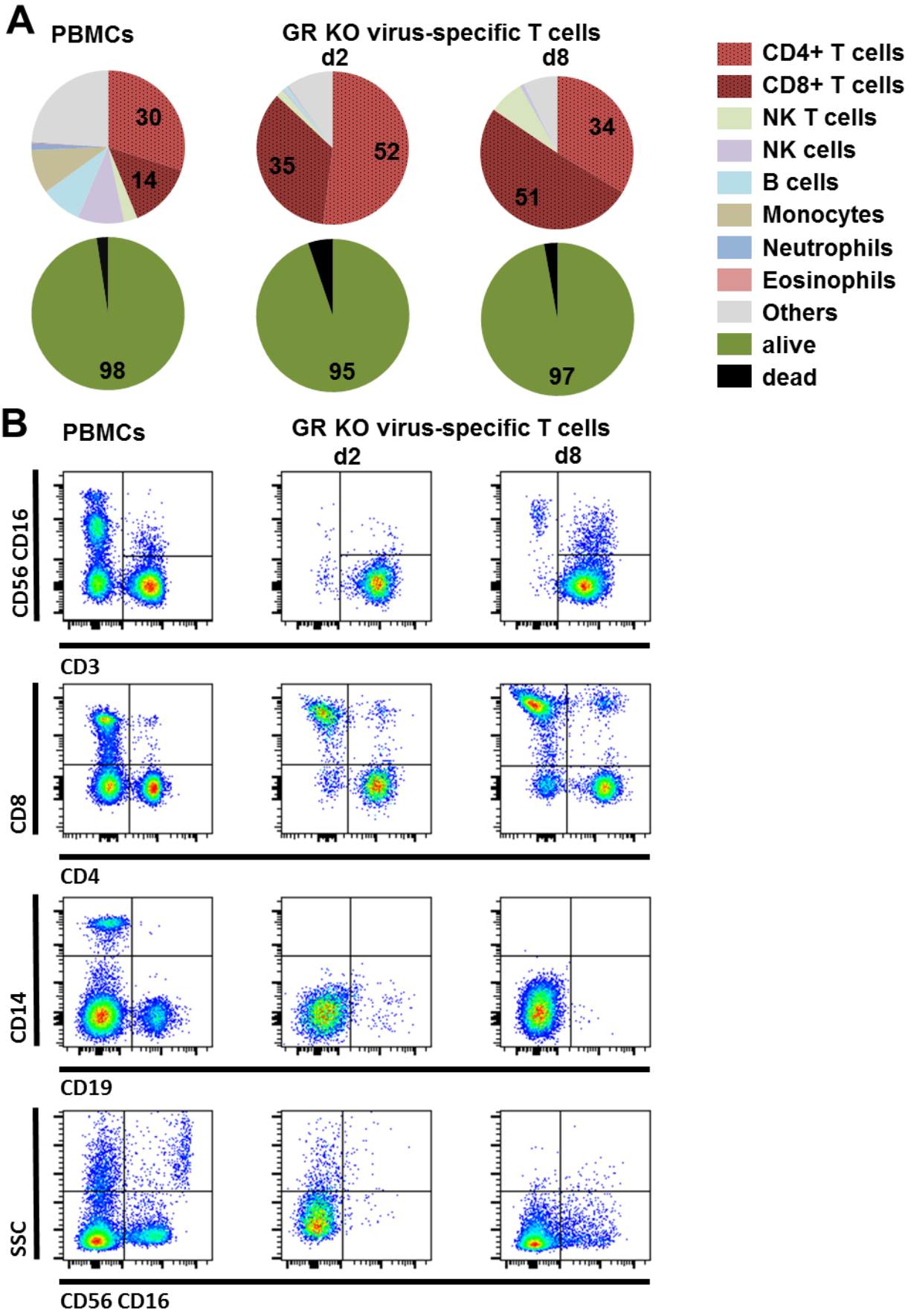
A
PBMCs
GR KO virus-specific T cells
d2
d8
30
14
98
35
52
95
34
51
97
CD4+ T cells
CD8+ T cells
NK T cells
NK cells
B cells
Monocytes
Neutrophils
Eosinophils
Others
alive
dead
B
PBMCs
GR KO virus-specific T cells
d2
d8
CD56 CD16
CD3
CD8
CD4
CD14
CD19
SSC
CD56 CD16

Figure 11 Purity of the cell product over time (A[1])

Distribution of cell types among viable cells as well as proportion of viable cells in the starting material and the virus-specific GR KO cells on d2 and d8. Mean of n=3 is pictured (A). Representative flow cytometry plots of one donor. Pre-gated on CD45+ leukocytes (B).

