TECHNISCHE UNIVERSITÄT MÜNCHEN

Fakultät für Medizin

Interleukin-21 mediated generation of allorestricted central memory cytotoxic T cells directed against Ewing tumour-specific antigens

Christina Müller-Spahn

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität zur Erlangung des akademischen Grades eines

Doktors der Medizin

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. E. J. Rummeny
Prüfer der Dissertation :	1. Univ.-Prof. Dr. S. Burdach
	2. Univ.-Prof. Dr. A. Krackhardt

Die Dissertation wurde am 20.08.2013 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 02.04.2014 angenommen.

Table of contents

Abbreviations

APC	Allophycocyanin
BM	Bone marrow
BSA	Bovine serum albumin
cALL	Common acute lymphoblastic leukaemia
CD	Cluster of differentiation
CHM	Chondromodulin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EBV	Epstein-Barr-virus
EICESS	European Intergroup Cooperative Ewing's Sarcoma Study
ELISpot	Enzyme linked immune sorbent spot
ERG	Ets related gene
ES	Ewing sarcoma
ET	Ewing tumour
ETV1	ETS variant 1
EWS	Ewing's sarcoma oncogene
EZH	Enhancer of Zeste, Drosophila, Homolog
FBS	Fetal bovine serum
FCS	Fetal calf serum
FEV	Fifth Ewing variant
FITC	Fluorescein isothiocyanate
FLI	Friend leukemia integration
GB	Granzyme B
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPR	G Protein-coupled Receptor
G*v*HD	Graft *versus* host disease

G*v*L	Graft *versus* leukaemia
G*v*T	Graft *versus* tumour
HDT	High dose chemotherapy
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
LCL	Lymphoblastoid cell line
MART	Melanoma antigen recognised by T cells
MHC	Major histocompatibility complex
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PNET	Primitive neuroectodermal tumour
PRC2	Polycomb repressor complex 2
RCC	Renal cell carcinoma
SCT	Stem cell transplantation
STEAP	Six-Transmembrane Epithelial Antigen of Prostate
TAA	Tumour associated antigen
TCR	T cell receptor
TIL	Tumour infiltrating lymphocyte
TRAIL	TNF-related apoptosis-inducing ligand
TSA	Tumour self antigen

1. Introduction

1.1 Ewing`s sarcoma family of tumours

The Ewing`s sarcoma family of tumours (Ewing sarcoma, ES) comprises a group of tumours that all originate from the same primordial, presumably bone marrow-derived mesenchymal stem cell (Suvà et al., 2009; Tirode et al., 2007), which is possibly derived from a neural crest cell origin (Staege et al., 2004). It includes the classic Ewing sarcoma, the primitive neuroectodermal tumour (PNET), peripheral neuroepithelioma, adult neuroblastoma, peripheral neuroblastoma and the Askin tumour (Ewing sarcoma of the thoracic wall) (Delattre et al., 1994; Whang-Peng et al., 1984; Askin et al., 1979). The Ewing sarcoma is named after James Ewing, who first described it in 1921, and is also referred to as Ewing tumour (ET).

1.1.1 Incidence and symptomatology

Ewing`s sarcoma family of tumours is with 10% the second most common malignant primary bone tumour of children and accounts for 3% of all childhood malignancies (Lahl et al., 2008). It has an annual incidence of 3 / 1'000'000 in Caucasian children under 15 years of age (Paulussen et al., 2001) and has remained unchanged for the past three decades (Esiashvili et al., 2008). ES occurs nine times more often in Caucasians than African Americans (Jawad et al., 2009) and the ratio of male patients to female ones is 3:2. ET occurs most often in the second decade of life, the time of the highest bone growth, with a mean age of 15 of afflicted patients. It is very uncommon in people over 40 (Pieper et al., 2008) and only a few cases have been described in neonates and infants (Kim et al., 2008; van der Berg et al., 2008). Analysis of 975 Patients from the European Intergroup Cooperative Ewing's Sarcoma Study Group showed that the most common primary bone tumour site is the pelvis with 25%, followed by the femur with 16%, the ribs with 12% and the spine with 8% (Cotterill et al., 2000). The most common tumour location of extraosseous Ewing's sarcoma is the trunk with 32%, followed by the extremities with 26%, the head and neck with 18% and the retroperitoneum with 16% as presented by 130 patients registered on three Intergroup Rhabdomyosarcoma Study clinical trials (Raney et al., 1997).

The most common symptoms of ET are pain in the bone, which may vary in presence and intensity, and a swelling. A pathological fracture due to weakening of the bone mass by the tumour and local inflammation signs may occur. Less common symptoms include fever, lethargy, a tingling pain accompanied by numbness, weight loss and loss of appetite. The symptoms may increase slowly over weeks or appear suddenly. Occasionally, the patients experience intervals of symptom freeness. Due to the unspecificity of the symptoms it often takes months before the right diagnosis is made as ET is often mistaken for trauma, tendonitis, osteomyelitis or growing pain. About 25% of patients have metastases at the time of diagnosis. Common sites of metastatic spread include lung, bone and bone marrow (Lahl et al., 2008).

1.1.2 Diagnosis

The first diagnostic tools include blood analysis as ES can be accompanied by a rise in lactate dehydrogenase which has prognostic significance (Bacci et al., 1999). The X-ray shows a permeative lytic lesion and periosteal sclerotization because of new bone formation in 85% of cases. Commonly, the periosteal reaction is lamellated and has onion shape. The tumour is confined to soft tissue in 15% of cases. Sonography, magnetic resonance imaging and X-ray computed tomography reveal the bony and soft tissue extent of the tumour. Also bone szintigraphy may be used. However, none of those can provide a specific diagnosis. Thus, a biopsy of the tumour is necessary.
Immunhistochemical tests detect a strong expression of the surface glycoproteins CD99 (Delaplace et al., 2011; Chen et al., 2010) and p30/32MIC2 (Weidner et al., 1994). Those tests are still not specific enough, therefore a cytogenetic analysis is essential as 95% of ET have a t(11;22)(q24;q12) or t(21;22)(q22;12) chromosome translocation, resulting in EWS-FLI1 or EWS- ERG fusion genes, respectively (Li et al., 2012; Delattre et al., 1994). In rare cases (< 1%) EWS can also fuse with ETV1 (Jeon et al., 1995), E1AF (Urano et al., 1998) or FEV (Llombart-Bosch et al., 2000). A study by Li et al. indicates that the chimeric protein of EWS-FLI1 might impair p53 acetylation “to inhibit its transcriptional function and protein stability“ (Li et al., 2012).
As the identification of those fusion genes by use of polymerase chain reaction (PCR) of amplified complementary DNA from tumour-derived messenger RNA is also feasible, it provides a precise and practical diagnostic tool.

1.1.3 Prognosis

Prognosis depends on the individual pre-treatment factors and the treatment response.

Pre-treatment factors include site and size of the tumour, gender, age and the presence of metastases at the point of diagnosis. Most favourably for the prognosis seems to be a location at the distal extremities followed by a position in the proximal extremities, then a central site (Cotterill et al., 2000) and most unfavourably is a location in the spine and sacrum (Bacci et al., 2009). Patients with smaller tumour volumes (< 100 ml) have a better prognosis than those with intermediate (100 – 200 ml) or large tumour volumes (> 200 ml) (Ahrens et al., 1999). Girls have a better prognosis than boys among the Caucasian population (Jawad et al., 2009) and younger children (< 15 years) a better one than older ones (Cotterill et al., 2000; Rodríguez-Galindo et al., 2007). Certainly the most important prognostic factor is the presence of metastases at diagnosis, which occurs roughly in 27% of cases (Esiashvili et al., 2008). A survival analysis of 171 patients from the EICESS studies revealed an event-free survival, four years after diagnosis, for isolated lung metastases of 34%, for bone/bone marrow (BM) metastases of 28%, and for combined lung plus bone/BM metastases of 14% (Paulussen et al., 1998). Patients who suffer from a recurrent Ewing sarcoma have a very poor prognosis with a five-year overall survival of 13.8% (Bacci et al., 2003).

Treatment response appears to be dependent on biological factors of the tumour such as an overexpression of p53, which seems associated with poor prognosis (Abudu et al., 1999).

1.1.4 Treatment

Treatment involves systemic chemotherapy and the use of surgery and/or radiation for local tumour control. Generally, patients are given preoperative chemotherapy to shrink the size of the tumour before applying local control measures.

The duration of primary chemotherapy can last from six to twelve months. European protocols involve the use of vincristine, ifosfamide, doxorubicin and etoposide (Juergens et al., 2006), whereas patients in the United States receive courses of vincristine, doxorubicin cyclophosphamide, and dactinomycin alternating with courses of ifosfamide and etoposide (Grier et al., 2003). An Italian study has shown that the use of high dose chemotherapy (HDT) with Busulfan and Melphalan with stem cell

support as a maintenance treatment after induction chemotherapy can considerably improve five-year event-free survival in poor responders (72% vs. 33% who did not receive HDT) (Ferrari et al., 2011).

With a five-year disease-free survival of 63.8% vs. 47.6% surgery has proven to be superior to radiation in extremity tumours (Bacci et al., 2006). Also in ETs located in the torso surgery has been preferred in the past when the tumour was resectable (Hoffmann et al., 1999; Shamberger et al., 2000), possibly because radiation in children might inhibit growth and cause infertility. However, radiation has proven to be an effective treatment option (La et al., 2006). As published in Burdach et al. the use of total body magnetic resonance imaging-governed involved compartment irradiation followed by high-dose chemotherapy and stem cell rescue prove superior over chemotherapy as described in the European Intergroup Cooperative Ewing Sarcoma Study-92 (EICESS-92) protocol in high-risk Ewing tumour patients with multiple primary bone metastases. The five-year survival rate was 45% vs. 8% (Burdach et al., 2010). In the use of radiation the dose is important for the outcome as reduced doses (< 40 Gy) resulted in a high return (19%) rate at ten years whereas standard doses (> 40 Gy) had no local recurrence as observed in an American study with 79 patients (Krasin et al., 2004). Unfortunately, higher doses are associated with second malignancies such as osteosarcoma, fibrosarcoma, malignant fibrous histiocytomas and others. In a retrospective study no second malignancies were observed under 48 Gy, whereas among patients who had received between 48 and 60 Gy 25 cases per 10000 person-years and at equal or above 60 Gy 130 cases per 10000 person-years were observed (Kuttesch et al., 1996).

High confidence has been placed on allogeneic stem cell transplantation. It was assumed that the graft *versus* host disease (G*v*HD) that is commonly observed in allogeneic transplantation patients, when immune cells of the donor attack the foreign cells of the recipient, might also result in a graft *versus* tumour (G*v*T) effect. Such an effect has indeed been observed in Ewing tumour patients as metastases have shrunk significantly after allogeneic stem cell transplantation (SCT) (Lucas et al., 2008; Koscielniak et al., 2005). However, even though chronic *Gv*HD but not acute G*v*HD seems to decrease relapse rate significantly, both types are associated with higher nonrelapse mortality due to treatment toxicity (Cho et al., 2012). A study by Burdach et al. comparing autologous and allogeneic SCT observed that the incidence of death of

treatment related toxicity “was more than twice as high after allogeneic (40%) compared to autologous (19%) SCT” (Burdach et al., 2000).

The aggressive nature of the treatment that still has a high failure rate and the tremendous incidence of metastases with their poor prognosis make it necessary to look into alternative treatment options such as adoptive immunotherapy that might target the cancer cells much more specifically and efficiently.

1.2 Immunotherapy

Immunotherapy functions either through passive immunization which means applying antibodies or immune cells directed specifically against the cancer cells or alternatively, by using vaccines that stimulate the immune system systematically.
The use of antibodies has proven to be effective in some cancers. E.g. Rituximab is directed against CD20 that is expressed on all B cells and is now in combination with chemotherapy the standard therapy for diffuse large B-cell lymphoma (Mey et al., 2012).
Adoptive T-cell therapy signifies “the transfusion of autologous or allogeneic T cells into tumour-bearing hosts, i.e., patients” (June, 2007). For this purpose tumour-specific antigens (TSAs) on the cancer cells or tumour-associated antigens (TAAs) that are expressed on cancer cells in much higher numbers than on healthy tissue have to be identified. Then cytotoxic T lymphocytes (CTLs) that are directed against these antigens are generated e.g. with the help of dendritic cells.

1.2.1 Dendritic cells

Dendritic cells (DCs) are cells of the immune system, which recognise and ingest foreign and self-material and present it on their cell surface to other immune cells such as B or T lymphocytes. They are present in immature form in tissues in contact with the external environment, such as the skin and the mucous membranes of the respiratory and gastrointestinal systems and the outer genitalia. These immature DCs take in the foreign material by use of antigen-capturing Fcγ and Fcε receptors or other methods such as phagocytosis, macropinocytosis or adsorptive pinocytosis (Banchereau and Steinman, 1998). Then they develop into mature cells with the typical dendritical branches that give the cells their name. These mature DCs express a high

number of CD86 on their surface, which is very important for T cell activation (Caux et al., 1994). They can open the tight junctions between epithelial cells to reach bacteria outside the epithelium while maintaining the integrity of the epithelial barrier with their tight-junction proteins such as claudin 1, occludin, and zonula occludens 1 (Rescigno et al., 2001). Once activated the mature dendritic cells migrate towards the lymphatic tissue (Cyster, 1999) where they gather in the T cell region and present their ingested peptides on their major histocompatabiliy compounds (MHC) I or II to cytotoxic T cells or T helper cells, respectively. With those they form highly efficient clusters as only one DC is needed to stimulate 100 to 3.000 T-cells (Banchereau and Steinman, 1998).

In vitro it has become possible to generate mature dendritic cells from $CD14^+$ cells. For that purpose the peripheral blood mononuclear cells (PBMCs) of the donor have to be obtained and $CD14^+$ monocytes extracted by immunomagnetic selection. These monocytes can be developed into mature dendritic cells by adding a cytokine cocktail (Meyer-Wentrup et al., 2003). Due to their feasibility and promising results in the stimulation of T cells, vaccinations of tumour patients with dendritic cells bearing tumour peptide on their MHC I have been evaluated. In a study with stage III melanoma vaccinations of patients resulted in a three-year overall survival rate of 68.2% versus 25.7% in the control group. The figures for three-year disease-free survival rate were 40.9% versus 14.5%, respectively (Markowicz et al., 2012). A systematic review and meta analysis of various studies regarding vaccinations with peptide-loaded dendritic cells in patients with prostate and renal cell carcinoma (RCC) revealed a clinical benefit rate (combined percentages of objective responses and stable diseases) of 54% in prostate cancer and 48% in RCC (Draube et al., 2011). The quality of life remains high in this treatment modality, as there is little toxicity (Leonhartsberger et al., 2012).

Instead of vaccinating the patient with loaded dendritic cells to stimulate the patients' T cells dendritic cells are similarly used to stimulate cytotoxic T cells *in vitro* and those T cells are then transferred into the patient.

1.2.2 CTL

In a variety of cancers TSA or TAA suitable for pulsing dendritic cells have been identified and successfully used to stimulate cytotoxic T cells. Tumour-specific

antigens are found on tumours presenting non-self material such as the EBV^+ Hodgkin lymphoma (HL). The treatment with cytotoxic T cells specific for those EBV-derived peptides led to complete remissions in EBV^+ HL patients and was also well tolerated (Merlo et al., 2008; Merlo et al., 2011). Tumour associated antigens have been identified on a variety of tumours such as melanoma. In a study with ten patients with metastatic melanoma who were poor responders to chemotherapy, transfusion with autologous $CD8^+$ T cell clones targeting the tumour-associated antigens MART1/MelanA or gp100 led to an antigen-specific immune response. The CTLs migrated to tumour sites and eliminated tumour cells leading to a regression of individual metastases and disease stabilization in five patients and minor or mixed responses in three patients. As the first infusions showed a median survival of $CD8^+$ T cells of about 6.7 days, Interleukin-2 (IL-2) was added to all subsequent infusions to prolong their existence *in vivo.* (Yee et al., 2002). However, in an independent trial an anti-tumour response was seen in only three of eleven patients after adoptive transfer of autologous Melan-A-specific CTL and courses of IL-2. A raised frequency of circulating Melan-A tetramer+ T cells was observed for up to 2 weeks (Mackensen et al., 2006). These trials illustrate some common problems associated with immunotherapy such as the use of autologous instead of allogeneic T cells and their short persistence *in vivo*.

In past *in vitro* experiments, almost all autologous CTL that were HLA-A2-restricted and peptide-specific were unable to recognise HLA-A2-positive tumour cell lines expressing those peptides, whereas their allorestricted (i.e alloreactive and peptide-specific) counterparts, on the other hand, recognised those tumour cell lines successfully (Krönig et al., 2009; Pirson, 2009). The reason is that autologous cytotoxic T cells have a limited repertoire based on central tolerance mechanisms. Central tolerance refers to the process of negative selection by which thymocytes that bind to MHC molecules presenting self-peptides are eliminated thus preventing self-reactivity and inducing self-tolerance. Several mechanisms of negative selection have been described such as clonal deletion, meaning induced cell death by apoptosis (McCaughtry and Hogquist, 2008), and agonist selection, in which thymocytes bearing self-reactive TCR are recruited into T cell lines involved in immune regulation (Baldwin et al., 2004) such as $TCR\alpha\beta^+CD8\alpha\alpha^+$ intraepithelial lymphocytes (Lambolez et al., 2007) and regulatory T cells (Jordan et al., 2001). Another method is receptor editing which describes the process of modification of self-reactive TCR by gene rearrangement at

the TCR α locus (McGargill et al., 2000). As tumour-derived peptides are mostly self-peptides presented by MHC molecules, in most cases a patient`s T cell repertoire has been rendered incapable of a functional response by central tolerance mechanisms.

On the other hand, allogeneic T cells that recognise tumour-peptides presented on a host MHC molecule may represent an extensive repository for tumour-specific T cells (Felix and Allen, 2007). Allogeneic T cells have also shown superior responses over their autologous counterparts to the stimulation with dendritic cells *in vitro* (Gervais et al., 2009). Mismatch by design is usually the HLA-A*0201 allele as it is the most frequent allele in Caucasians (Sanchez-Velasco et al., 2001) and also very common in Asian and Black populations (Cao et al., *2001*). The HLA-A*0201 allele frequency is currently estimated to be 49.9% in the German population (Middleton *et al.*, 2003).

Since the well known Graft *versus* leukaemia (G*v*L) effect after allogeneic stem cell transplantation is accompanied by a highly toxic G*v*HD, the more specific allorestricted cytotoxic T cell therapy is explored as a possible future treatment option for leukaemia therapy (Vincent et al., 2011). Also in research with melanoma-derived peptides (Dutoit et al., 2002) and other TAAs (Krönig et al., 2009) adoptive allorestricted T cell therapy has proven to be very effective. Thiel, Pirson et al. have shown that it is possible to generate allorestricted cytotoxic effector T cells that recognise Ewing tumour antigens *in vitro* and inhibit tumour growth *in vivo* (Thiel et al., 2011). Stefan Pirson investigated several Ewing tumour antigens in his thesis, previously identified through microarray analysis (Staege et al., 2004). The following antigen-derived peptides were proven to be good binders to HLA-A*0201 and were specifically recognised by the cytotoxic effector T cells: Chrondomodulin 1 – 139 (CHM1-139), G Protein-coupled Receptor 64 – 135 (GPR64-135), Enhancer of Zeste, Drosophila, Homolog 2 – 666 (EZH2-666) and Six-Transmembrane Epithelial Antigen of Prostate 1 – 86 (STEAP1-86) (Pirson, 2009). They were used as Ewing tumour-specific antigens in this thesis.

1.2.3 Ewing tumour antigens

1.2.3.1 Chrondomodulin 1

Chondromodulin 1 is an anti-angiogenic glycoprotein that “stabilizes the chondrocyte phenotype by supporting chondrogenesis but inhibiting chondrocyte hypertrophy and

endochondral ossification" (Klinger et al., 2011). It is specifically localised at the extracellular matrix of the avascular mesenchyme including cartilage (Shukunami et al., 2008) and cardiac valves (Yoshioka et al., 2006). CHM1 is strongly overexpressed in Ewing tumour tissue and may be associated with its reduced microvessel density (Thiel et al., 2011).

1.2.3.2 G Protein-coupled Receptor 64

GPR64 comprises seven transmembrane-spanning domains characteristic of G protein-coupled receptors and has an extended extracellular N terminus. The receptor protein is found mainly in the ductuli efferentes and epididymis (Osterhoff et al., 1997; Kirchhoff et al., 2008). Richter et al. showed that GPR64 expression in Ewing sarcoma promotes less sensitivity to TRAIL-induced apoptosis and endorses cellular invasiveness and metastatic spread by inducing placental growth factor and matrix metalloproteinase (Richter et al., 2013).

1.2.3.3 Six-Transmembrane Epithelial Antigen of Prostate 86

Hubert et al. identified the gene named Six-Transmembrane Epithelial Antigen of Prostate that "encodes a 339-amino acid protein with six potential membrane-spanning regions flanked by hydrophilic amino- and carboxyl-terminal domains". It is strongly expressed in advanced human prostate cancer and Ewing sarcoma and is up-regulated in various other cancer cell lines. In healthy tissues, however, its cell-surface expression is limited to the prostate and the bladder, making it a promising target for immunotherapy (Hubert et al., 1999). Grunewald et al. demonstrated the high expression of STEAP1 in ET and showed that it is involved in the proliferation, cellular invasiveness and colony formation of Ewing tumour cells, thus supporting tumorigenicity and metastatic spread. Furthermore, STEAP1 seems to be connected to the oxidative stress phenotype of Ewing tumours and is regulated by EWS-FLI1 (Grunewald et al., 2012).

1.2.3.4 Enhancer of Zeste, Drosophila, Homolog 2

"The histone methyltransferase enhancer of Zeste, *Drosophila*, Homolog 2 (EZH2) […] is part of the polycomb repressor complex 2 (PRC2), together with embryonic

ectoderm development (EED) protein and suppressor of Zeste (SUZ12)". Within PRC2, EZH2 silences target genes by use of its methyltransferase activity. EZH2 is overexpressed in a variety of cancer cells including Ewing tumour and is associated with tumorigenicity and metastatic spread. EZH2 is induced by EWS/FLI1, which binds to its promoter in vivo and preserves a stem cell phenotype by inducing "stemness genes such as nerve growth factor receptor (NGFR), as well as [suppressing] genes involved in neuroectodermal and endothelial differentiation" (Richter et al., 2009). Stemness is maintained by epigenetic mechanisms as down-regulation of EZH2 led to reduced overall histone H3K27-trimethylation and augmented histone H3-acetylation (Burdach et al., 2009).

1.3 Aim of this study

Although effector T cells have proven to inhibit tumour growth and prolong the recipients´ time of survival, complete tumour clearance has rarely been achieved. This is partly due to their short persistence in the patient. A study measuring the response rate in patients with metastatic melanoma who received transfusions with tumour infiltrating lymphocytes (TILs) showed that the patients who responded well to the treatment had twice as many traceable adoptively transferred T cells in their blood after 5 to 15 days than the non-responders and even five times as many after 1 to 2 months. It was indicated that the response rate correlated with the persistence of the TILs in peripheral blood (Robbins et al., 2004). Hence, research on adoptive T cell therapy is focussing increasingly on memory T cells. They have been shown to be preserved and functional for life in response to persistent latent infection (Lang et al., 2011). Memory T cells can be divided into two subsets namely central memory T cells (Tcm) and effector memory T cells (Tem) (Sallusto et al., 1999).

Tcm express CD127 and high levels of CD62L on their cell surface and reside mostly in lymph nodes, whereas Tem cells express CD127 but only very low levels of CD62L and are located mostly in peripheral tissues. Tcm have a higher proliferative potential than Tem, supplying a reservoir of antigen-specific T cells that is reactivated upon secondary antigen encounter, multiplies and obtains a more active phenotype (Bachmann et al., 2005; Perret and Ronchese, 2008). There has already been some success in creating peptide-specific central memory T cells directed against melanoma cell lines. For that purpose naive $CD8^+$ T cells were stimulated with peptide pulsed

dendritic cells and exposed to Interleukin-21 (IL-21), Interleukin-7 (IL-7) and Interleukin-15 (IL-15) sequentially. The resulting antigen-specific and tumour-reactive T cells showed a central memory like phenotype that highly expressed the lymph node homing receptors CD62L and CCR7 (Wölfl et al., 2011). A crucial role in the development of central memory T cells seems associated with Interleukin-21, which belongs to the IL-2 γ-chain receptor cytokine family together with IL-2, IL-4 IL- 7, IL-9 and IL-15. IL-21 has been found to influence primarily naive T cells resulting - upon stimulation with antigen presenting DCs - in an exceedingly proliferative T cell population with a strong CD45RO and CD28 expression and high affinity and target cell avidity. The ideal IL-21 concentration for this purpose was 30 ng/ml (Li et al., 2005).

Thus, the aim of this study is to find the best combination and dosages of IL-21, IL-15 and IL-7 in order to generate allorestricted central memory cytotoxic T cells capable of recognising and attacking Ewing tumour antigen-bearing cancer cells *in vitro*. For this purpose various approaches were conducted of which the three most successful are described further in this thesis.

First of all, an established approach producing allorestricted effector cytotoxic T cells (Thiel et al., 2011; Pirson, 2009) was conducted in order to ascertain a reference protocol for the IL-21 approaches. This established approach is hereafter also referred to as the standard or classical approach.

In the next step, two approaches with regard to the generation of allorestricted central memory cytotoxic T cells were performed by isolating $CD8^+$ T cells from an HLA-$A*0201^-$ adult donor and stimulating them with peptide-loaded HLA-$A*0201^+$ DCs. The T cells were then stained with peptide-specific pentamer and sorted for specificity. Afterwards, these cells were expanded in the presence of various dosages of IL-21, IL-7 and IL-15 and tested in an ELISpot assay for allorestricted antigen recognition. They were further expanded in the presence of various dosages of IL-21, IL-7 and IL-15 and tested. These two approaches, differing in their dosages, are hereafter referred to as IL-21 approach *a* and *b*.

The third IL-21 approach - henceforth referred to as IL-21 approach *c* - followed the same general method as IL-21 approach *a* and *b* but differed in two points: Different dosages of cytokines were used and the $CD8^+$ cells were isolated from blood of the umbilical cord rather than from an adult donor. IL-21 approach *c* revealed that it was possible to generate allorestricted central memory cytotoxic T cells out of $CD8^+$ cells

isolated from blood of the umbilical cord. This is important for subsequent *in vivo* tests of such T cells in a murine model, which was not conducted in this thesis but rather provides an outlook for future research: For this purpose $CD34^+$ stem cells would be isolated from HLA-A*0201$^-$ blood of the umbilical cord and injected into a $Rag2^{-/-}\gamma c^{-/-}$ mice to establish and simulate a human immune system. Then $CD8^+$ T cells from the same donor would be isolated, stimulated with peptide-loaded HLA-A*0201$^+$ DCs and expanded in the presence of IL-21, IL-7 and IL-15. The mice would subsequently be challenged with HLA-A*0201$^+$ Ewing tumour cells and receive allorestricted Tcm, simulating immunotherapy with allorestricted Tcm in a HLA-A*0201$^+$ Ewing tumour patient.

2. Materials

2.1 Instruments and Equipment

Type of device	Model	Manufacturer
Autoclave	Systec V – 95	Systec GmbH
Balance	EW 3000-2M	Kern
Cell separation system	BD IMAG™	BD Biosciences
Cell separation system	MIDIMACS™ separator MACS® cell separation columns LS MACS MultiStand	Miltenyi Biotec
Cell counting chamber	Neubauer improved	Marienfeld
Centrifuge	Multifuge 3 S-R	Heraeus
Centrifuge	Biofudge fresco	Heraeus
Controlled-freezing box		Nalgene
Degas system	Elmasonic 560 H	Elma
ELISpot reader	AID iSpot reader unit	AID GmbH
Flow cytometer	FACSCalibur™	Becton Dickinson
Freezer (-80°C)	Hera freeze	Heraeus
Freezer (-20°C)	Cool vario	Siemens
Fridge (+4°C)	Cool vario	Siemens
Gamma irradiation facility	BIOBEAM 8000	STS Steuerungstechnik und Strahlenschutz GMBH
Ice machine	AF 100	Scotsman
Incubator	Hera cell 150	Heraeus
Liquid nitrogen tank	L-240 K series	Taylor-Wharton
Multichannel pipette	(10 - 100µl)	Eppendorf
Micropipettes	(0.5 - 10 µl; 50 - 200 µl) (5 - 20 µl; 20 - 100 µl; 200 - 1000 µl)	Eppendorf
Microscope		Leica

Original perfusor syringe	OPS (50ml)	Luer Lock
Protection glasses		Uvex
Sterile bench		Heraeus
Storing chamber (+4°C)		Viessmann Kältetechnik
Turning wheel		GLW
Vortexer		Heidolph Reaxtop
Water bath		GFL
Water purification system	TKA GenPure	TKA GmbH

2.2 Commonly used materials

Cell strainer, nylon (40 µm)	Falcon
Cryovials	Greiner bio-one
Flasks for cell culture (25 cm^2, 75 cm^2, 150 cm^2)	TPP
Gloves (cryo)	Tempershield Inc.
Gloves (latex)	Sempermed
Kleenex	
Pipettes (5, 10, 25)	Sarstedt
Pipette tips with filter (10, 20, 100, 200, 1000)	ART Molecular BioProdukt
Pipette tips without filter (10, 200, 1000)	Sarstedt
Plates for cell culture 96 well	TPP
Rack for FACS™ tubes	Pharmacia Diagnostics
Rack for 10 cell culture tubes	Hartenstein
Rack for 24 cell culture tubes	Nalgene®
Reservoir for reagents, polystyrene (50 ml)	Corning Inc. Costar®
Reservoirs, pipette, 12 channel, polypropylene	VWR International
Sigmaware wash bottle	Sigma
Tubes for cell culture, polypropylene (15 ml)	Greiner bio-one
Tubes for cell culture, polypropylene (50 ml)	Sarstedt
Tubes for molecular biology (1.5 ml)	Eppendorf
Tubes for FACS™, polystyrene (5 ml)	Falcon

2.3 Chemicals and biological reagents

AIMV medium	Invitrogen
Albumin bovine fraction V	Serva
Anti-human CD14 Magnetic Particles	BD Biosciences
β_2 – macroglobulin	Sigma
BD IMAG™ buffer (10x)	BD Biosciences
$CD8^+$ T cell isolation kit human	Miltenyi Biotec
Deoxyribonuclease I	Invitrogen
Dimethylformamid	ROTH
DMEM medium	Invitrogen
DMSO (dimethylsulfoxide)	Sigma - Aldrich
Erythrocyte lysis buffer	Pharmacy MRI
Ethanol	Pharmacy MRI
FACS™ Clean	BD Biosciences
FACS™ Flow	BD Biosciences
FACS™ Rinse	BD Biosciences
Ficoll – Paque	GE Healthcare
FBS (fetal bovine serum)	Biochrom AG
Gentamicinsulfate	Biochrom AG
GM – CSF	Bayer Healthcare Pharmaceuticals
Human male AB serum	Lonza
Interleukin-1β (IL-1β)	R&D Systems, Immunotools
Interleukin-2 (IL-2)	R&D Systems, Immunotools
Interleukin-4 (IL-4)	R&D Systems, Immunotools
Interleukin-6 (IL-6)	R&D Systems, Immunotools
Interleukin-7 (IL-7)	R&D Systems, Immunotools
Interleukin-12 (IL-12)	R&D Systems, Immunotools
Interleukin-15 (IL-15)	R&D Systems, Immunotools
Incidur spray	Ecolab
L-glutamine	Invitrogen
MACS® BSA stock solution	Miltenyi Biotec GmbHautoMACS™
Rinsing solution	Miltenyi Biotec GmbH
Na – pyruvate	Invitrogen

100x non – essential amino acids	Invitrogen
PBS (10x) (phosphate buffered saline)	Invitrogen
PGE2 (prostaglandine E2)	Caymen Chemical Company
RPMI 1640	Invitrogen
TCR Vβ analysis kit (IOTest® Beta Mark)	Beckman Coulter
TNF α (tumour necrosis factor α)	R&D Systems
Trypan blue	Invitrogen
Trypsin / EDTA	Invitrogen
Tween 20	Sigma
Whole blood CD15 microbeads, human	Miltenyi Biotec
X-vivo 15 medium	Lonza

2.4 Cell culture media and solution

T cell medium:

- AIM V
- 5% human male AB serum
- 2 mM L- glutamine (200 mM)
- 50 µg/ml gentamicin

Dendritic cell medium:

- X-Vivo 15
- 0.1% human AB serum

LCL/T2 medium:

- RPMI 1640
- 10% FCS (fetal calf serum)
- 1x non- essential amino acids (100x)
- 1 mM Na-pyruvate (100 mM)
- 2 mM L- glutamine (200 mM)
- 50 µg/ml gentamicin

A673, SB-KMS-KS1 and TC-71 :

- RPMI 1640
- 10% FCS
- 2 mM L-glutamine (200mM)
- 50 µg/ml gentamicin

K562, 697:

- RPMI 1640
- 20% FCS
- 2 mM L- glutamine (200 mM)
- 50 µg/ml gentamicin

Standard freezing medium:

- 90% FCS
- 10% DMSO

Freezing medium for CTL, DC, LCL and T2:

- 90% human AB serum
- 10% DMSO

Trypsin / EDTA 1x :

- 90% PBS
- 10% Trypsin-EDTA (10x)

2.5 Cells

Table 1: Cell lines and sources

Name	Description	Source	References
A673	Ewing tumour cell line, HLA-A*0201^{+}	ATCC	Giard et al., 1973
DCs	Differentiated from CD14^{+} monocytes of an adult HLA-A*0201^{+} donor	Generated in the laboratory	
K562	Erythroid leukaemia cell line, HLA-A*0201^{-}	A. Knuth* E. Jäger*	Lozzio and Lozzio, 1975
LCLs	EBV- immortalized B cells	S. Pirson	Pirson, 2009
SB-KMS-KS1	Ewing tumour cell line, HLA-A*0201^{-}	Generated in the laboratory	Richter et al., 2009
TC-71	Ewing tumour cell line, HLA-A*0201^{+}	DSMZ	Whang-Peng et al., 1984
T2	hybrid of a T and a B lymphoblastoid cell line TAP-deficient, HLA-A*0201^{+}	P. Cresswell°	Salter et al., 1985
697	cALL cell line HLA-A*0201^{+}	DSMZ	Findley et al., 1982
SK-Mel-29	Melanoma cell line HLA-A*0201^{+}	L. Old#	Carey et al., 1976

ATCC: LGC Standards GmbH, Wesel,Germany

DSMZ: German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

*Krankenhaus Nordwest, Frankfurt, Germany

°Yale University School of Medicine, New Haven, CT

Memorial Sloan-Kettering Cancer Institute, New York, NY

2.6 ELISpot Reagents

2.6.1 Antibodies

anti-h-INFγ mAb 1-D1K, purified	Mabtech
anti-h-INFγ mAb 7-B6-1, biotinylated	Mabtech
anti-h-Granzyme B mAb GB10, purified	Mabtech
anti-h-Granzyme B mAb, GB11,biotinylated	Mabtech
anti-h-IL2 mAb IL-2 1/249	Mabtech
anti-h-IL2 mAb IL-2 11, biotinylated	Mabtech

2.6.2 Plates, enzymes, buffers

MultiScreen-HA filter plate, 0.45 µm, clear, sterile	Millipore
Streptavidin-horse radish peroxidase	Mabtech
3-Amino-9-ethyl-carbazole (AEC) tablets	Sigma

Wash buffer:	1 x PBS / 0.05% Tween
Antibody buffer:	1 x PBS / 0.5% BSA
Acetate buffer:	37.5 ml H_2O dest. + 3.75 ml 0,2 N acetic acid
	+ 8.8 ml 0,2 N sodium acetate
AEC solution:	one 3-Amino-9-ethyl-carbazole (AEC) tablet
	in 2.5 ml Dimethylformamid (DMF)
	+ 47.5 ml acetate buffer

Developing solution/ plate: 10 ml AEC solution + 25 µl 30% H_2O_2

2.7 Peptides

Peptides were ordered and synthesized by Thermo Scientific.
(http://www.thermohybaid.de/cgi-bin/start.app)

Table 2: *In silico* predicted peptides and their scores

Gene	HLA Allele	Sequence	Position (first N-terminal aa)	NetCTL1.2	SYFPEITHI	BIMAS
CHM1	A*0201	VIMPCSWWV	319	1.33	22	2481.34
EZH2	A*0201	YMCSFLFNL	666	1.49	25	3.60
GPR64	A*0201	FIMCATAEA	135	1.29	20	11.63
STEAP	A*0201	FLYTLLREV	86	1.35	29	470.95
Influenza (FLU)	A*0201	GILGFVFTL	-	1.29	30	550.93