2.2 PHENOTYPE

To further examine the T-cell subsets during the protocol, cells were harvested at various time points and stained for the phenotypic markers CD45RO, CD62L and CD95.[1] T cells were classified into five different phenotypic subsets according to their maturation stage: Naïve T cells (Tn; CD45RO- CD62L+ CD95-), stem-cell like memory T cells (Tscm; CD45RO- CD62L+ CD95+), central memory T cells (Tcm; CD45RO+ CD62L+ CD95+), effector memory T cells (Tem; CD45RO+ CD62L- CD95+) and effector T cells (Teff; CD45RO- CD62L- CD95+).[1] **Figure 12**A shows the high frequency of naïve T cells (57.1%) and a low number of effector memory (18.6%) and effector cells (7.0%) in freshly isolated PBMCs.[1] After the CC on day 0 a change in phenotype occurred, as effector memory (51.9%) and effector T cells (21.9%) were enriched in the positive fraction.[1] There were no drastic changes due to electroporation observable, except the disappearance of naïve T cells and an increase in stem cell like memory T cells. The final cell product on day 2 contained 14.0% Tscm, 27.2% Tcm, 39.2% Tem and 19.6% Teff cells. During culture central memory T cells showed a tendency to increase up to 32.6% again on day 8. The GR KO had no impact on the phenotype compared to the MOCK control.[1]

To confirm that the mature phenotype is due to CC and not caused by high expansion rates, cells were counted at the same time points as the phenotype was measured. As the phenotype mainly changed between day -2 and day 0 but remained stable during expansion, it can be concluded, that expansion didn´t influence the phenotype of the T cells negatively. **Figure 12**B shows the expansion of GR KO and MOCK control cells. The GR KO did not influence the expansion of the cells, which was 17-fold from day 0 to day 8, respectively.

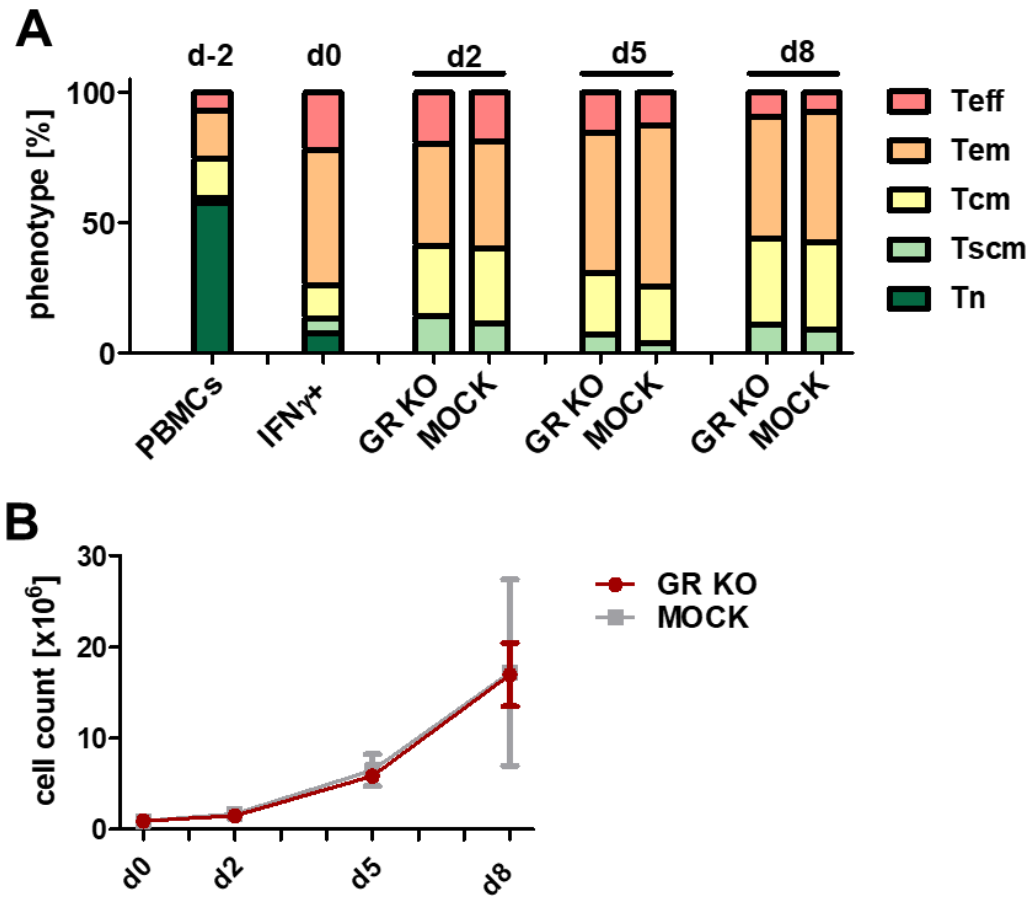

Figure 12 T-cell phenotype and expansion of the cell product (A[1])

Phenotype and cell count assessment *via* flow cytometry and microscopic count at various time points during CC after SEB stimulation and culture. (A) Phenotypes of T cells, $CD4^+$ and $CD8^+$ T cells averaged. Each section of a bar represents the mean of n=3. (B) Expansion of the cell product. Mean and SD of n=3.