2.8 Flow cytometry reagents

2.8.1 Reagents

staining buffer: 2% FCS + 0.05% sodium azide in PBS

fixing solution: 1% paraformaldehyde in PBS

2.8.2 Antibodies

Table 3: Antibodies used in flow cytometry

Specificity	Clone	Fluorophore	Manufacturer
mouse IgG1 κ	MOPC-31C	FITC	BD Biosciences
mouse IgG1 κ	MOPC-31C	PE	BD Biosciences
mouse IgG1 κ	MOPC-21	APC	BD Biosciences
mouse IgG2b κ	MPC-11	FITC	BD Biosciences
CD3	HIT3a	FITC	BD Biosciences
CD8	RPA-T8	FITC	BD Biosciences
CD27	M-T271	FITC	BD Biosciences
CD28	CD28.2	FITC	BD Biosciences
CD34	8G12	PE	BD Biosciences
CD38	HIT2	APC	BD Biosciences

CD45RO	UCHL1	PE	Miltenyi Biotec
CD56	NCAM16.2	PE	BD Biosciences
CD62L	Dreg 56	APC	BD Biosciences
CD83	HB15e	APC	BD Biosciences
CD86	2331 (FUN-1)	FITC	BD Biosciences
CD127 (IL7R)	40131	APC	R&D
IL21 R	17A12	APC	BD Biosciences
HLADR	L243 (G46-6)	PE	BD Immunocytometry systems
HLA A2	BB7.2	FITC	BD Biosciences

2.9 List of manufacturers

AID GmbH	Straßberg, Germany
ART Molecular Bioproduct	San Diego, California, USA
ATCC	Rockyville, Maryland, USA
Bayer HealthCare Pharmaceuticals	Leverkusen, Germany
BD Biosciences	Heidelberg, Germany
BD Immuno cytometry systems	Heidelberg, Germany
Beckman Coulter	Krefeld, Germany
Becton Dickinson	Jersey City, New Jersey, USA
Biochrom	Berlin, Germany
Cayman Chemical Company	Ann Arbor, Michigan, USA
Corning Inc. Costar	Corning, New York, USA
DSMZ	Braunschweig, Germany
Ecolab	Beckum. Germany
Elma	Singen, Germany
Eppendorf	Hamburg, Germany
Falcon	Oxnard, California, USA
GE Healthcare	Uppsala, Sweden
GFL	Burgwedel, Germany
Gilson	Middleton, Wyoming, USA
GLW	Würzburg, Germany

Hartenstein	Würzburg, Germany
Heraeus	Hanau, Germany
Heidolph Reaxtop	Schwabach, Germany
Immunotools	Friesoythe, Germany
Invitrogen	Karlsruhe, Germany
Kern	Balingen-Frommern, Germany
Leica	Wetzlar, Germany
Lonza	Basel, Switzerland
Mabtech	Nacka Strand, Sweden
Marienfeld	Neu-Isenburg, Germany
Millipore	Billerica, Massachusetts, USA
Miltenyi Biotec GmbH	Bergisch Gladbach, Germany
Nalgene	Rochester, New York, USA
Pharmacia Diagnostics	Freiburg, Germany
R&D Systems	Minneapolis, Minnesota, USA
(Carl) ROTH	Karlsruhe, Germany
Sarstedt	Nümbrecht, Germany
Scotsman	Milan, Italy
Sempermed	Wien, Austria
Serva	Heidelberg, Germany
Siemens	Germany
Sigma	St. Louis, Missouri, USA
STS Steuerungstechnik und Strahlenschutz GMBH	Braunschweig, Germany
Systec GmbH	Wettenberg, Germany
Taylor-Wharton	Husum, Germany
Tempershield Inc.	Mount Desert, Maine, USA
TKA GmbH	Niederelbert, Germany
TPP	Trasadingen, Switzerland
Thermo Fisher Scientific	Ulm, Germany
Uvex	Fürth, Germany
Viessmann Kältetechnik	Hof/Saale, Germany
VWR International	Darmstadt, Germany

3. Methods

3.1 Cell culture

3.1.1 Cell counting

Cells were counted using an improved Neubauer haemocytometer. It contains a counting chamber consisting of four big corner squares, each divided into 16 smaller squares. 10 µl of cell suspension were applied into the chamber and live cells in all four corner squares were counted. In this process cells touching the top and left gridlines but not the right and bottom lines were included in order to avoid counting cells twice. Higher cell numbers had to be diluted so that there were not more than approximately 200 cells in each big corner square. For this purpose cells were diluted with trypan blue in a 1:10 solution. Trypan blue has the advantage that dead cells are coloured but not live cells hence facilitating the determination of the real live cell number. This number was calculated using the following formula:

Table 4: Principle to determine the total number of cells using a Neubauer haematocytometer

Cell concentration per ml =
average number of cells counted per square x dilution factor x chamber factor
average number of cells counted per square = cells counted in all 4 corner squares ÷ 4
dilution factor = for example 10 in a 1: 10 trypan blue solution
chamber factor = 10^4
total number of cells = cell concentration x volume of cell suspension

3.1.2 Cryopreservation of cells

Cells were pelleted in the centrifuge and then re-suspended in the appropriate precooled freezing media. The media consisted either of FCS with 10% DMSO for tumour cells and pool PBMCs (see 3.10) or of human AB serum with 10% DMSO for T cells, dendritic cells, lymphoblastoid cell lines (LCLs) and T2 cells. Aliquots were made with roughly 5 x 10^6 cells per 500 µl for tumour cell lines and 5 x 10^7 cells per 500 µl for pool PBMCs. T cells and dendritic cells were frozen at a concentration of 1 x 10^6 in 100 µl for faster freezing. These aliquots were put in cryovials which were immediately placed in a controlled freezing box. This box was stored in a -80°C freezer overnight. Here the temperature of the cell suspension decreased at a rate of one degree Celsius per minute. The next day the cells were transferred to the liquid nitrogen freezer, which preserved the cells at -196°C for long-term storage.

3.1.3 Thawing of cryopreserved cells

A tube containing 10 ml of appropriate warmed culture medium was prepared. The cryovial containing the frozen cells was retrieved from the liquid nitrogen freezer and 500 µl of the warm medium were added to its content. The vial was then warmed in hand until a small lump of frozen cells floated in the medium. The content of the cryovial was then poured into the tube containing warm culture medium and then the tube was immediately placed in the centrifuge, where it was centrifuged at 1500 rpm for five minutes. The resulting cell pellet was re-suspended in the appropriate medium which had been pre-warmed to 37°C.

3.1.4 Culture of adherent tumour cell lines

The adherent tumour cell lines used were the Ewing tumour cell lines A673, TC-71 and SB-KMS-KS1.

The tumour cells were thawed in the appropriate medium as described above and then pipetted into a cell culture flask. Two sizes of culture flasks were used depending on the number of cells needed and their division rates. In the medium culture flask containing 75 cm^2 adherence surface the tumour cells were suspended

in 20 ml medium. In the large culture flask with 175 cm^2 adherence surface the tumour cells were suspended in 40 ml medium. The cell lines were grown in a moisturized incubator at 5% CO_2. Usually within an hour adherence of the cells to the walls could be seen in the microscope. TC-71 could need several days to adhere. The growth of the cells was controlled microscopically and tumour cells were split when 80% of the adherence surface was covered. In this procedure the used medium was collected in a tube and the cells were washed with warm PBS, which was also added to the tube. Then 3 – 5 ml of warm 10% trypsin in PBS solution was added to the cells and the culture flask was placed in the incubator for several minutes. When all the cells had detached, which was controlled microscopically, the cell suspension was added to the used medium in the tube and centrifuged at 1500 rpm. The cell pellet was then re-suspended in the appropriate warm cell medium and transferred to new culture flasks. In this process cells were diluted at 1:2 to 1:9 to their previous concentration according to their individual growth rates. Track of the cell age and proximal division rate was protocolled by passage number.

3.1.5 Culture of suspension cell lines

Suspension cell lines are T2 cells, LCLs, K562 and 697.
Cells were thawed as described in 3.1.3 and re-suspended in the appropriate cell culture medium. 25 ml of cell suspension was pipetted into a medium culture flask and placed standing up in the incubator. Every day the colour of the medium was controlled. When the medium had turned yellow - a sign that the nutrients had been metabolised -, the same amount of fresh medium as already contained in the flask was added. When the flask was full, the content was split, centrifuged and suspended in new medium.

3.2 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from either blood of an adult blood donor or from blood of the umbilical cord. The fresh adult blood was obtained from the DRK - Blutspendedienst in Ulm, Baden-Würtemberg-Hessen, in form of buffy coats. For these most of the plasma and erythrocytes had been removed so that 500 ml of blood had been reduced to 20 to 25 ml of thick cell

suspension consisting mostly of leukocytes and platelets. The blood of the umbilical cord was collected daily before pickup by midwives in the Klinikum rechts der Isar in Munich and outcome could vary greatly between 20 and over 100 ml. Only donations with at least 50 ml were used.

First of all, the blood was carefully transferred from the blood unit bag into 50 ml collection tubes. Then the blood of the umbilical cord was diluted with PBS in a ratio of 1:2, the adult blood in a ratio of 1:4 since cell concentration was higher. After this step both blood types were processed in the same way. Firstly, 30 ml of diluted blood were layered upon 20 ml Ficoll-Paque (which is characterized by its high density) in 50 ml collection tubes so slowly and carefully that mixing of blood and Ficoll was avoided. Then the tubes were centrifuged at 400 g for 30 minutes without brake. Upon retrieval from the centrifuge a lymphocyte layer of one cm width could be seen swimming above Ficoll. The plasma on top of this layer was aspirated and discarded and the lymphocytes were collected with a 5 ml pipette and placed into 50 ml tubes. The cell suspension was then adjusted with PBS to 50 ml and centrifuged at 1500 rpm for 5 minutes. The resulting cell pellet was re-suspended into 10 ml of pre-warmed erythrocyte-lysis-buffer in order to remove the erythrocytes. This reaction was stopped after two minutes by diluting up to 50 ml with PBS. The cells were then washed twice with PBS. After the final washing step the cells representing the peripheral blood mononuclear cell fraction of the blood were suspended in 25 ml PBS and counted.

The number of PBMCs in the adult blood unit bags varied between 2 and 6 x 10^8.

The number of PBMCs in the umbilical cord blood fluctuated between 5 x 10^7 and 2 x 10^8 depending on the age of the bag (up to three days) and its volume. Only PBMCs with at least 1 x 10^8 were processed further.

3.3 Generation of dendritic cells

3.3.1 Isolation of $CD14^+$ cells

$CD14^+$ cells were isolated from HLA-A*0201^+ adult PBMCs using a BD Anti-Human CD14 Magnetic Particles set and a BD™ IMagnet. The instructions of the manufacturer were followed exactly:

Firstly, PBMCs were counted and washed with BD IMAG buffer which had to be diluted 1:10 with sterile distilled water before use. The supernatant was removed completely, the BD Anti-Human CD14 Particles were vortexed and 50 µl of particles were added for every 10^7 total cells. The cell suspension was mixed thoroughly and incubated at room temperature for 30 minutes for immuno-magnetic labelling. The BD IMAG-particle labelling volume was adjusted to 1-8 x 10^7 cells/ml with diluted BD IMAG buffer resulting in six tubes with 3 ml content each, which were immediately placed on the BD™ IMagnet and incubated for 10 minutes for magnetic separation. After this step the labelled cells could be seen as a brownish mass clinging to the walls of the tubes. With the tubes left on the magnet the supernatant comprising the $CD14^-$ cells was carefully aspirated. The labelled cell fraction was re-suspended in 3 ml buffer per tube and incubated on the magnet for 3 minutes. This washing step was repeated four times. Afterwards the $CD14^+$ monocytes were re-suspended in 10 ml X-Vivo medium and counted.

3.3.2 Differentiation of $CD14^+$ cells into dendritic cells

$CD14^+$ cells are monocytes that can differentiate into macrophages or dendritic cells depending on the signals they get.

The $CD14^+$ cells were isolated as described in 3.3.1. The monocytes were suspended in X-Vivo with 1% human AB serum at a concentration of 1 x 10^6 cells/ml and transferred to medium cell culture flasks together with IL-4 (30 ng/ml) and GM-CSF (800 U/ml). The flasks were placed in an incubator at 37°C and 5% CO_2 lying down. After two days half the medium was replaced and IL-4 and GM-CSF were added at the same concentrations as before. After another two days half the medium was replaced again and a cytokine cocktail was added consisting of IL-6 (1000 U/ml), IL-1β (10 ng/ml), TNFα (10 ng/ml), and PGE_2 (1 µg/ml). At this point immature dendritic cells could be seen under the microscope. After 48 hours the maturity of the dendritic cells could be verified microscopically and was controlled by flow cytometry. The cells were mature when they expressed a strong positivity for CD83, CD86 and HLA-DR. The number of harvested mature DCs varied between 7 x 10^6 and 2,75 x 10^7. The mature dendritic cells were loaded with Ewing tumour peptides in the presence of β2-microglobulin and used for *in vitro* priming of $CD8^+$ cells as described in 3.5.

3.4 Isolation of CD8$^+$ cells

3.4.1 Isolation of CD8$^+$ cells from blood of an adult donor

CD8$^+$ cells were isolated from HLA-A*0201$^-$ adult PBMCs using a CD8$^+$ T Cell Isolation kit, a LS column and a MidiMACS^{TM} seperator.

Firstly, PBMCs were isolated from the blood as described in 3.2. For magnetic labelling and separation instructions of the manufacturer were followed accordingly:

The cells were washed once with rinsing buffer. The cell suspension was then centrifuged at 300x g for 10 minutes. The supernatant was aspirated completely and the cell pellet was re-suspended in 40 µl of buffer per 10^7 total cells. Then 10 µl of CD8$^+$ T Cell Biotin-Antibody Cocktail per 10^7 total cells were added, mixed and incubated for 10 minutes at 4°C on a rotating wheel. Subsequently, 30 µl buffer and 20 µl of CD8$^+$ T Cell Microbead Cocktail per 10^7 total cells were added, mixed and incubated for 15 minutes at 4°C on a rotating wheel. Then 2 ml of buffer were added and the cell suspension was centrifuged at 300 g for 10 minutes. The supernatant was aspirated completely and the cell pellet was re-suspended in 500 µl of buffer per 10^8 cells. For magnetic separation the LS column had to be prepared by rinsing with 3 ml buffer. In order to avoid clogging of the LS column by cell clots or cell debris a 40 µl cell strainer was placed on top of the column which was attached to the MidiMACS^{TM} seperator and the cell suspension was applied onto it. The column was then washed three times with 3 ml of buffer with new buffer only being applied when the column reservoir was empty. The total unlabelled effluent was collected comprising the CD8$^+$ cells. They were counted and used for *in vitro* priming experiments.

3.4.2 Isolation of CD8$^+$ cells from blood of the umbilical cord

The process was the same as for adult blood except that CD15$^+$ cells had to be depleted in order to improve CD8$^+$ T cells when injected into the humanized mice. For this purpose the PBMCs that had been labelled with the CD8$^+$ T Cell Microbead Cocktail were supplemented with 20 µl CD15$^+$ microbeads per 10^7 PBMCs for 15 minutes at 4°C. The cells were washed and then magnetic seperation was performed as described in 3.4.1

3.5 In vitro priming of CD8$^+$ cells

HLA-A*0201$^+$ dendritic cells were loaded with a selected Ewing tumour-specific peptide and then used to prime HLA-A*0201$^-$ CD8$^+$ cells. The peptides utilized for priming were CHM1-319, STEAP1-86, GPR64-135 and EZH2-666. The following method of processing the cells was the same for the classical and the IL-21 approach. They varied only in the cytokines added:

For loading DCs with tumour peptides, cells were suspended in T cell medium (AIM V, 5% human male AB serum, 2 mM L- glutamine, 50 µg/ml gentamicin) at a concentration of 5 x 10^6/ml and pulsed with a selected peptide at a concentration of 30 µM in the presence of 20 µg/ml β_2-microglobulin for 4 hours at 37°C and 5% CO_2. The cell suspension was vortexed every 15 minutes during that time. Was the number of DCs lower than 5 x 10^6/ml the same amount of peptide and β2-microglobulin was used. Afterwards the dendritic cells were washed three times with T cell medium to remove any unbound peptide. For *in vitro* priming the dendritic cells and CD8$^+$ cells were co-cultured together with IL-6 (1000 U/ml) and IL-12 (10 ng/ml) in T cell medium and placed in 96 well round bottom plates at a concentration of 5 x 10^3 DCs/ well and 1 x 10^5 CD8$^+$ cells/well in 200 µl per well. The plates were left in the incubator for a week.

After that the CD8$^+$ cells were re-stimulated by exchanging 100 µl/well with fresh T cell medium containing 5 x 10^3 peptide-pulsed DCs/well and cytokines. For the standard approach those were IL-2 (100 U/ml) and IL-7 (5 ng/ml). For the IL-21 approaches IL-21 (30 ng/ml) and IL-7 (10 ng/ml) were used. The plates were then left in the incubator for another week. When the medium had turned yellow during that time, half the T cell medium was exchanged but no new cytokines were added. After that CD8$^+$ cells were ready for cell sorting.

3.6 Cell sorting

For cell sorting the CD8$^+$ cell were stained with their corresponding peptide-MHC-pentamer, e.g cells that had been primed with STEAP1-86-loaded DCs were stained with STEAP1-HLA-A*0201-Pentamer-PE.

Firstly, the CD8$^+$ cells were pooled, counted and washed twice with wash buffer (0.1% BSA + 0.1% sodium azide in PBS). For staining a 96 well round bottom plate was used. Three staining samples with 2 x 10^5 cells each were allocated (see Table

5). Those would later be used to set up the parameters for fluorescence-based cell sorting. All $CD8^+$ cell groups were given their own well according to their priming. No more than 2 x 10^7 cells per group were used for sorting, the rest was frozen down. The cells were washed with washing buffer and re-suspended in 50 µl buffer each.

Table 5: Staining scheme for cell sorting

sample	1. sample	2. sample	3. sample	4. sample
Cell number	2 x 10^5	2 x 10^5	2 x 10^5	all cells of the same peptide group
fluorescence	unstained	CD8 FITC	CD8 PE	CD8 FITC Pentamer-PE

0.5 µl peptide-HLA-pentamer-PE was added for every 2 x 10^6 cells to be sorted, mixed by pipetting and incubated at room temperature for 10 minutes. Then the cells were washed and re-suspended in 50 µl washing buffer. Pro5® Fluorotag was centrifuged at 13 000 x g for 3 minute before use to precipitate any protein aggregate that would lead to unspecific staining. Only the supernatant was subsequently used for staining. 2 µl Pro5® Flurotag for every 2 x 10^6 cells, which had been stained with peptide-HLA-pentamer-PE, and anti-human CD8-FITC mAb and anti-human CD8-PE mAb were added to the sorting samples as indicated in Table 5. The cells were then incubated on ice for 20 minutes in the dark. Afterwards the cells were washed twice with buffer and each sample was suspended in one ml PBS for sorting. All solutions were precooled and stored on ice during the staining procedure. The staining samples were transported on ice to the flow cytometry facility where they were passed though a filter and stained with 10 µl propidium iodide each (stock concentration: 50 µg/ml) in order to label and exclude the dead cells. Sorting was executed by Lynette Henkel on a BD FACS Aria in the flow cytometry facility in the laboratory of Prof. Dr. Dirk Busch, Klinikum rechts der Isar, Technische Universität München.

3.7 Limiting dilution

The sorting process resulted in 200 – 2000 peptide-HLA-pentamer-specific T cells per group. Those cells were subsequently cloned and expanded by limiting dilution in order to obtain individual cell clones.

The $CD8^+$ cells were expanded in 96 well round bottom plates with 1 cell/well, 2 cells/well, 3 cells/well, 5 cells/well or 10 cells/well. For this purpose the T cells were mixed with irradiated feeder cells consisting of pool PBMCs (5 x 10^4/well) and LCLs (1 x 10^5/well). The cell suspension was then co-cultured with anti-CD3 (30 ng/ml) and different cytokines (depending on the individual approach) in T cell medium at a volume of 200 µl/well:

Table 6: Comparison of different approaches with varying cytokines for expansion after sorting

Classical approach	**IL-21 approach *a***	**IL-21 approach *b***	**IL-21 approach *c***
IL-2 (50 U/ml) IL-15 (2 ng/ml)	IL-21 (30 ng/ml) IL-15 (2 ng/ml)	IL-21 (30 ng/ml) IL-15 (10 ng/ml) IL-7 (10 ng/ml)	IL-21 (30 ng/ml) IL-15 (2 ng/ml)

Seven days later 100 µl/well of T cell medium was exchanged and new cytokines were added as described in Table 6. After an additional seven days the T cell clones were tested for specificity in an ELISpot assay.

3.8 ELISpot

In an ELISpot assay the specificity of a T cell clone can be tested by measuring the T cell release of certain cytokines as a reaction to the presence of peptide-loaded T2 cells or tumour cells. The whole procedure takes 3 days.

On the first day the multiscreen plates were covered with the capture antibody. For this purpose 50 µl capture antibody solution were diluted with 5 ml PBS per plate and 50 µl/well was added as indicted in Table 7. The plates were then left at 4°C over night to allow the antibodies to attach to the bottom membranes of the plates.

Table 7: Antibodies used in ELISpots

cytokine	Capture antibody		Detection antibody	
	name	*concentration*	*name*	*concentration*
INFγ	D-1K	10 µg/ml	7B6-1Biotin	2 µg/ml
Granzyme B	GB 10	10 µg/ml	GB 11 Biotin	2 µg/ml

On day two the plates were washed four times with PBS by filling the wells with 200 µl/well for 15 minutes. After the final washing step the plate was filled with T cell medium (150 µl/well) to block unspecific binding sites and left in the incubator (37°C, 5% CO_2) for one hour.

When the T cells had been expanded in 96 well plates after sorting, they were counted, transferred to conical bottom plates (1000 cells/well), washed three time with T cell medium and then transferred to the ELISpot plates (50 µl/well).

When the clones had been expanded in culture flasks, they were washed three times and transferred to the ELISpot plates directly.

For granzyme B ELISpots the T cell clones had to be at a much higher concentration in order to obtain analysable results. Hence the clones were pipetted in duplicates into the wells starting with 200,000 cells per well. The concentration of target cells allocated per well remained at 20,000 cells per well.

After allocation of the clones the plates were incubated for 30 minutes at 37°C.

T2 cells were loaded with peptide by coculturing 1 x 10^7 cells/ml with selected peptide at a concentration of 30 µM in T cell medium for 2 hours in the incubator. During that time the cell suspension was mixed by vortexing every 15 minutes. Subsequently, cells were washed three times. Tumour cells had been incubated with INFγ (100 U/ml) 48 hours prior to the assay in order to enhance HLA-A*0201 expression and were washed twice with T cell medium before use.

The target cells were then pipetted onto the T cell suspension at a concentration of 20,000 cells/well in 50 µl/well. This had to been done carefully so that the T cells clinging to the membrane surface of the plate were not dispersed. Afterwards the plates were incubated at 37°C for 20 hours during which cytokines were released by the T cells in reaction to the target cells and captured.

On day three the plates were washed six times with washing buffer (PBS/0.05% Tween) and then drained of residual fluid by tipping the plates upside down on paper

towels. The detection antibody was diluted by adding 22 µl antibody to 10 ml PBS and 1 ml PBS/0.5% BSA per plate and then pipetted on the plates at a volume of 100 µl/well. Then the plates were incubated at 37°C for two hours. Afterwards the plates were washed again six times with washing buffer and drained of residual fluid by tipping the plates on paper towels. The Streptavidin Horse Radish Peroxidase was diluted by adding 100 µl peroxidase to 10 ml PBS and 1ml PBS/0.5% BSA per plate and then pipetted on the plates at a volume of 100 µl/well. Then the plates were incubated at room temperature for an hour in the dark. During that time the developing solution was prepared. For this purpose one tablet 3-Amino-9-ethyl-carbazole (AEC) was dissolved in 2.5 ml dimethylformamid and then added to an acetate buffer (see 2.6.2)
Subsequently, the plates were washed three times with washing buffer and three times with PBS and carefully drained of residual fluid. Per 10ml developing solution 25 µl H_2O_2 were added. The solution was immediately passed though a 40 µl cell strainer to remove any aggregate and then pipetted on the plates at a volume of 100 µl/well. The reaction was stopped after seven minutes under running tap water. The plates were dried and evaluated.

3.9 Expansion of CTLs

The T cells clones that were considered the most specific in the screening ELISpot, i.e. the first ELISpot after expansion in limiting dilution, were expanded further for subsequent testing. For this purpose 5 x 10^4 to 1 x 10^5 cells were mixed with irradiated feeder cells consisting of pool PBMCs (2,5 x 10^7) and LCL (5 x10^6) in 25 ml T cell medium and co-cultured with anti-CD3 mAb (30 ng/ml) in small cell culture flasks. The next day different cytokines were added as shown in Table 8 depending on the individual approach.

Table 8: Varying cytokine concentrations used for T cell expansion after the screening ELISpot.

Time of expansion	**Classical approach**	**IL-21 approach a**	**IL-21 approach b**	**IL-21 approach c**
Expansion after screening ELISpot	IL-2 (50 U/ml) IL-15 (2 ng/ml)	IL-21 (30 ng/ml) IL-15 (10 ng/ml) IL-7 (10 ng/ml)	IL-21 (30 ng/ml) IL-15 (10 ng/ml) IL-7 (10 ng/ml)	IL-21 (30 ng/ml) IL-15 (2 ng/ml)

Cytokines were added every other day and medium was exchanged when necessary.

No new cytokines were added on the last three days before the ELISpot assay.

Clones that had not been used for testing or staining were frozen down in aliquots of 1 x 10^5 cells.

3.10 Feeder cells

Pool PBMCs and lymphoblastoid cell lines (LCLs) are feeder cells that were used for every expansion of T cell clones since their cell interaction with T cells provided growth signals for the T cells.

For generating pool PBMCs, PBMCs from three adult donors were isolated as described in 3.2, mixed in equal numbers and frozen down in aliquots of 5 x 10^7 cells. Before use in T cell expansion they were thawed and irradiated at 30 Grey. Many cells died during irradiation and their released DNA potentially clogged live cells. Therefore such cells were pre-incubated with 1 µl DNAse (260 U/µl) in a warm water bath (37°C) until no more lumps were visible. Afterwards cells were washed three times with T cell medium and were ready for use.

LCLs are human B cells that have been immortalized by Epstein-Barr virus. Before use in expansion they were irradiated with 100 Grey and washed three times.

Irradiation was necessary to inhibit growth of feeder cells.

3.11 Flow cytometry

Flow cytometry is a method for detecting surface markers of cells by staining them with fluorescent-labeled antibodies.

For cell staining a 96 well round bottom plate was used. For each staining sample 2 x 10^5 cells were allocated, washed in staining buffer (PBS/2% FCS /0.5% sodium azide) and re-suspended in 40 µl buffer. Unspecific binding sites were blocked by adding 10 µl human IgG (stock concentration: 100 µg/ml) and incubating the mix for 20 minutes at 4°C. 2 µl antibody solution was added per sample. One sample was stained with the isotype controls of the fluorophores used in order to set up the parameters for the flow cytometer. Then the cell suspension was incubated for another 30 minutes at 4°C. Afterwards the cells were washed twice with staining buffer (200 µl/well). If the staining samples were evaluated in the cytometer right away, they were suspended in 400 µl PBS. If the evaluation was postponed, the samples were fixed in 200 µl fixing solution (PBS/ 1% PFA) and stored at 4°C.

3.12 Vβ analysis

The Vβ analysis is a method used to distinguish between oligoclonal and monoclonal T cells by employing flow cytometry. For this purpose the IOTest® Beta Mark kit was used which covers about 70% of the normal human TCR Vβ repertoire. It contains eight vials (A – H) each covering three Vβ segments by staining either with PE- or PE+FITC- or FITC-labelled antibodies. The staining procedure is consistent with the protocol described in 3.11. As 30% of the TCR Vβ repertoire is not covered by the kit it is possible to exclude monoclonality by a double or multiple positivities of Vβ segments but no positivity does not prove monoclonality.

The following table provided by the manufacturer of the kit shows the covered Vβ specificities.

Table 9: Content of Vβ analysis staining kit

Tube	Vβ	Fluorochrome	Clone	Isotype (species)
A	Vb 5.3 (TRBV5-5)	PE	3D11	IgG1 (mouse)
	Vb 7.1 (TRBV4-1, TRBV4-2, TRBV4-3)	PE+FITC	ZOE	IgG2a (mouse)
	Vb 3 (TRBV28)	FITC	CH92	IgM (mouse)
B	Vb 9 (TRBV3-1)	PE	FIN9	IgG2a (mouse)
	Vb 17 (TRBV19)	PE+FITC	E17.5F3	IgG1 (mouse)
	Vb 16 (TRBV14)	FITC	TAMAYA1.2	IgG1 (mouse)
C	Vb 18 (TRBV18)	PE	BA62.6	gG1 (mouse)
	Vb 5.1 (TRBV5-1)	PE+FITC	IMMU157	IgG2a (mouse)
	Vb 20 (TRBV30)	FITC	ELL1.4	IgG (mouse)
D	Vb 13.1 (TRBV6-5, TRBV6-6, TRBV6-9)	PE	IMMU222	IgG2b (mouse)
	Vb 13.6 (TRBV6-6)	PE+FITC	JU74.3	IgG1 (mouse)
	Vb 8 (TRBV12-3, TRBV12-4)	FITC	56C5.2	IgG2a (mouse
E	Vb 5.2 (TRBV5-6)	PE	36213	IgG1 (mouse)
	Vb 2 (TRBV20-1)	PE+FITC	MPB2D5	IgG1 (mouse)
	Vb 12 (TRBV10-3)	FITC	VER2.32	IgG2a (mouse
F	Vb 23 (TRBV13)	PE	AF23	IgG1 (mouse)
	Vb 1 (TRBV9)	PE+FITC	BL37.2	IgG1 (rat)
	Vb 21.3 (TRBV11-2)	FITC	IG125	IgG2a (mouse
G	Vb 11 (TRBV25-1)	PE	C21	IgG2a (mouse)
	Vb 22 (TRBV2)	PE+FITC	IMMU546	IgG1 (mouse)
	Vb 14 (TRBV27)	FITC	CAS1.1.3	IgG1 (mouse
H	Vb 13.2 (TRBV6-2)	PE	H132	IgG1 (mouse)
	Vb 4 (TRBV29-1)	PE+FITC	WJF24	IgM (rat)
	Vb 7.2 (TRBV4-3)	FITC	ZIZOU4	IgG2a (mouse)

The table is the original table found in the manual of the Vβ analysis staining kit but without the references provided by the manufacturer

The manufacturer stated that the nomenclature for the Vβ families such as Vb 5.1 was taken from Wei, S., Charmley, P., Robinson, M.A., Concannon, P., "The extent of the human germline T-cell receptor V β gene segment repertoire", 1994, Immunogenetics, 40, 27-36.

The nomenclature such as TRBV5-5 was the IMGT nomenclature from

Lefranc, M.P., Giudicelli, V., Ginestoux, C., Bodmer, J., Muller, W., Bontrop, R., Lemaitre, M., Malik, A., Barbie, V.,Chaume D., "IMGT, the international ImMunoGeneTics database", 1999, Nucleic Acids Res., 27, 209-212. and

Lefranc, M.P., "IMGT, the international ImMunoGeneTics database", 2003, Nucleic Acids Res., 31, 307-331

4. Results

4.1 Dendritic cells

Before loading dendritic cells with peptide their maturity was checked microscopically:

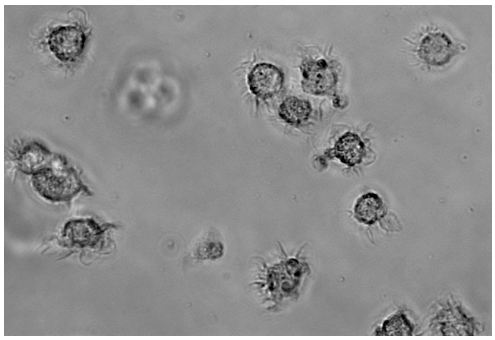


Figure 1: Picture of mature dendritic cells

In this picture the many dendrites of mature dendritic cells can be observed which are their eponym.

Mature DCs express several characteristic surface markers such as HLA-DR, CD83 (Zhou and Tedder, 1995) and CD86 (Caux et al., 1994). Hence, maturity was verified by determining expression of these maturity surface markers using flow cytometry:

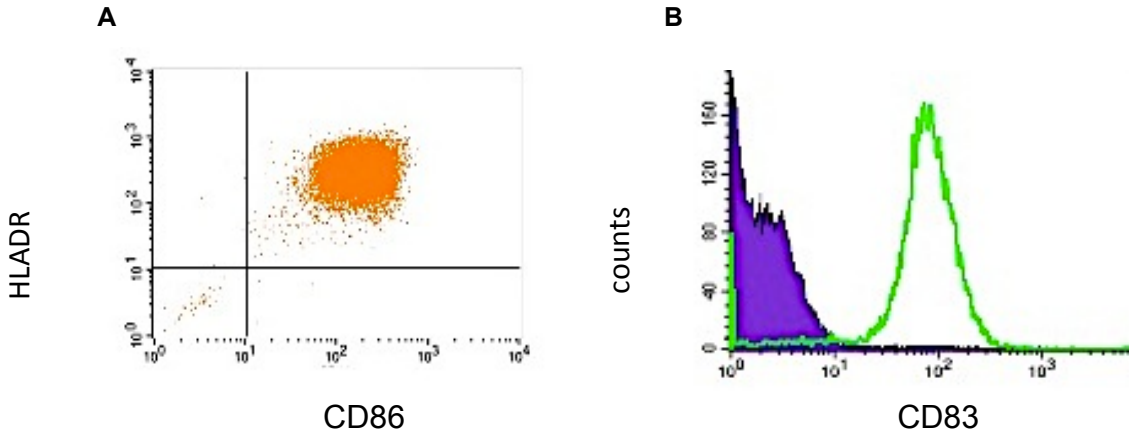


Figure 2: A demonstrates the expression of HLA-DR and CD86**, B** shows the overlay of CD83 (green) and the isotype (purple)

Only dendritic cells that demonstrated this maturity phenotype microscopically and cytometrically were used for the priming of T cells.

4.2 Standard approach

The HLA-A*0201-binding peptides CHM1-319 (VIMPCSWWV) and EZH2-666 (YMCSFLFNL) have been successfully used to generate allorestricted effector T cells (Thiel et al., 2011; Pirson, 2009). Although the use of STEAP1-86 (FLYTLLREV) and GPR64-135 (FIMCATAEA) produced effector T cells with peptide-specificity in the past, a specificity for HLA-A*0201^{+} Ewing tumour cell lines expressing those peptides has not yet been successfully demonstrated (Pirson, 2009).

For this study, T cells have been primed with either CHM1-319 or STEAP1-86 or GPR64-135 and then expanded according to the standard protocol for the production of effector T cells in order to establish a reference point for the IL-21 approaches.

4.2.1 CHM1-319

In two different standard approaches CHM1-319-specific effector T cells were produced and tested in ELISpot assays. An example is shown in Fig. 3.

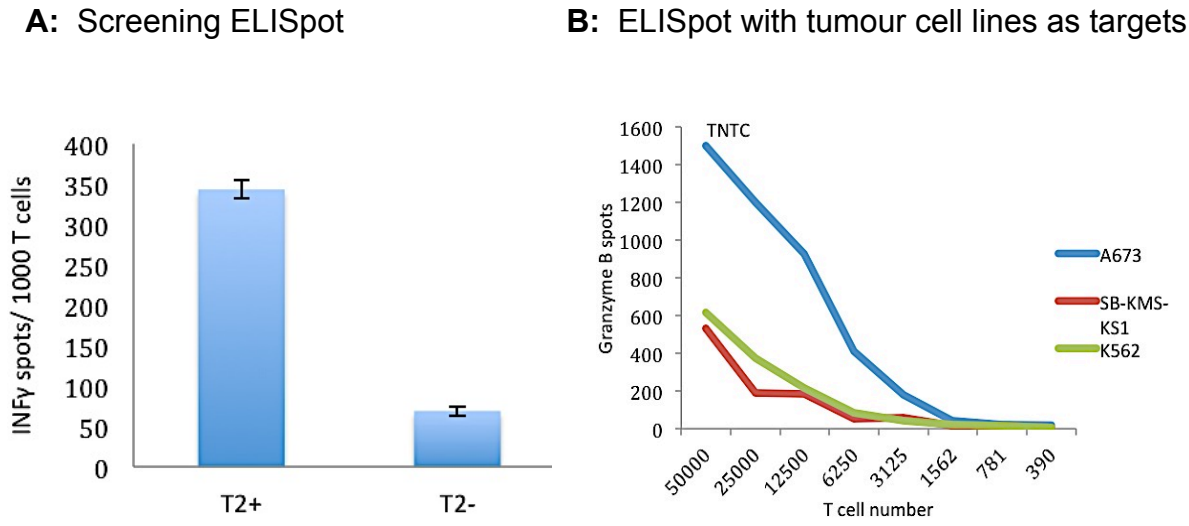


Figure 3: ELISpots of CHM1-319-primed T cells. A shows the peptide-specificity of the T cell clone CHM_D6 measuring INFγ release. **B** shows a granzyme B ELISpot of the same clone with tumour cell lines as targets. (TNTC = too numerous to count)

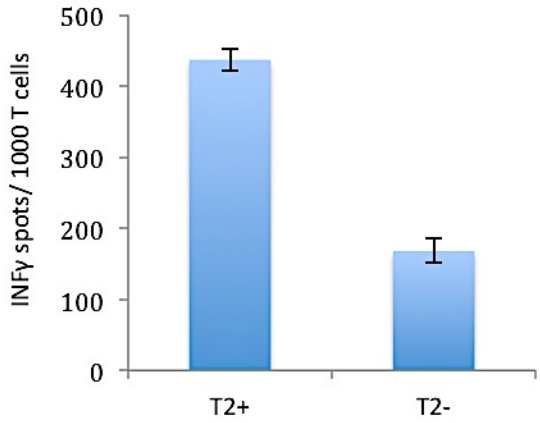
T2+ here means that the T2 cells have been pulsed with the CHM1-319 peptide hence presenting it to the T cells on their cell surface. T2- here means the T2 cells have been pulsed with influenza-derived peptide (GILGFVFTL) that the T cells do not recognise.