2.3 DRUG SENSITIVITY AND RESISTANCE

To assess whether the GR KO really leads to glucocorticoid resistance, GR KO and MOCK cells were treated with 200 µM dexamethasone.[1] Lymphocyte count was measured *via* flow cytometry. Protein lysates were generated before and after the treatment and Western Blot was performed. GR KO cells proliferated during five days of high-dose dexamethasone treatment (from $7.5x10^4$ cells on day 0 to $22.6x10^4$ cells on day 5), whereas MOCK cells showed stagnation in growth (from $7.5x10^4$ cells on d0 to $10.7x10^4$) and cell death ($4.5x10^4$ on day 2; **Figure 13**A).[1] This difference was highly significant on day 5 ($p=0.002$).[1] Furthermore, the KO rate was increased due to dexamethasone treatment (**Figure 13**B).[1] Facing safety issues, the effect of GR-independent immunosuppressive drugs on GR KO cells was investigated.[1] As cyclosporin A (CsA) is known to be a commonly used drug in the *post*-HSCT setting and suppresses T-cell proliferation, the virus-specific GR KO cells were treated with a clinically low peak level of CsA (400 ng/ml).[1] **Figure 13**C shows the effect of 400 ng/ml CsA on GR KO and MOCK control cells. Whereas 82.0% of $CD4^+$ and $CD8^+$ T cells were proliferating after 72 h in the virus-specific GR KO cells without CsA, only 25.6% did in the CsA treated condition. With 82.4% proliferating T cells without CsA and 37.2% with CsA, the MOCK control cells showed similar results. Thus, CsA significantly inhibited proliferation in GR KO as well as MOCK cells without significant difference between both cell types.[1] The representative flow cytometry histogram in **Figure 13**C shows the difference in proliferation between GR KO control-treated cells and GR KO cells upon CsA treatment.