The granzyme B ELISpot demonstrates that although CHM1-319 is expressed on both ET cell lines A673 and SB-KMS-KS1[1] (Pirson, 2009) the T cells require the presence of HLA-A*0201$^+$ MHC molecules for which they have been primed to bind to the target cells and subsequently release cytokines. A673 is HLA-A*0201$^+$ but not SB-KMS-KS1. The erythroleukaemia cell line K562 does not express HLA-molecules and serves as a natural killer (NK) cell control.

4.2.2 STEAP1-86

CD8$^+$ cells were primed with dendritic cells loaded with STEAP1-86 in a standard approach. The clones did recognise STEAP1-86-loaded T2 cells and the HLA-A*0201$^+$ and STEAP-expressing (Pirson, 2009) ET cell line A673 but did not recognise SB-KMS-KS1 cells which also express STEAP (Pirson, 2009) but are HLA-A*0201$^-$. An example of a specific clone is shown below.

A: Screening ELISpot



B: ELISpot with ET cell lines as targets

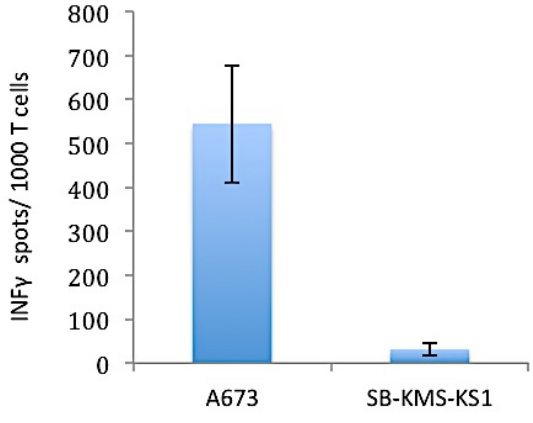


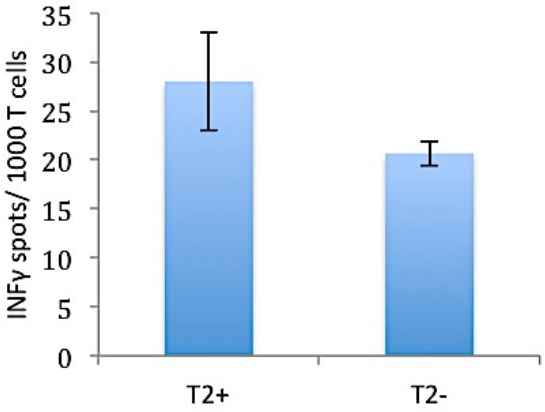
Figure 4: A demonstrates the peptide-specificity of clone STEAP_D3, **B** shows the same clone`s HLA-A*0201$^+$-specific recognition of the ET cell line A673 versus SB-KMS-KS1.

[1] SB-KMS-KS1 has been recently renamed and is referred to as SBSR-AKS in the paper by Thiel et al. 2011, Richter et al. 2009, and the thesis by Pirson

4.2.3 GPR64-135

$CD8^+$ cells were primed with GPR64-135-loaded dendritic cells in a standard approach. The clones were specific for T2+ cells and the Ewing tumour cell line A673. A673 and SB-KMS-KS1 both express GPR64 (Pirson, 2009). An example of a clone is shown below.

A: Screening ELISpot



B: ELISpot with tumour cell lines as targets

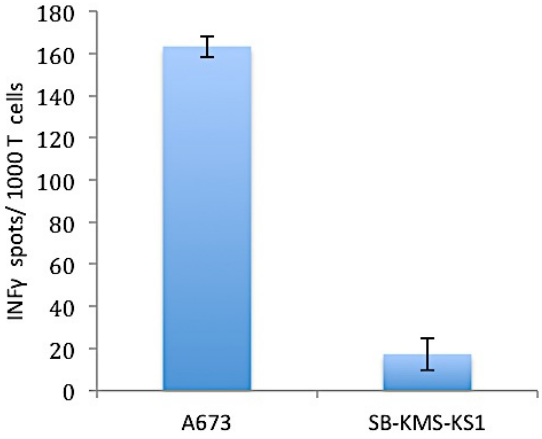


Figure 5: A demonstrates the peptide-specificity of the clone GPR64_F5**, B** shows the same clones HLA-A*0201$^+$ specific recognition of the Ewing tumour cell line A673 versus SB-KMS-KS1.

4.2.4 EZH2-666

It has been established before that $CD8^+$ cells can be primed with EZH2-666-loaded dendritic cells and develop into effector T cells that specifically recognise T2+ cells and A673 cells in the standard approach (Thiel et al., 2011; Pirson, 2009). This experiment was not repeated for this thesis.

4.3 IL-21 approach a

4.3.1 Dendritic cells and priming

The dendritic cells were checked for their maturity using flow cytometry before loading with peptide. Mature DCs highly expressed CD86, HLA-DR and CD83.

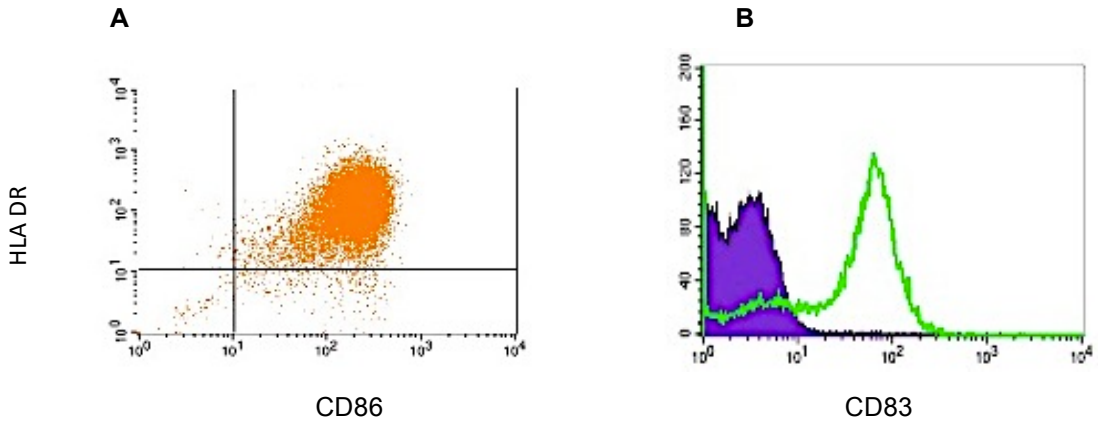


Figure 6: A shows the expression of HLA-DR and CD86, **B** demonstrates the overlay of CD83 (green) and the isotype (purple), illustrating the high expression of CD83

DCs were loaded with either CHM1-319 or STEAP1-86 or GPR64-135 and used to prime $CD8^+$ cells. After cell sorting the T cells were expanded as shown in Table 10:

Table 10: Combination of cytokines used to expand the T cell clones

Expansion in limiting dilution after cell sorting	IL-21 (30 ng/ml) IL-15 (2 ng/ml)
Expansion after the screening ELISpot	IL-21 (30 ng/ml) IL-15 (10 ng/ml) IL-7 (10 ng/ml)

Unfortunately, the screening ELISpots of the clones specific for STEAP1-86 or GPR64-135 were unsuccessful but the screening of the CHM1-319-directed clones revealed a high number of specific clones of which the best four clones, CHM1_A9, CHM1_F3, CHM1_G2 and CHM1_ A5 were expanded further for subsequent testing (see 4.3.3).

4.3.2 Phenotype

After the screening ELISpot the selected clones were expanded in cell culture flasks and the phenotype of the T cells was determined. For that purpose the cells were stained with antibodies specific for CD27, CD28, CD45RO, CD62L, CD127 and CD44 (Figure 7). The presence or absence of these surface markers categorizes T cells into naive T cells, effector T cells or memory T cells (Table 11).

Table 11: Comparison of the expression of certain surface markers of T_E, T_{CM} and T_{21a}

Surface marker	**Effector T cells**	**Central Memory T cells**	**IL-21 approach *a* T cells**
CD62L	-	+	+
IL-7 Rα (CD127)	-	+	-
CD45RO	-	+	+
CD28	-	+	+
CD27	-/+	+	+
CD44	-	+	+

The cells were also stained with CHM1-319-HLA-A*0201-pentamer or irrelevant pentamer demonstrating their strong positivity for CHM1-319 (Fig. 7).
The strong presence of the surface markers typical for central memory cells detected by flow cytometry revealed that IL-21 approach *a* successfully generated T cells closely resembling the memory phenotype illustrated in Table 11.

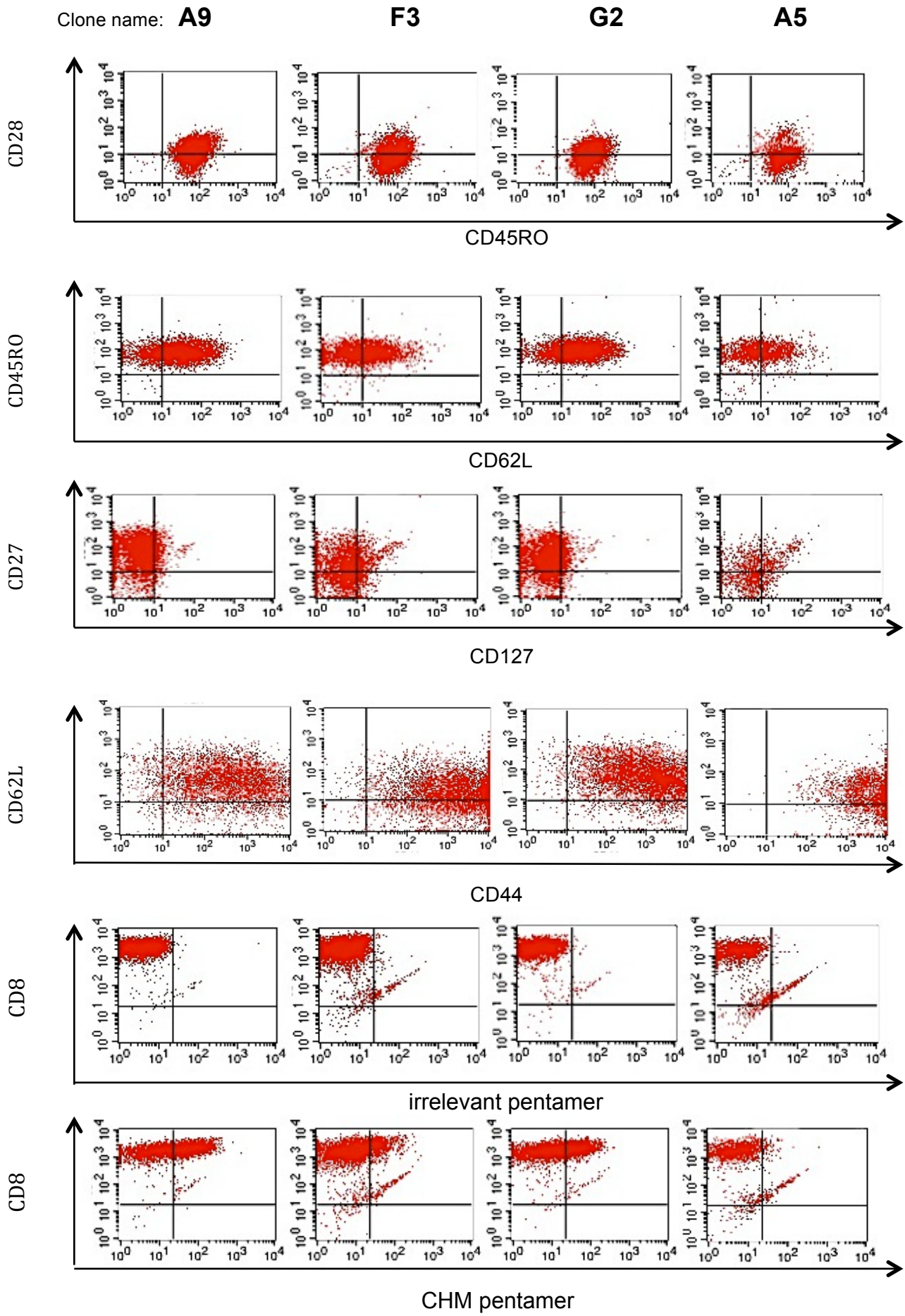


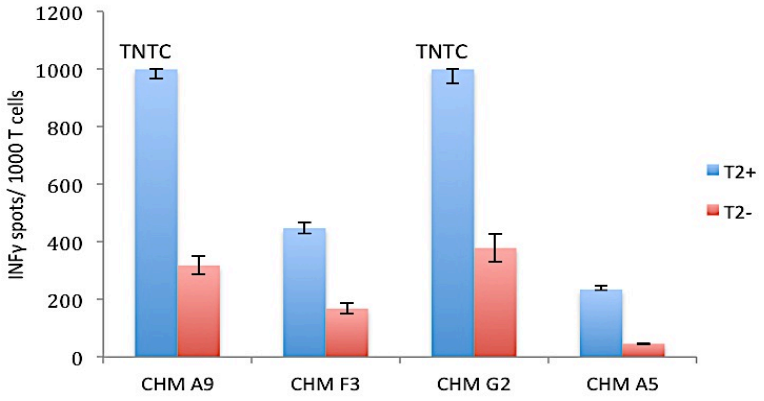
Figure 7: T cell clones were stained and analysed in flow cytometry.

The absence of IL-7 Rα on the cell surface of the IL-21 approach T cells could be explained by the fact that IL-7 was involved in the stimulation process and hence an internalization of the receptor as a regulatory response might have taken place. This theory is supported by the fact that hardly any IL-21 receptor could be detected by staining with the corresponding antibody (data not shown) and IL-21 was involved in almost every step of the T cell generation process.

4.3.3 Specificity

The specificity of the IL-21 approach *a* clones was determined in an ELISpot by measuring their INFγ release in the presence of T2+ cells (T2 cells loaded with CHM1-319 peptide) and T2- cells (T2 cells loaded with influenza peptide as a negative control). The four most specific clones were expanded further for subsequent testing of the recognition of the tumour cell lines A673, SB-KMS-KS1 and the HLA-A*0201^{+} melanoma cell line Sk-Mel-29 (Fig. 8).

A



B

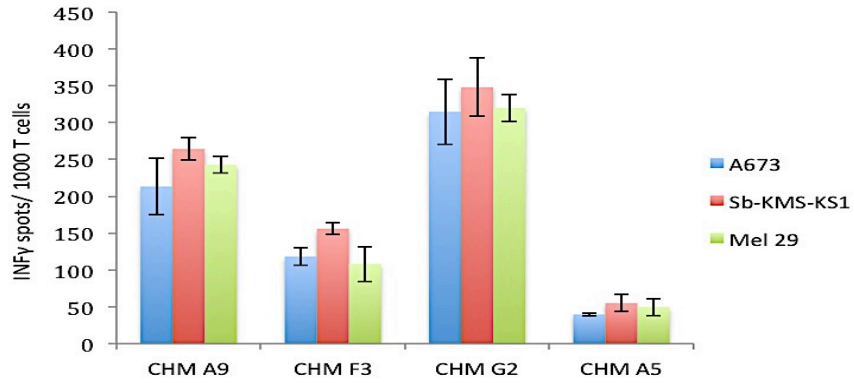


Figure 8 : A shows the high peptide-specificity of the IL-21 *a* clones; **B** demonstrates that the clones are nonspecific towards the tumour cell lines

As shown in Figure 8 the clones were very specific for T2 cells loaded with CHM1-319 peptide but not for A673. In order to clarify, whether the tumour unspecificity was due to the nature of the cells or the screening process, generated T cell clones in IL-21 approach *b* and *c* (see below) were screened for loaded T2 cells as well as for the ET lines A673 and SB-KMS-KS1.

4.3.4 Expansion with IL-2 and IL-15

Furthermore, in order to determine whether the memory T cells could be turned into effector cells with the right signals an expansion according to the standard approach was executed with IL-2 (50 U/ml) and IL-15 (2 ng/ml). For comparison, the four clones were also expanded simultaneously with IL-21 (30 ng/ml), IL-15 (10 ng/ml) and IL-7 (10 ng/ml). The phenotype of the IL-2/IL-15 clones was determined using flow cytometry (Fig. 9).