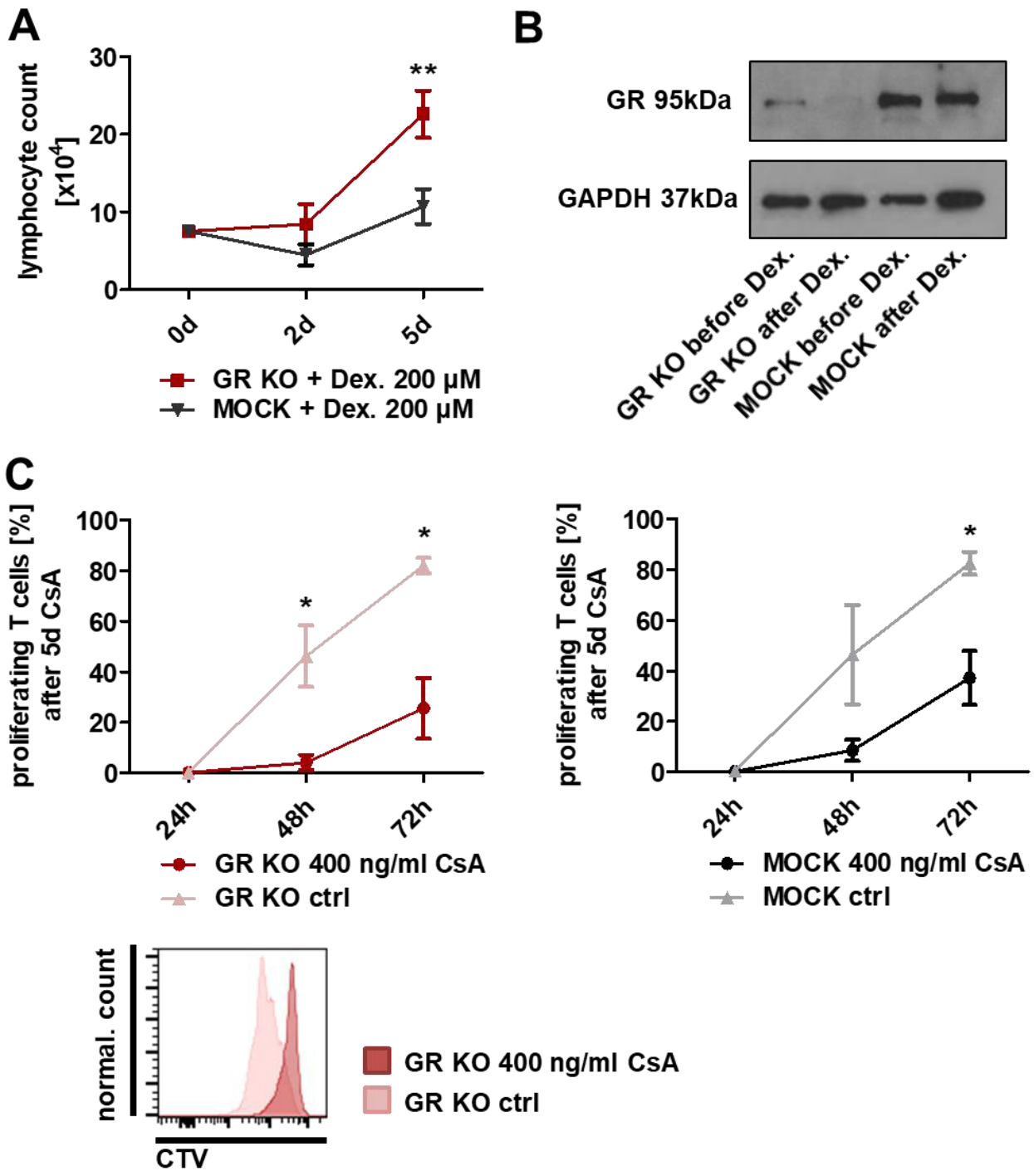

Figure 13 Influence of dexamethasone and cyclosporin A on GR KO cells [1]

(A) and (B) The cell product was enriched upon SEB stimulation and was cultured with 200 µM of dexamethasone starting on d8. After two and five days of dexamethasone incubation 150 µl of cell suspension was washed and lymphocyte count was assessed in flow cytometry. Mean and SD of n=3 (5d) or n=2 (2d); paired t-test; ** = $p<0.01$ (A). Western Blot of GR KO and control cells before and after dexamethasone (200 µM) treatment. Lysates were generated before start (d8) and after five days of dexamethasone incubation (B). Virus-specific GR KO and control cells were cultured from d10 on with 400 ng/ml cyclosporin A. On d15 CTV labelling and CD3 CD28 restimulation took place. Measurement of proliferating CD4+ and CD8+ T cells *via* flow cytometry 24 h, 48 h and 72 h after re-stimulation. CD4+ and CD8+ proliferating cells were averaged. Mean and SD of n=3 is shown. Paired t-test; * = $p<0.05$. Representative overlay histogram of GR KO CD8+ cell proliferation on d3, CsA or control-treated (C).

2.4 FUNCTIONALITY

To test the functionality of the virus-specific GR KO T cells, IFN-γ secretion upon specific restimulation, cell proliferation and cytotoxic capacity was evaluated.[1] To this end GR KO and MOCK cells, each of positive and negative fraction, were specifically restimulated with CMV pp65 and IFN-γ secretion and cytotoxicity were investigated, as well as proliferation capacity.[1] **Figure 14**A and B show, that the virus-specific GR KO T cells specifically secret IFN-γ upon restimulation (11.3%), compared to 1.5% in the unstimulated control (p=0.0249).[1] The cells of the negative fraction secreted very low IFN-γ levels upon pp65 restimulation (0.8% IFN-γ^+ cells). Compared to the KO samples of the negative fraction, the IFN-γ secretion in the KO sample of the positive fraction was highly significantly increased (p=0.005). In addition, the positive control shows that all samples still have a very high IFN-γ secretion capacity. When looking at the phenotype of IFN-γ secreting cells, effector memory (71.1%) and effector T cells (26.0%) were present (**Figure 14**C). This phenotypic pattern is comparable with the pattern observed after initial stimulation and enrichment, as in both cases effector memory T cells and effector T cells are the biggest part (compare **Figure 12**A).

Figure 14D gives an overview of the cell counts during T-cell product generation and expansion. While in the mean $13.9x10^7$ PBMCs were used for stimulation, $3x10^5$ cells were selected as CMV-specific. The virus-specific cells were activated and expanded until GR KO and MOCK electroporation on day 0. Until day 8 the virus-specific GR KO cells expanded up to 88-fold ($5.3 \pm 2.3x10^7$) and the MOCK control up to 130-fold ($7.8 \pm 3.3x10^7$) cells. The virus-specific GR KO cells had no significant disadvantage in expansion compared to the MOCK control.

To investigate the cytotoxic effect of the T-cell product, the generated cells of one donor were co-cultured with PHA blasts generated from autologous PBMCs.[1] Blasts were either pulsed with CMV pp65 Peptivator or remained unpulsed and were used in effector-to-target ratios of 4:1; 2:1 and 1:1. At the highest E:T ratio target cell lysis was 32.9% for pp65-pulsed blasts, compared to -8.7% in the co-culture with unpulsed blasts, which indicates a specific and highly significant cytotoxic effect (p=0.0042). A significant difference of 13.1% versus -8.3% between pp65-pulsed and unpulsed target cell lysis could be detected at E:T 2:1 (**Figure 14**E).

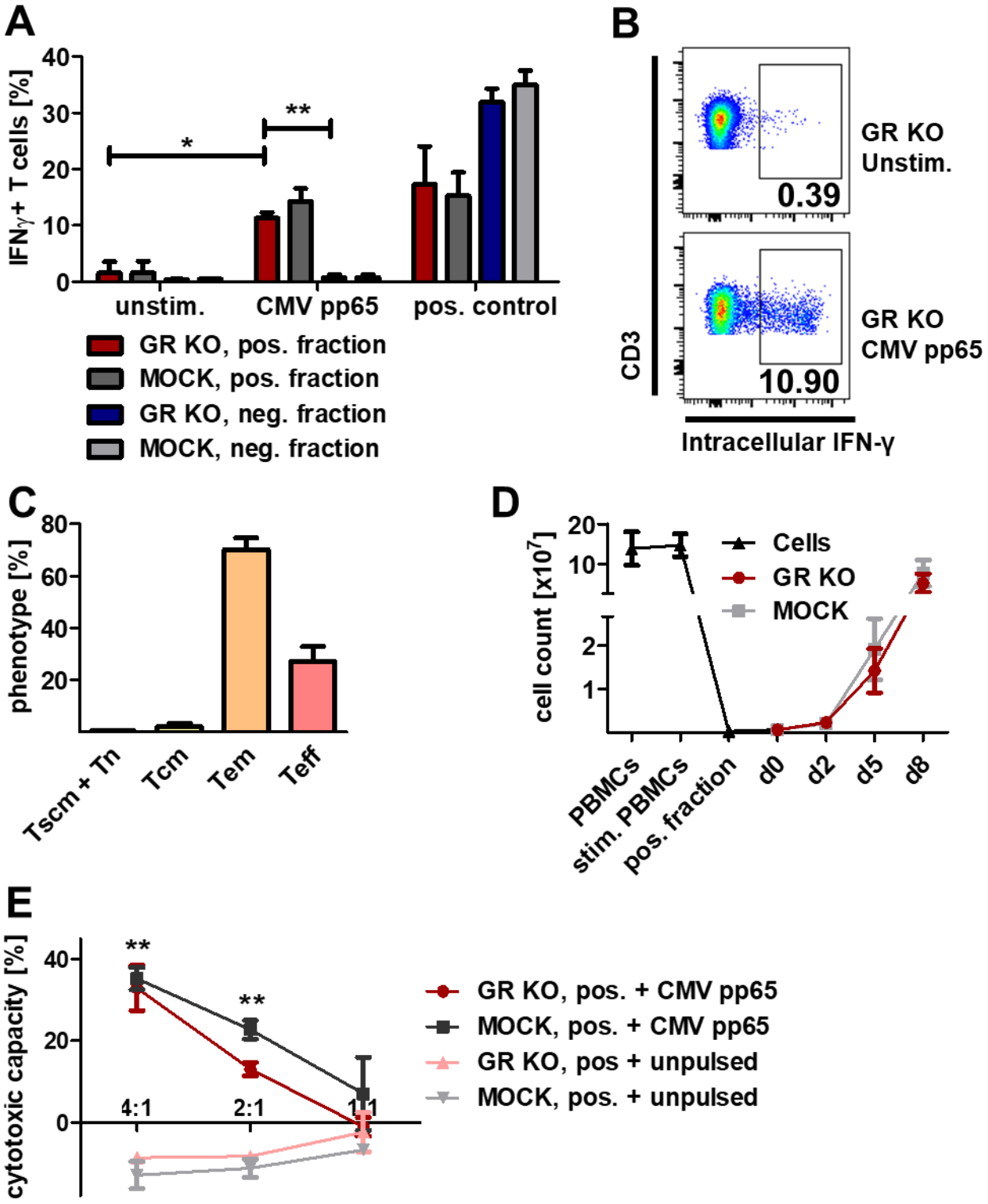

Figure 14 IFN-γ secretion, proliferation capacity and cytotoxicity of virus-specific GR KO cells (A based on [1])

Virus-specific GR KO T cells were restimulated with pp65 on d9. As negative controls unstimulated cells and pp65-stimulated cells of the negative fraction were included; as positive control, cells were stimulated *via* CD3 CD28. Cumulated IFN-γ was stained intracellularly. (A) Mean and SD of n=3; paired t-test; *= p< 0.05; **= p< 0.01 (B) Representative flow cytometry plots. Pre-gated on CD3+ CD56- T cells. (C) Phenotype of IFN-γ+ cells in the cell product. Mean and SD of n=3. (D) Cell counts during T-cell production process. Mean and SD of n=3. Cytotoxic capacity of virus-specific GR KO and MOCK T cells against CMV pp65-pulsed or unpulsed autologous PHA blasts. E:T ratios 4:1; 2:1; 1:1. Mean and SD of technical triplicates; n=1. Paired t-test; **= p< 0.01 (E).