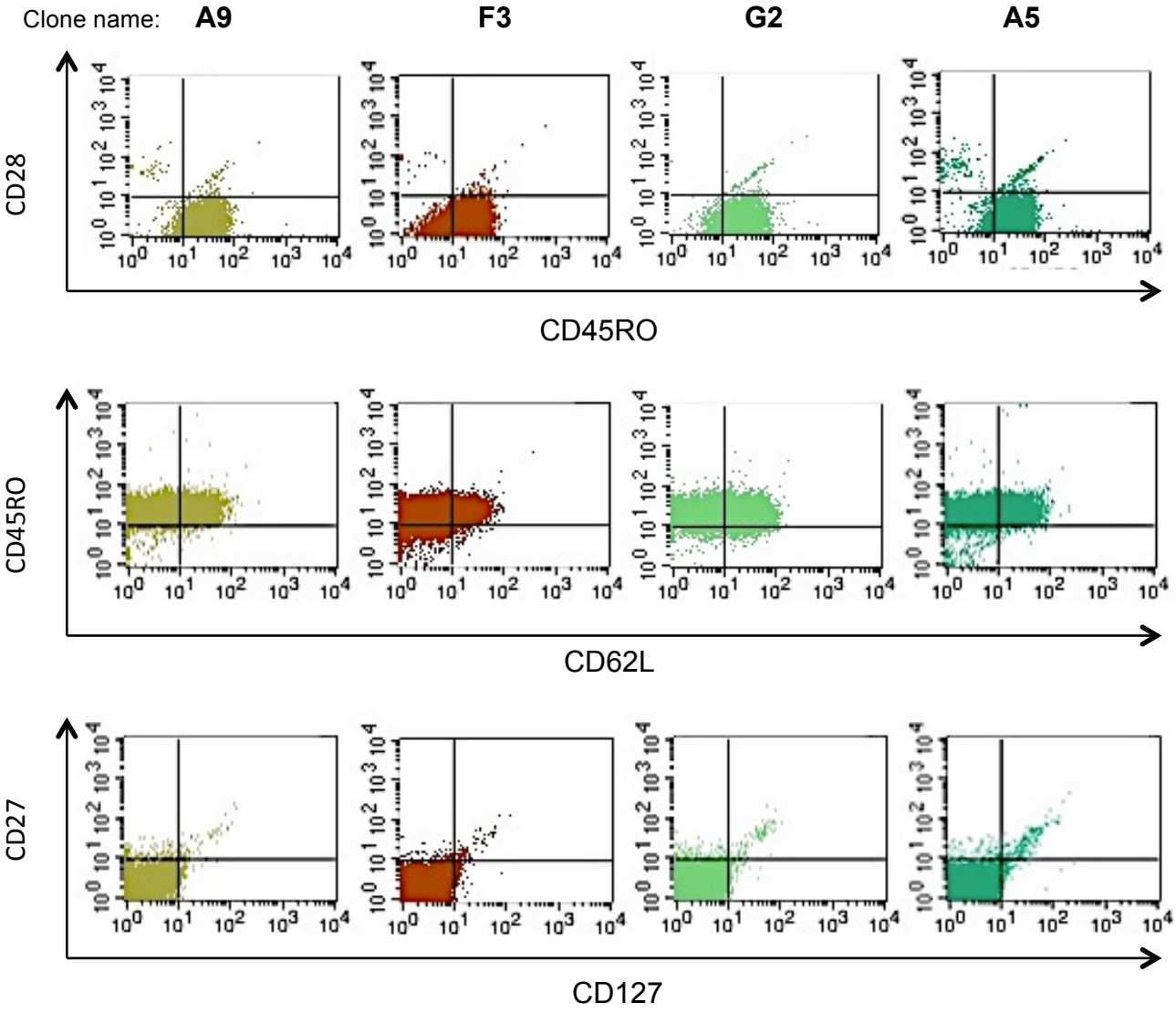


Figure 9: Analysis of the surface markers detected in flow cytometry

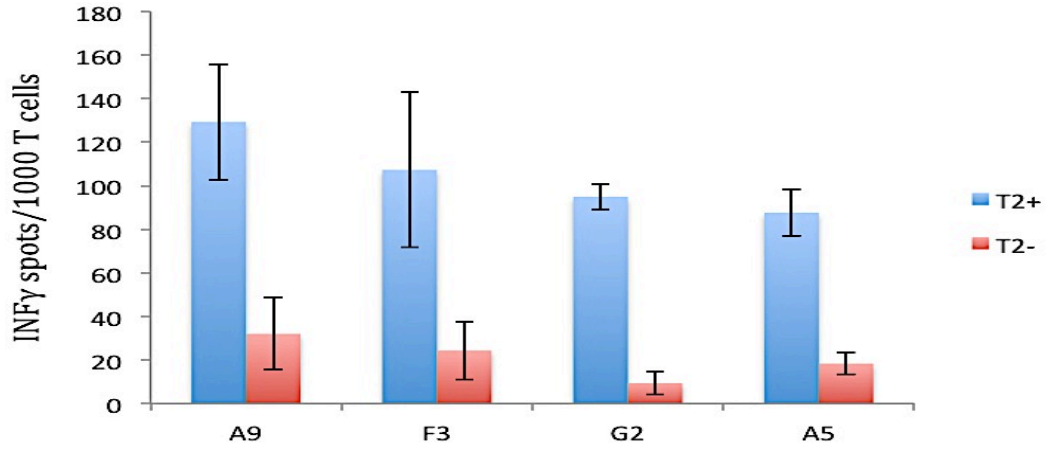
Table 12 compares the expression of surface markers of the central memory phenotype and the expression of surface markers after expansion with IL-2 and IL-15 which promote an effector phenotype. It shows that the positivity for CD28 and CD27 was decreased to almost nil in all clones and CD62L expression was reduced by 25 – 69%.

Table 12: Comparison of surface marker before and after expansion with IL-2 and IL-15.

Clone	CD28 positivity in %		CD62L positivity in %		CD27 positivity in %	
	Before IL-2/IL-15	**After** IL-2/IL-15	**Before** IL-2/IL-15	**After** IL-2/IL-15	**Before** IL-2/IL-15	**After** IL-2/IL-15
A9	70	1	78	32	80	3
F3	38	1	51	16	46	1
G2	55	1	80	60	68	3
A5	40	2	47	27	37	2

The specificities of the clones were determined by an ELISpot assay by measuring their INFγ release in the presence of T2+ cells and T2- cells. Furthermore, the INFγ and Granzyme B release in the presence of tumour cell lines was measured.
Both, the clones expanded with IL-21, IL-15 and IL-7 and the clones expanded with IL-2 and IL-15, were tested simultaneously.

Expansion with IL-21, IL-15, IL-7



Expansion with IL-2 and IL-15

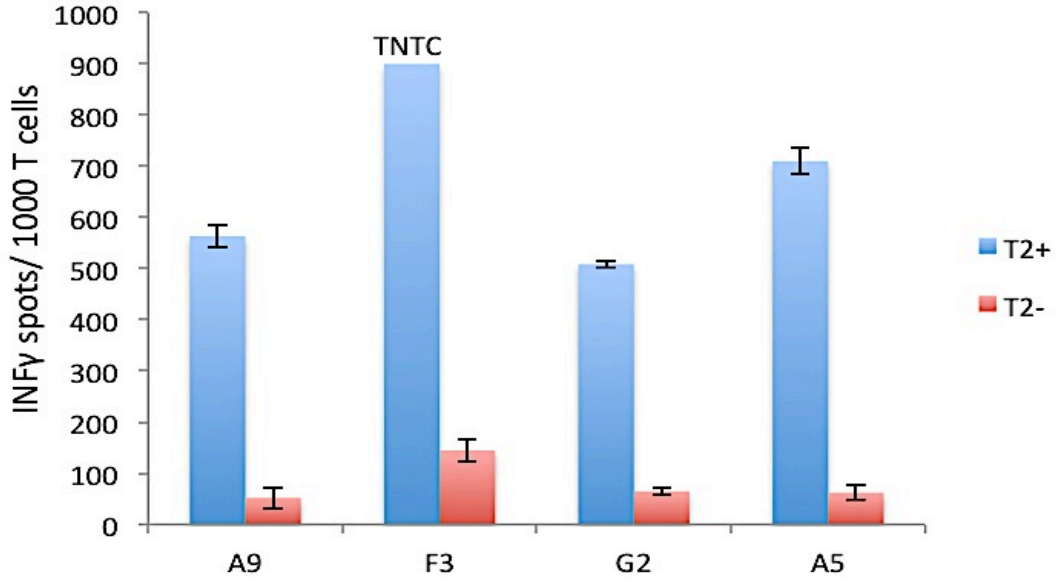


Figure 10: Comparison of the INFγ release in the presence of peptide-loaded T2 cells by the T cell clones expanded either with IL-21, IL-15 and IL-7 or with IL-2 and IL-15

The specific reactivity of the IL-2/IL-15 clones to T2+ cells was on average was six times stronger than that of the IL-21/IL-15/IL-7 clones. The reaction to tumour cells was also much stronger but still very unspecific (not shown).

4.4 IL-21 approach b

4.4.1 Dendritic cells and priming

The dendritic cells were checked for their maturity using flow cytometry before loading with peptide.

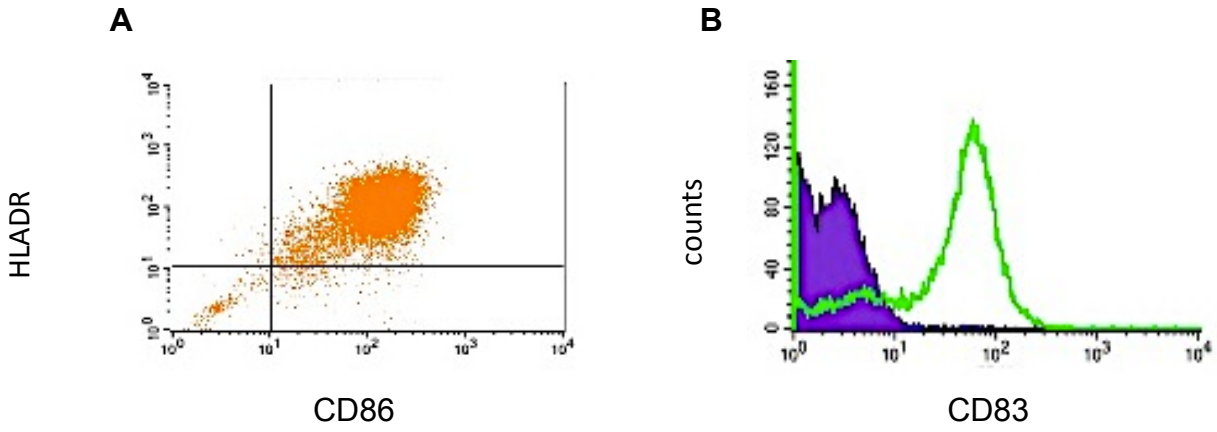


Figure 11: A: expression of HLA-DR and CD86**; B:** overlay of CD83 (green) and the isotype (purple), illustrating the high expression of CD83

DCs were loaded with either CHM1-319 or STEAP1-86 or EZH2-666 and then used to prime $CD8^+$ cells. After cell sorting the T cells were expanded using the combination of cytokines shown in Table 13:

Table 13: Combination of cytokines used in the expansion of T cell clones in approach *b*

Expansion in limiting dilution after cell sorting	IL-21 (30 ng/ml) IL-15 (10 ng/ml) IL-7 (10 ng/ml)
Expansion after the screening ELISpot	IL-21 (30 ng/ml) IL-15 (10 ng/ml) IL-7 (10 ng/ml)

The clones CHM_E10, STEAP_H9 and EZH_G3 were selected in the screening process and expanded further for subsequent testing.

4.4.2 Phenotype

The T cell clones were stained with CD28, CD45RO, CD62L, CD27, CD127, CD56 and CD8 and analysed in flow cytometry (Figure 12)

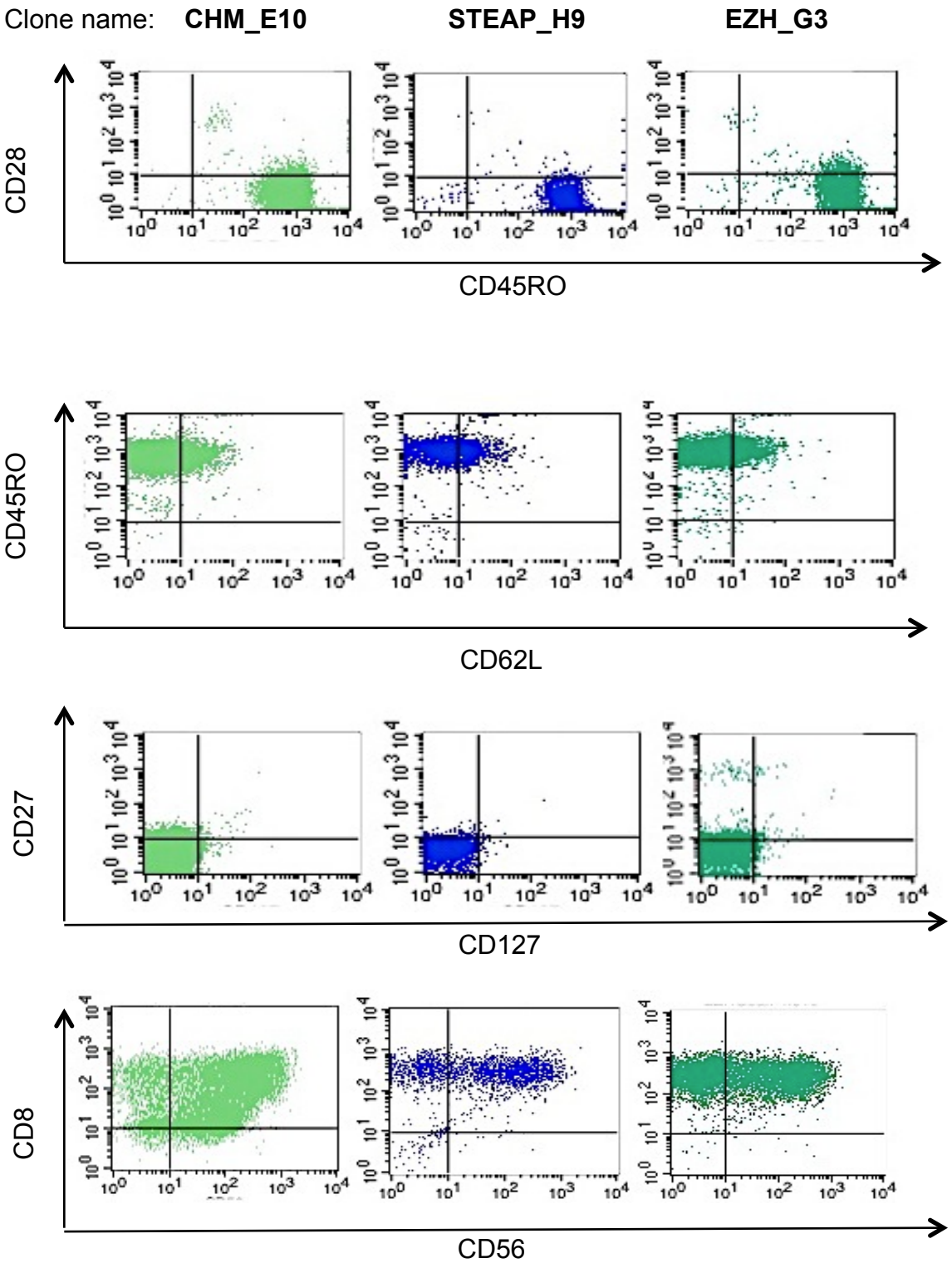


Figure 12: The T cell clones were stained and analysed in flow cytometry.

The T cell clones in approach *b* exhibited the surface markers found on central memory phenotypic cells much less strongly than the ones in approach *a*.

Vβ analysis was conducted for each clone (Table 14). It is used to test cells for mono- or oligoclonality (see 3.12).

Table 14: Vβ analysis of the clones

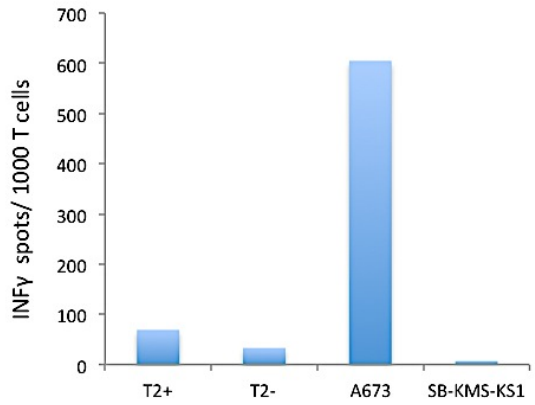
clone	Positivity / Fluorochrome	
CHM_E10	Vβ 14	FITC
STEAP_H9	Vβ 14	FITC
EZH_G3	Vβ 14	FITC

As the T cell clones were positive for Vβ 14 but negative for all other tested Vβ families, they might be monoclonal.

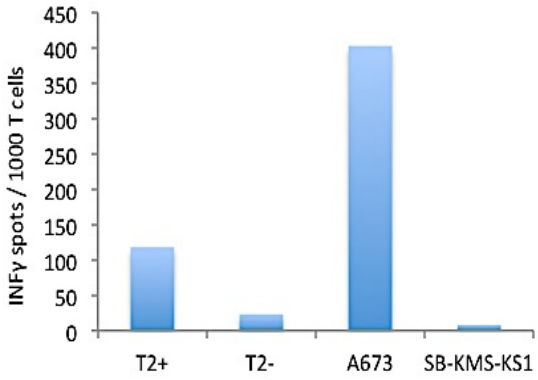
4.4.3 Specificity

In the screening ELISpot the clones were tested for specificity by measuring their INFγ release in the presence of T2+, T2-, A673 and SB-KMS-KS1 (Fig. 13). Both A673 and SB-KMS-KS1 express CHM1, STEAP and EZH2 albeit at different levels (Pirson, 2009). Only the most specific clones were expanded further:

CHM_E10



STEAP_H9



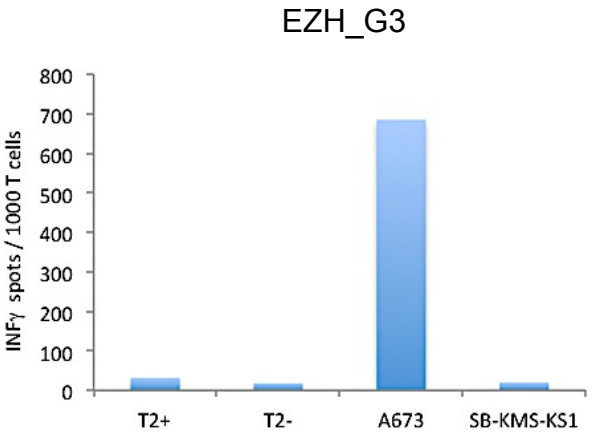


Figure 13: Screening ELISpot of the three T cell clones

Table 15 demonstrates the specifity of the clones by correlating the INFγ spots released in reaction to various target cells.

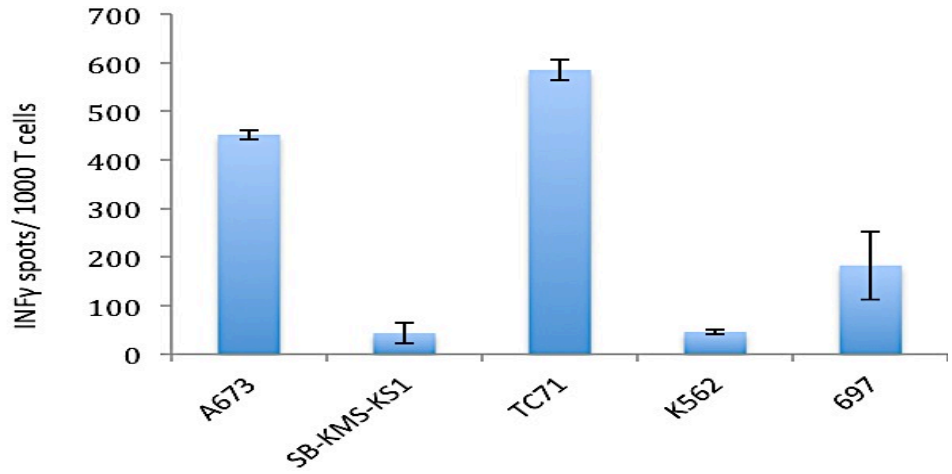
Table 15: Ratios of the number of spots in reaction to the tested target cells

Clone	T2+ : T2-	A673 : SB-KMS-KS1
CHM_E10	2:1	86:1
STEAP_H9	5:1	50:1
EZH_G3	2:1	34:1

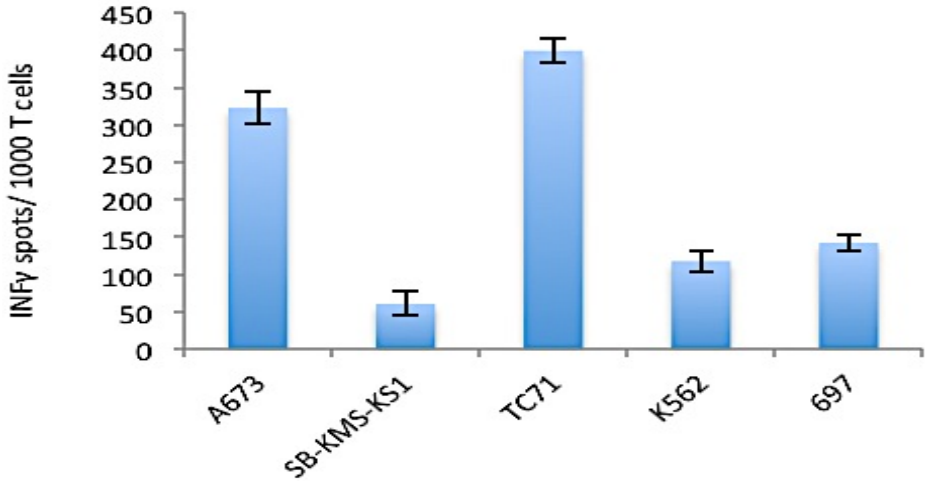
After the second expansion these clones were tested for the specific recognition of various tumour cell lines (Figure 14).

TC-71 is a HLA-A*0201^{+} ET cell line just like A673 and expresses CHM1, STEAP, GPR64 and EZH2 (Pirson, 2009). The erythroid leukaemia cell line K562 is HLA-negative and served as a NK cell control. The common acute lymphoblastic leukaemia (cALL) cell line 697 is HLA-A*0201^{+} but expresses no CHM1, STEAP1, GPR64 and only small amounts of EZH2 (Pirson, 2009) and served as a negative control.

CHM_E10



STEAP_H9



EZH_G3

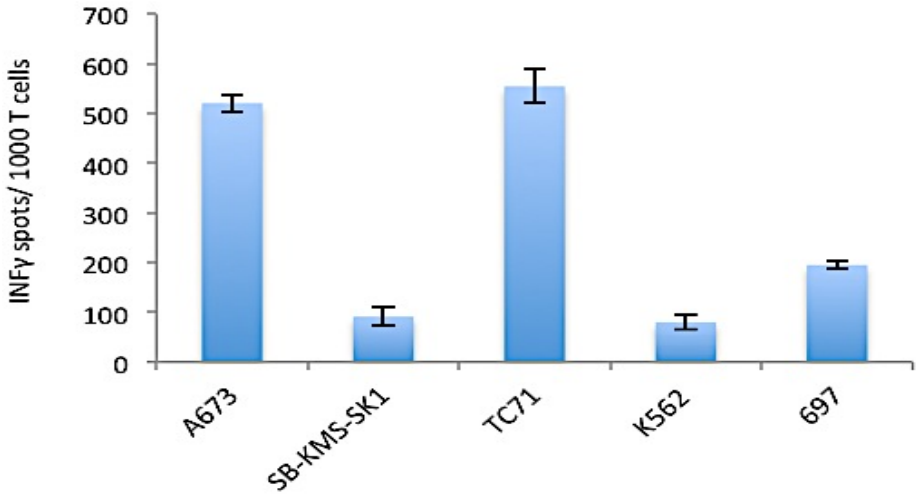


Figure 14: INFγ release in reaction to various tumour cell lines.

The T cells were also tested again for the recognition of peptide-loaded T2 cells (not shown) in the same ELISpot. Unfortunately, INFγ-release was rather unspecific. However, given the high specificity for HLA-A*0201^{+} tumour cell lines presenting those peptides on their cell surface, loss of specificity in the presence of T2 cells

might be due to other factors such as the age of the T2 cells. The T cells also showed considerable INFγ-release in the presence of the HLA-A*0201^{+} melanoma cell line SK-Mel-29 (not shown). It is not known, whether Sk-Mel-29 presents CHM1, STEAP1 or EZH2 on its cell surface. Therefore, the recognition of this melanoma cell line does not allow conclusions to be drawn about the specificity of the T cells.

Table 16 clarifies the high specificity of the cells by demonstrating the high ratios of INFγ release in the presence HLA*0201^{+} ET cell lines A673 and TC-71 compared to HLA*0201^{-} ET cells (SB-KMS-KS1), the negative control 697 and the NK cell control K 562 which were only marginally recognised.

Table 16: Comparison of INFγ release of the three T cell clones in reaction to various tumour cell lines

clone	A 673 : SB-KMS-KS1	TC-71 : SB-KMS-KS1	TC-71 : K562	TC-71 : 697
CHM_E10	11 : 1	14 : 1	13 : 1	3 : 1
STEAP_H9	5 : 1	7 : 1	3 : 1	3 : 1
EZH_G3	15 : 1	16 : 1	15 : 1	2 : 1

4.5 IL-21 approach c

This approach involved the CD8^{+} cells from the HLA-A*0201^{-} blood of the umbilical cord of two different donors.

4.5.1 Dendritic cells and priming

The dendritic cells were generated and checked for their maturity using flow cytometry before loading with peptide (Fig. 15).

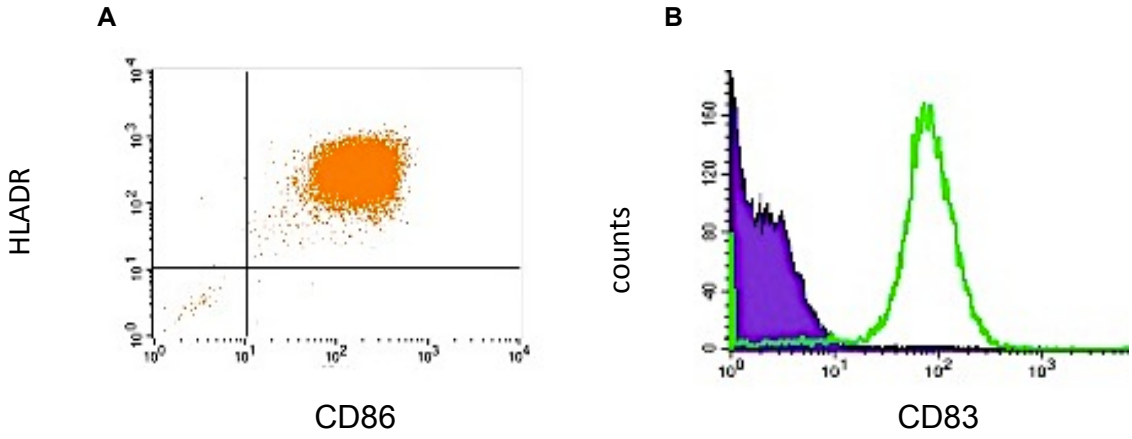


Figure 15: A: expression of HLA-DR and CD86**; B:** overlay of CD83 (green) and the isotype (purple), illustrating the high expression of CD83

The dendritic cells were loaded with CHM1-319, STEAP1-86 or EZH2-666 and used to prime $CD8^+$ cells using the combination of cytokines shown in Table 17:

Table 17: Combination of cytokines used to expand the T cell clones

Expansion after cell sorting in limiting dilution	IL-21 (30ng/ml) IL-15 (2ng/ml)
Expansion after the screening ELISpot	IL-21 (30ng/ml) IL-15 (2ng/ml)

The clones CHM_G12 and STEAP_F10 - both originating from the same donor - and EZH_D12 - originating from another donor - were selected in the screening process and expanded further for subsequent testing:

4.5.2 Phenotype

The T cell clones were stained with CD28, CD45RO, CD62L, CD27, CD127, CD56 and CD8 and analysed in flow cytometry (Figure 16)

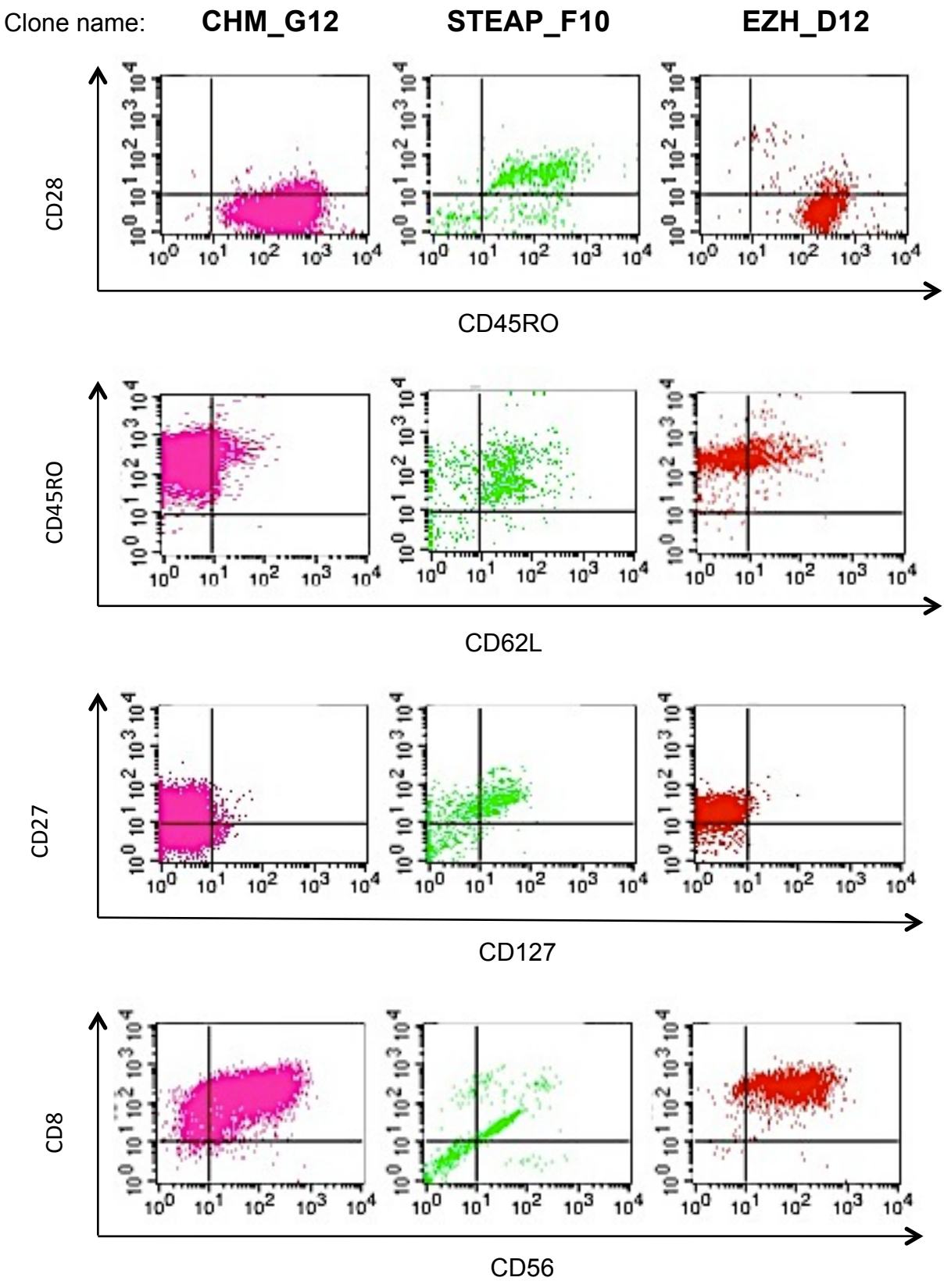


Figure 16: Analysis of the surface markers detected by flow cytometry

The T cell clones in approach *c* exhibited the surface markers found on a central memory phenotype. Unfortunately, the T cells of STEAP_F10 died in great numbers in repetitive attempts to stain them for FACS analysis. Thus, the FACS analysis was greatly compromised. Nonetheless, the few living cells detected reveal a similar phenotype to CHM_G12 and EZH_D12.

4.5.3 Specificity

The clones were tested in the screening ELISpot by measuring their INFγ release in the presence of peptide-loaded T2 cells, A673 and SB-KMS-KS1 (Figure 17).

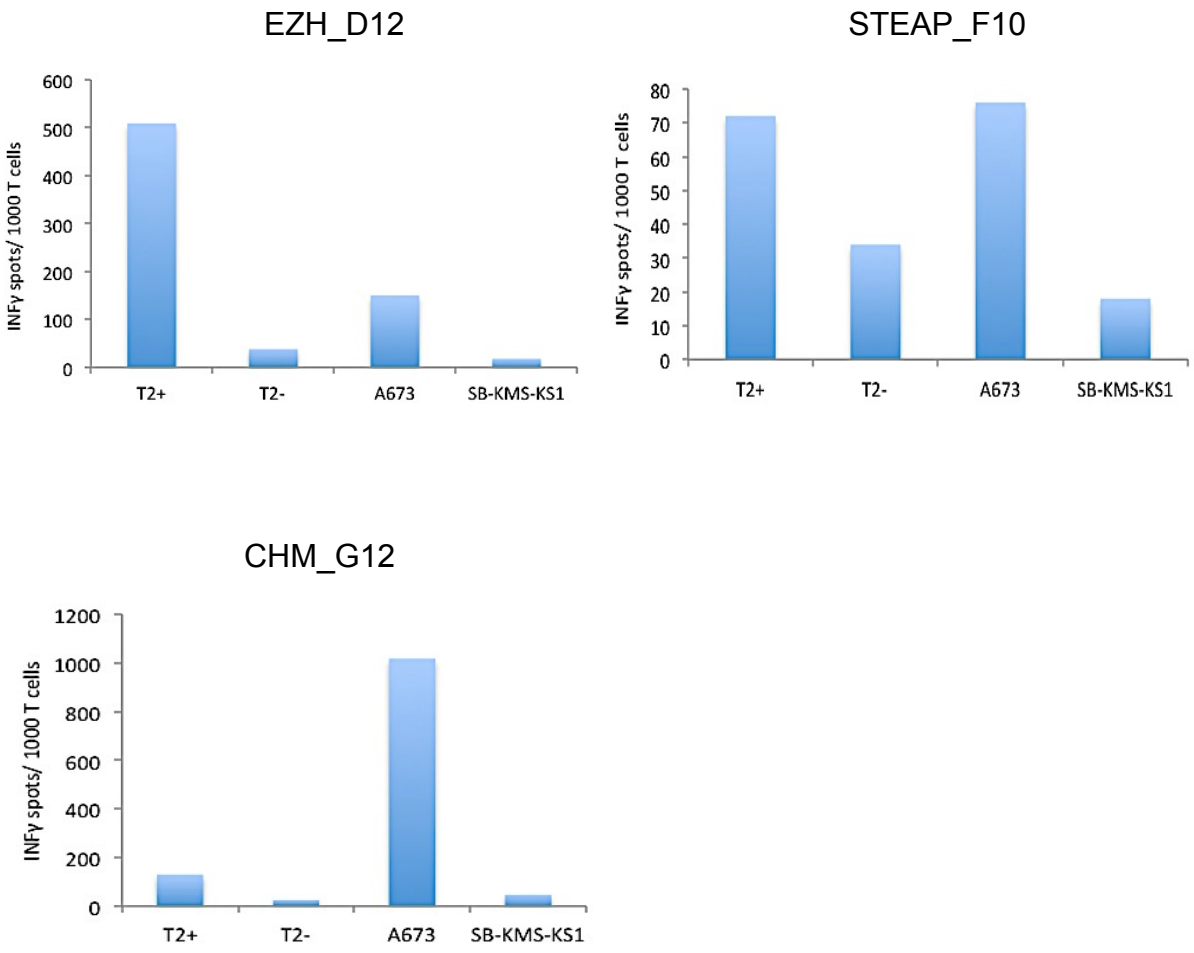


Figure 17: Screening ELISpot of the three T cell clones

The T cell clones showed great specificity for the specific tumour peptide (T2+ cells) and A673.

Table 18 illustrates the specifity of the $CD8^+$ cells by putting the number of counted spots in relation to each other.

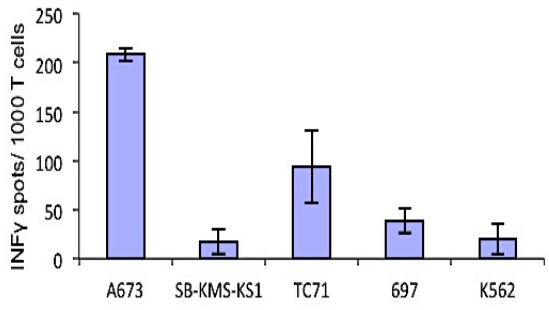
Table 18: Ratios of the number of spots in reaction to the tested target cells

Clone	T2+ : T2-	A673 : SB-KMS-KS1
EZH_D12	13 : 1	8 : 1
STEAP_F10	2 : 1	4 : 2
CHM_G12	5 : 1	22 : 1

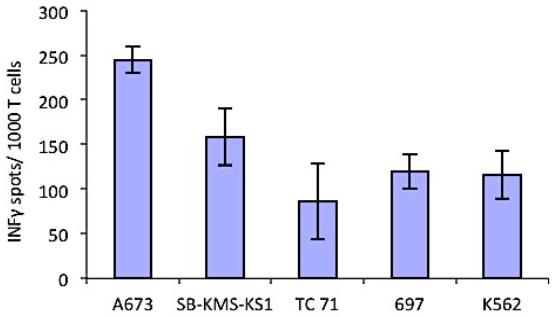
Unfortunately, the selected clones revealing high specificity must have been contaminated by unspecific T cells which were more proliferative as demonstrated by the increase of unspecific results in subsequent ELISpot assays.