E. DISCUSSION

As there is still the need for new therapeutic options in the context of viral infections in immunocompromised patients, adoptive T-cell transfer with glucocorticoid-resistant virus-specific T cells could be a promising therapy. A previous study using streptamer technique for isolation and TALEN for T-cell editing was done by *Menger* et al.[74] In the present study, the focus was on establishing a cytokine capture-based isolation and CRISPR/Cas9-mediated knock-out, which is attractive for a straightforward implementation into clinical procedures, and on the extensive characterization of the generated cell product. Following this study, *Amini* et al. generated Tacrolimus-resistant T cells whereas *Basar* et al. and *Koukoulias* et al. developed multi-virus specific glucocorticoid-resistant T cells using *in vitro* stimulation.[75-77]

1. KEY FINDINGS

Enrichment of pp65-specific T cells *via* CC was performed. With a short stimulation and expansion period in between, a subsequent CRISPR/Cas9-mediated knock-out of the GR revealed over 80% KO rate on protein level. The final cell product consisted of 87% T cells with a stable effector-memory phenotype. The virus-specific glucocorticoid-resistant T cells were highly functional in terms of IFN-γ release upon restimulation, proliferation and cytotoxicity against specific target cells. Dexamethasone resistance of GR KO cells, as well as cyclosporin A sensitivity was shown.

2. ENRICHMENT AND GR KO

2.1 ENRICHMENT RATE IN DIFFERENT METHODS

Feuchtinger et al. were the first to treat patients with pp65-specific T cells generated with the CC method on CliniMACS device. The mean donors' T-cell response was 0.56% IFN-γ^+ in the original fraction. With a donors' T-cell response of 0.3±0.2% in the original fraction the present results were in the range of published data. The positive fraction contained 65% IFN-γ^+ cells in the study of *Feuchtinger* et al.[4] Averaged enrichment rates between 42% and 72% of virus-specific T cells using CC are reported in different studies for CMV, AdV and EBV.[78-80] This is highly donor-dependent, as enrichment varies from 10 – 97%.[4] With about 50% enrichment for pp65-specific cells the results are within the range of previous studies. *Amini* et al. recently used a very similar protocol resulting in higher enrichment rates.[75] A longer initial stimulation time may be the reason. However, in contrast to studies using CliniMACS device, present data were generated manually, which may explain differences in enrichment rates. In comparison with studies using streptamer technique, CC tend to result in lower purities of virus-specific T cells. For streptamer technique mean enrichment rates up to 98.8% are reported,[34] but also varieties in purity from 13.4% to 99.5%.[81] Studies using *in vitro* expansion show purities of up to 96% pp65-specific CD8$^+$ T-cells[82] and streptamer-positive T cells around 30% with ranges from 6% to 75%.[83,84] However, no correlation between clinical outcome and enrichment rates was observed.[4] With a range of 29.9% (36.6% minimum and 66.5% maximum) the results show donor-dependencies similar to other publications.

2.2 GR KO ON DNA AND PROTEIN LEVEL

GR KO efficacy was higher on protein level than on DNA level. One explanation could be the difference between gene integrity and gene expression, for which transcription, translation and trafficking is needed. Whereas the GR gene may be still present in about 40% of the cells, only 10-20% are expressing the GR on protein level. The previous publications using CRISPR/Cas9 system for gene knock-outs in primary human T cells show efficacy rates between 10% and 80% KO on protein level.[85,86] The GR has been targeted first *via* TALEN, where a high KO rate of 98% in T cells was shown.[74] In a recent study, lower KO rates for the GR using CRISPR/Cas9 with a single crRNA were shown.[77] *Basar* et al. reached KO efficacies on DNA and protein level of 82 – 98% using two crRNAs to target the GR simultaneously.[76] With 80 – 90% KO on protein level the present results are in the upper range of published results, reaching similar efficacies than with using two crRNAs or TALEN method. Recently, the location of crRNA reaching the highest GR KO rates was confirmed to be in the middle of

exon 2.[77] In comparison with published data the present protocol was confirmed to be stable and highly efficient. As the enrichment rate of CMV-specific cells was about 50%, there were a mix of CMV-specific and non-CMV specific cells for the KO induction. However, no difference in the KO efficacy between specific and non-specific cells in the positive fraction is expected, as the KO worked comparable in the positive and the negative fraction, which was used as a control. Since it is easily applicable and possible to implement into a closed automatic system, CRISPR/Cas9 is a promising tool for generating therapeutic T-cell products.[87]

2.3 gRNA-DEPENDENT INDEL PROFILE

DSBs are repaired by NHEJ, which induces small insertions and deletions and thereby is used for KO induction.[35] There is a donor independent InDel profile for different gRNAs which is in line with previous observations. These findings underline that each gRNA has its own recognisable specific InDel pattern. Small +1 insertions and deletions of a few base-pair size, which peak around the expected CRISPR cut site 3-4 base pairs upstream of the PAM, are most frequent. This is in line with the present results, as crRNA GR1 resulted mainly in +1 insertions and crRNA GR2 in 2-base deletions. Insertions are typically composed of one nucleotide, specific for the gRNA. How one nucleotide is selected by the repair system is still not known.[73] Insertions due to crRNA GR1 consistently were thymine (about 70% of insertions) and adenine (25% of insertions; data not shown) additions, which confirms previous observations. The location and sequence of the gRNA may be responsible for this reproducible InDel formation. Furthermore, also the delivery method of the CRISPR/Cas9 system may influence the InDel formation. Delivering the RNP complex into the cell *via* electroporation leads to early detectable InDels and stable efficacies with a maximum at 2-3 days after electroporation.[88] High and stable KO efficacies at day 5 and day 8 in the present project support published theories. These results underline the advantages of CRISPR/Cas9 system. It is easy to adjust to specific requirements, as new gRNAs and therefore new target sites can be designed very fast and price effective. The KO efficacies are stable, high and donor independent.

3. CHARACTERIZATION OF VIRUS-SPECIFIC GR KO T CELLS

3.1 DESIRABLE PHENOTYPES AND PURITIES FOR T-CELL THERAPY

As Tcm have a high proliferation capacity and the rapid differentiation possibility into effector stages, this T-cell subset is described as the preferable in cell products for adoptive T-cell therapy.[89] Early and specific proliferation is known to be needed *in vivo* for a successful viral clearance.[4] A product consisting of all maturation stages with a predominance of effector memory and effector T cells, followed by central memory T cells, is reported for CC.[4,78,80] Products generated *via in vitro* stimulation show effector and effector-memory T cells with only small numbers of less differentiated phenotypes.[83,90] As challenging with their antigen drives the T-cell differentiation, CC and *in vitro* stimulation result in more mature phenotypes. Using streptamer technique, staining and isolation do not change the T cells' phenotype and functionality, when performed at low temperatures (4°C).[30] In line with published CC results, the data also show about half effector-memory T cells, followed by effector T cells after the enrichment.[75] Central memory and stem-cell like memory T cells were present in smaller fractions. However, the final cell product had a stable amount of less mature phenotypes, probably due to expansion of less differentiated stages, which could be underlined by a recent study.[75] Though CC and *in vitro* expansion usually show more mature phenotypes, there has not been reported any correlation with outcome in clinical studies.[33] This may be explained by the fact that even one specific T cell with an immature phenotype is enough to proliferate and mediate immunity.[89,91] Furthermore, distribution of phenotypes did not differ between KO and control samples, suggesting no negative effects of CRISPR technology on T-cell phenotype. This was reproduced by recent studies,[75,76] which is a promising result in the perspective of adoptive cell transfer.

Regarding cellular composition of the cell product, clinical studies with *in vitro* stimulated products show over 90% $CD3^+$ cells, with predominantly $CD8^+$ T cells.[83,84] Small numbers below 1% of contaminating B cells and monocytes, and higher amounts of NK cells with huge donor-dependent differences (0.1% – 68%) are reported.[83] In contrast, studies using CC show a higher fraction of $CD4^+$ T cells.[78,79] With 87% $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells present results are similar. With more than half $CD4^+$ T cells on day 2 and increasing rates of $CD8^+$ T cells during culture, the results confirm published CC data. As product generation requires no long culture-period, contaminating NK cells, B cells and monocytes in this product are very low (less than 2%).

3.2 DEXAMETHASONE AND CSA EFFECTS ON VIRUS-SPECIFIC T CELLS

Previous studies have shown the long-term persistence of immunity through transferred virus-specific T cells after HSCT.[84,92] After solid organ transplantation however, persistence is more limited due to ongoing needed immunosuppression.[93] In the context of co-administration of glucocorticoids for GvHD prevention or treatment and virus-specific T cells, there is no controlled clinical study available so far. Some studies reported patients, where the co-administration of glucocorticoids during the T-cell transfer didn't influence the *in vivo* efficacy of the T cells.[80,94] However, because of the absence of studies, glucocorticoid treatment was usually stopped or decreased before T-cell transfer to increase the risk of a successful T-cell therapy. This led to a reactivation of GvHD in several patients.[84,94] Furthermore, CMV reactivation in responding patients correlated with glucocorticoid treatment. *Peggs* et al. reported two patients with high T-cell counts, who nevertheless had a CMV reinfection after receiving high doses of glucocorticoids. *Feuchtinger* et al. had a similar case with one patient.[4,78] To study the adverse effects of glucocorticoids on virus-specific T cells *in vitro*, *Campidelli* et al. treated virus-specific T cells with methylprednisolone and found a significant decrease in early and specific proliferation of the cells,[95] which is known to be crucial for a successful viral clearance.[4]

GR KO cells showed resistance with 2 to 3-fold expansion under treatment *in vitro*. In contrast, MOCK cells were inhibited with many apoptotic cells, especially until day 2. Not all control cells died during the treatment, which could be due to the different sensitivities of different activation and maturation stages. Results show consistency with effects described by other groups.[74,76,96] *Koukoulias* et al. used similar dosage of dexamethasone (100µM) and observed superior proliferation and vitality of KO cells in comparison to control cells.[77] The KO rate even was increased during dexamethasone treatment in the present study, which may be the result of outgrowth of drug-resistant clones. This mechanism was also shown *in vivo* for human T-ALLs.[97] *Basar* et al. recently confirmed dexamethasone resistance of GR KO cells in an *in vivo* mouse model.[76]

CsA inhibited activation GR-independent as published previously,[70] whereas pretreatment of T cells with CsA before restimulation led to a suppression of the T-cell response.[72] Doses of cyclosporin A, that are in the range between peak and trough levels of clinical immunosuppressive therapy,[71] were enough to strongly suppress the proliferation of GR KO T cells *in vitro*.

3.3 IFN-GAMMA SECRETION AND CYTOTOXICITY

11% IFN-γ^+ T cells were detected in ICS after specific restimulation. *Rauser* et al., and more recently *Basar* et al. show higher rates of IFN-γ^+ cells in the ICS (27% and up to 50%, respectively).[98] This difference could be explained by the different protocols and enrichment methods. As an additional anti-CD3-CD28 stimulation was required for expansion and activation before KO induction in this project, this was a stronger pre-stimulation as well as one stimulation more compared to previous protocols. This may cause the decreased cytokine secretion capacity upon the third stimulation in the ICS.

Cytotoxicity assay of the cell product showed more than 30% specific cell lysis of CMV-pulsed PHA blasts. *Koukoulias* et al. report a similar experimental setup with consistent cytotoxic effects but with higher E:T ratios needed. However, in this study the E:T ratio was calculated on used bulk effector T cells and thus the number of real virus-specific effectors was lower. Only specific and functional T cells in a late effector stage can mediate target cell lysis. As target cells, pp65-pulsed or unpulsed PHA blasts generated from autologous PBMCs were used. As autologous cells and not immortalized cell lines were used, this is closer to an *in vivo* situation than other previously described approaches.[74,98]

4. OUTLOOK

Functional experiments such as analysis of IFN-γ secretion capacity, the cytotoxic effect on target cells and the proliferation can be additionally performed during dexamethasone treatment, mimicking a more clinical setting. This data was added by our group following my fundamental work and all data was published.[1] To confirm efficacy and safety of the glucocorticoid-resistant virus-specific T-cell product *in vivo*, experiments in a humanized GvHD as well as CMV mouse model are needed. Our publication paved the way for other groups who recently confirmed findings of this study and published more functional and *in vivo* data.[75-77] As the protocol is easily adaptable, not only CMV-specific glucocorticoid-resistant T cells but also multivirus-specific other drug-resistant T cells can be produced likewise.[75-77] In future, glucocorticoid-resistant virus-specific T cells could have a variety of possible applications. Not only after HSCT, but also for patients after solid organ transplantation they could be a promising therapy. Even during viral infection, immunosuppressive treatment cannot be stopped because of possible graft loss. With glucocorticoid-resistant T cells immunosuppressive therapy could be switched to glucocorticoids only to cure the viral infection through the T-cell product. Afterwards normal immunosuppressive regimen could be started again. Another new aspect may be glucocorticoid-resistant SARS-CoV-2-specific T cells for patients with severe Covid19, as they are also treated with glucocorticoids.[99] In summary, glucocorticoid-resistant virus-specific T cells are a promising therapy for a variety of patients. First clinical trials are on the way.

5. CONCLUSIONS

A glucocorticoid-resistant virus-specific T-cell product was generated *via* cytokine capture technique and CRISPR/Cas9 to provide a treatment option for patients suffering from chemo-refractory viral infections under ongoing immunosuppression. The T-cell product was characterized in terms of purity and phenotype. T-cell functionality was confirmed based on IFN-γ secretion, cytotoxicity, and proliferation *in vitro*. Furthermore, resistance to glucocorticoids and sensitivity to rescue treatments was demonstrated. The developed protocol presents a high degree of automation capability and thus can be implemented straightforward into a GMP-conform system. Additional *in vitro* and *in vivo* investigations were published by different groups recently. First clinical trials are currently being planned. This project lays the foundation for a better treatment of glucocorticoid-treated patients suffering from severe viral infections.

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EIDESSTATTLICHE VERSICHERUNG

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