EZH_D12

1st ELISpot after screening

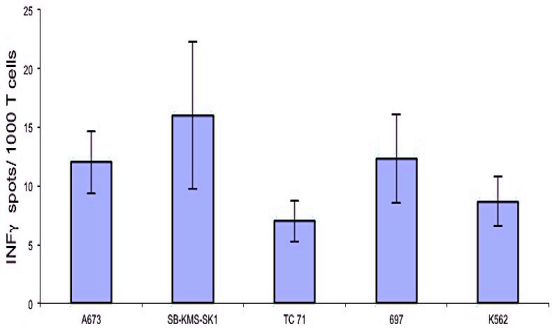


2nd ELISpot after screening



STEAP_F10

1st ELISpot after screening



The 2nd ELISpot showed no measurable results

CHM_G12

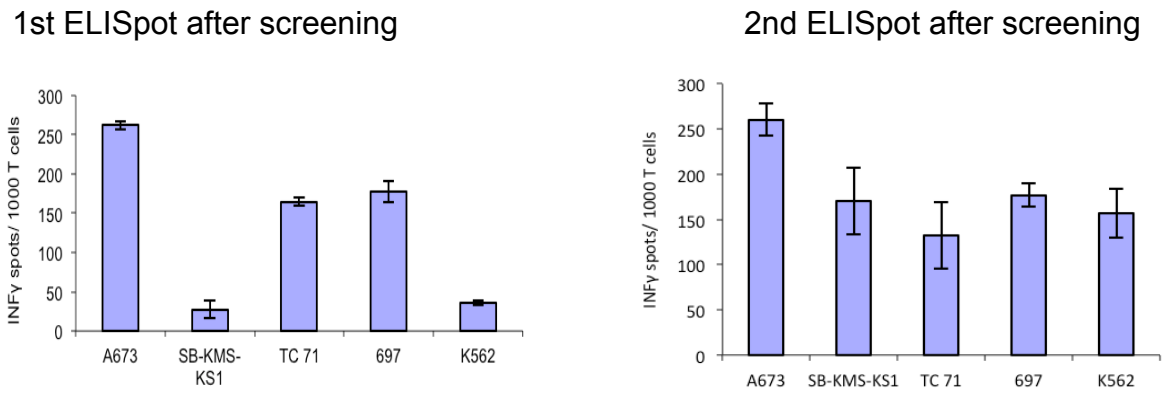


Figure 18: Comparison of the specificity of the T cell lines in two consecutive ELISpots.

In those subsequent ELISPots T cells were also tested again for the recognition of peptide-loaded T2 cells (not shown) and INFγ-release was rather unspecific. In the first ELISpot after the screening the T cells were also tested for SK-Mel-29 (not shown). The T cells showed considerable INFγ-release in the presence SK-Mel-29, the significance of which is at present left open (see 4.4.3).

As the T cells were very specific in the screening ELISpot their continuous deterioration of specificities indicates that they were either oligoclonal and the unspecific clones dominated the INFγ release in subsequent ELISpots or that the T cells lost their specificity due to an insufficient activation process.

Thus, Vβ analysis was conducted (Table 19).

Table 19: Vβ analysis of the T cells

clone	Positivity for fluorochrome
EZH_D12	-
STEAP_F10	-
CHM_G12	Vb 13.1 (TRBV6-5, TRBV6-6, TRBV6-9) PE

As the Vβ analysis did not demonstrate oligoclonality, it is possible that the T cells demonstrated Vβ types that are in the uncovered 30% of the Vβ kit.

The T cells were not used in a murine setting as they lacked specificity.

Nonetheless, approach *c* was a success as it showed that T cells from the umbilical cord blood can be developed into allorestricted central memory cytotoxic T cells just as well as T cells from an adult donor. However, as these T cells possibly lost specificity, they might require a more thorough priming/activation process.

4.6 comparison of IL-21 approaches

The generation of ET antigen-specific T cells is a highly selective process (Table 20). Starting with between ca. 1 x 10^6 to 1 x 10^7 cells before sorting only one to three clones are selected in the screening ELISpot.

Table 20: Illustration of the highly distinguishing process from cell priming to the selection of the best T cell clone(s)

approach		peptide	cell no before sorting	cell no after sorting	screening process			no of clones tested further
					no of tested clones	no of specific clones	no of selected clones	
Std app. 1		CHM1-319	2.5 x 10^6	1089	9	2	2	2
		STEAP1-86	1.8 x 10^6	328	12	2	2	2
		GPR64-135	1.9 x 10^6	271	19	3	3	1
Std. app. 2		CHM1-319	6.1 x 10^6	590	48	2	1	1
IL-21 app. *a*		CHM1 -319	2.4 x 10^7	1126	144	121	4	4
		STEAP1-86	2.4 x 10^7	217	96	0	-	-
		GPR64-135	1.9 x 10^7	235	96	0	-	-
IL-21 app. *b*		CHM1-319	1.1 x 10^7	1325	59	42	3	1
		STEAP1-86	1.2 x 10^7	1700	30	16	3	1
		EZH2-666	9.6 x 10^6	1189	32	10	4	1
IL-21 app. *c*	**donor a**	CHM1-319	3 x 10^6	1013	18	4	2	1
		EZH2-666	2.6 x 10^6	596	18	4	3	1
	donor b	CHM1-319	8.3 x 10^5	1800	54	10	2	1
		STEAP1-86	8.3 x 10^5	1113	30	4	2	2

There is no considerable difference between the T cells of the standard and IL-21 approaches in the selection process. In general, CHM1-319-specific T cells had a greater number of tested clones meaning that more cells proliferated from single cell suspensions to a sufficiently large number of cells for testing in the ELISpot assays compared to the T cells primed with DCs loaded with either STEAP1-86 or GPR64-135 or EZH2-666. This was true for both the standard and IL-21 approaches.

Nonetheless, great differences were observed in the phenotypes of each IL-21 approach despite the relatively small variances in use and doses of the cytokines (Table 21)

Table 21: Differences in the expression of certain surface markers in the varying IL-21 approaches

approach	expansion	cytokines	peptide	clone no	av. d.p.* of CD28 and CD45RO in %	av. d.p.* of CD45RO and CD62L in %	av. p. of CD27 in %
IL-21 app. *a*	1. exp.	IL-21 (30ng/ml) IL-15 (2ng/ml)	CHM	4	51	64	58
	2. exp	IL-21 (30ng/ml) IL-15 (10ng/ml) IL-7 (10ng/ml)					
IL-21 app. *b*	1. exp.	IL-21 (30ng/ml) IL-15 (10ng/ml) IL-7 (10ng/ml)	CHM	3	5	17	13
			STEAP	2	2	24	28
	2. exp	IL-21 (30ng/ml) IL-15 (10ng/ml) IL-7 (10ng/ml)	EZH	4	4	18	10
IL-21 app. *c*	1. exp.	IL-21 (30ng/ml) IL-15 (2ng/ml)	CHM	4	35	31	59
	2. exp.	IL-21 (30ng/ml) IL-15 (2ng/ml)	STEAP	1	29	40	56
			EZH	3	6	17	71

av. d.p. = average double positivity

Table 21 demonstrates that the central memory typical expressions of CD28, CD45RO, CD62L and CD27 were smallest in approach *b* where IL-7 and high dose IL-15 (10 ng/ml) were used in both expansions after sorting. T cells in approach *a* had the best outcome regarding the expression of central memory phenotypic surface markers.

It can be concluded that only IL-21 and low dose IL-15 (2 ng/ml) should be used in the first expansion after cell sorting. The use of IL-7 has probably no adverse effect on the central memory phenotype as discussed below.

5. Discussion

IL-21 has proven to be an effective tool in creating allorestricted cytotoxic central memory T cells. The method of generating Tcm was basically the same as producing Tef but IL-21 replaced IL-2 in every step, starting with the stimulation process of T cells with peptide-loaded dendritic cells. The result was a very specific phenotype expressing the typical surface markers such as CD28, CD27, CD45RO, CD62L and CD44.

5.1 Phenotype

IL-21 generated Tcm expressed high levels of CD28. CD28 interacts with CD86 on DCs (Caux et al., 1994) and B7 on B lymphocytes acting as a co-stimulatory for T cell activation (Linsley *et al.*, 1991). It also increases the expression of Bcl-XL considerably, enabling T cells to withstand apoptosis (Boise et al., 1995). More importantly, CD28 seems to be firmly linked to the ability to produce IL-2 (Topp et al., 2003), a growth and proliferation factor of T cells. CD28 is expressed on naive T cells and on early differentiation stages of antigen experienced cells (Appay et al., 2008). IL-21 generated cells also expressed high levels of CD27. Whereas CD28 assists cell proliferation by augmenting cell cycle entry as well as activity, CD27 enhances the numbers of TCR-activated T cells by supporting their survival (Hendriks et al., 2003). CD27 is the receptor for CD70 that is found on B lymphocytes and dendritic cells. It has been shown by Roberts et al. that CD27 stimulation is therapeutically effective in a murine model in the treatment of established solid melanomas by maintaining tumour-reactive $CD8^+$ T cells within the tumour and increasing their IFNγ release (Roberts et al., 2010).

The IL-21 generated cells also expressed high levels of CD45RO and CD62L. CD45RO is characteristic for memory cells (Merkenschlager and Beverley, 1989). High expression of CD62L is found only on central memory cells whereas effector memory cells express low levels of CD62L on their surface (Farber et al., 2002). CD62L (L-selectin) is an adhesion molecule required for cellular extravasation in high endothelial venules (Tedder et al., 1995). There have been indications that the ability to home to secondary lymphoid tissues, mediated by CD62L, was important for an optimal anti-tumour response (Klebanoff et al., 2005).

CD44 was also expressed by the IL-21 generated T cells. High CD44 expression has been found on $CD27^+$ and $CD28^+$ T cells. It is an adhesion molecule and seems to be linked to IFNγ and IL-2 release upon tumour stimulation (Zhou et al., 2011). The IL-21 generated T cells further expressed high levels of CD56. Although CD56 is a characteristic marker of natural killer cells, it is also expressed on a small subset of $CD4^+$ and $CD8^+$ T cells. It is a membrane glycoprotein belonging to the Ig superfamily and has been indicated to correlate with effector function of cytotoxic T cells (Pittet et al., 2000). However, it was shown by Thulesen et al. that the influence of IL-15 on thymocytes resulted in a $CD8^+$ $CD56^+$ and $CD45R0^+$ memory phenotype that exhibited high expression of the IL-15R-complex and displayed cytotoxic activity (Thulesen et al., 2001).

Thus, the use of IL-21, IL-15 and IL-7 in the generation of allorestricted cytotoxic T cells results in a central memory like phenotype endowed with great proliferative potential, lymph node homing receptors and cytotoxic activity.

However, the choice and dosage of cytokines in the generation process have considerable influence on the phenotype.

5.2 Cytokines

IL-21 has proven to be essential for generating central memory T cells.

It is a novel type I cytokine that is generated by activated $CD4^+$ T cells, consists of a 4-helix-bundle structure with significant similarity to IL-2, IL-4 and IL-15 and interacts with the newly described type I cytokine receptor, IL-21R (Parrish-Novak et al., 2000). The IL-21 receptor contains the common cytokine receptor gamma chain (gamma c), which is also a required subunit of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (Habib et al., 2002; Parrish-Novak et al., 2000), The IL-21 receptor is found on B and T lymphocytes and also on NK cells. For the latter its activation has a part in the augmentation of cytotoxic function and INFγ production (Kasaian et al., 2002). For B cells IL-21 has a role in the differentiation to plasma cells, cell activation and proliferation and the production of immunoglobulins (Kuchen et al., 2007). For T cells, however, it is a co-stimulator for proliferation resulting in an augmentation of memory cells (Nguyen and Weng, 2010). In this function it differs greatly from IL-2 although they are closely related.

IL-2 supports differentiation of $CD8^+$ cells into effector cells and augments IL-2Rα expression through a positive feedback loop (Kim et al., 2006). IL-21, on the other hand, was shown by Hinrichs et al. to have a negative regulatory influence on effector T cells by repressing the expression of IL-2Rα and Eomesodermin, a T-box transcription factor that promotes effector functions of $CD8^+$ T cells. Instead, it enhanced expression of CD62L found only on central memory phenotypes. Furthermore, they showed that cells that were antigen-primed in the presence of IL-21 showed superior anti-tumour response in the murine model than T cells primed in the presence of IL-2 or IL-15 (Hinrichs et al., 2008). Hence, in all IL-21 approaches IL-21 replaced IL-2 during the stimulation of T cells with peptide-loaded dendritic cells and every subsequent cell expansion for the generation of Tcm directed against Ewing tumour cell lines.

However, in the first attempts I tried to use IL-21 alone without addition of any other cytokines in the stimulation and expansion process. This resulted in approximately 25 – 50% fewer T cells in the IL-21 approach than the classical approach which were conducted simultaneously with $CD8^+$ T lymphocytes from the same donor. Therefore it was decided to add IL-15 and IL-7 to further support proliferation.

Although IL-15 has proven to be significant for the development and function of effector T cell, it has also been shown to be important in the generation and preservation of memory phenotype $CD8^+$ T cells (Schluns *et al.,* 2002). Zeng et al. have reported that IL-21 and IL-15 work synergistically to enhance T cell proliferation by increasing the cell division rate of both naive and memory-phenotype T cells. They also showed that both cytokines enhanced effector function of T cells measurable by an increase in INFγ release. These effects led to a potent anti-tumour response as treatment with both cytokines of melanoma bearing mice led to tumour regression. They also showed that the combination of both cytokines at lower doses was more effective than each cytokine alone at a higher dose (Zeng et al., 2005). However, IL-15 is also used in allorestricted effector T cell expansion simultaneously with IL-2 (Thiel et al., 2011). It promotes an effector phenotype by down-regulating CD28. It was shown by Alves et al. that IL-21 averts this entirely and was also able to counteract down-modulation of CD62L by IL-15 though at a lower extent (Alves et al., 2005).

IL-15 was used in every T cell expansion in all three IL-21 approaches as it works synergistically with IL-21 and has been shown to have a role in the maintenance of memory $CD8^+$ cells. Nevertheless at approach *b* in which IL-15 was used at a

concentration of 10 ng/ml in both expansions after cell sorting CD28 and CD62L expressions were the poorest of all results obtained. In approach *a* in which IL-15 was used at a concentration of 2 ng/ml in the first expansion after cell sorting the T cells had very high CD28 and CD62L expressions. Possibly IL-21 failed to counteract the down-regulatory effects of IL-15 at the higher dosage. Although IL-15 was used at a higher dosage in the second cell expansion of approach *a* it is likely that the cells were less susceptible to a phenotype change at this later stage than at the first cell expansion.

IL-7 has been shown to be crucial for the "homeostatic proliferation and survival of naive T cells" (Tan et al., 2001). A study by Goldrath et al. demonstrated that "memory T cells, on the other hand, could use either IL-7Ralpha- or IL-15-mediated signals for acute homeostatic proliferation" (Goldrath et al., 2002). IL-7 has been reported to also work synergistically with IL-21 to increase cell proliferation but this combination led to a cell yield of only about 30 to 60% of the cell numbers brought about by a combination of IL-21 and IL-15 in those studies (Zeng et al., 2005; Alves et al., 2005). There is no indication that the increased use of IL-7 is responsible for the poorer expression of memory phenotype surface markers in approach *b* as a study by Alves et al. showed that a seven day stimulation of naive T cells with IL-7 had no down-regulatory effect on the expression of CD28, CD27 and CD62L (Alves et al., 2005).

Therefore it can be concluded that IL-21 is essential in the process of creating central memory T cells and that IL-15 is preferably used at a lower dosage of 2 ng/ml at the first cell expansion after cell sorting. The addition of IL-7 might aid in the process of cell proliferation and has probably no adverse effect on the central memory phenotype.

5.3 Antigen specificity and differentiation capacity

CHM1-319, EZH2-666 and STEAP1-86 are equally recognised by both effector and central memory T cells and have proven to be suitable targets for allorestricted T cell immunotherapy. The suitability of GPR64-135 for the generation of allorestricted Tcm has yet to be shown. Indeed Tcm are no less specific than Tef in detecting HLA-A*0201^{+} ET cell lines as measured by their INFγ release in ELISpot assays.

Tcm are also characteristically known to secrete IL-2 upon stimulation (Sallusto et al., 1999). Unfortunately, IL-2 release was not successfully illustrated by an ELISpot since

it required at least 10^5 T cells for measurable results similarly to granzyme B. Such high cell numbers were not achieved by a IL-21 approach.

However, the T cells of approach *a* whose phenotype was closer to effector memory T cells after expansion with IL-2 and IL-15 secreted four to nines times as much INFγ in the presence of peptide-loaded T2 cells as central memory T cells. CD28 expression was reduced to almost nil in those T cells. As CD28 is needed for the production of IL-2, the loss of it in the differentiation process of memory T cells might make them more dependent on survival factors derived from other immune cells such a T helper cells and this might be an important regulatory tool for differentiated T cells upon antigen stimulation (Topp et al., 2003). The almost complete loss of CD27 indicates that the T cells were well differentiated at the effector stage (Appay et al., 2008).

The T cells of approach *a* that had been further expanded with IL-2 and IL-15 retained their memory phenotype as they kept their CD45RO expression, which is characteristic for memory T cells (Merkenschlager and Beverley, 1989). However, Tem, in contrast to Tcm have low expression of CD62L (Farber et al., 2002). Whereas the central memory T cells maintained a very high expression of CD62L, ranging from 47 to 80%, the T cell further expanded with IL-2 and IL-15 had lost CD62L expression by 25 to 70%. Interestingly, INFγ release correlated with the expression of CD62L meaning the clone with the lowest expression (16%) had innumerable spots whereas the clone with the highest expression (60%) revealed the lowest INFγ release. This indicates that the central memory T cells generated by the use of IL-21 are capable of differentiating into a more active effector phenotype.

A study by Bachmann et al. investigating Tcm, Tem and Tef during acute and chronic viral infection illustrated that the availability of antigen regulated the equilibrium between the three subsets: Low level Ag persistence resulted in long-term preservation of all three subsets whereas high antigen presence resulted in a majority of Ag-specific effector cells (CD62L–CD127–) which have the highest cytolytic potential. Was no more antigen measurable an almost full reversion to a CD62L+CD127+ phenotype which have the highest proliferative potential was observed over time (Bachmann et al., 2005). It is to be expected that the allorestricted central memory T cells would act similarly in Ewing tumour patients, providing them with a continuous stock of highly cytolytic effector T cells when needed or resting in

the lymph nodes capable of proliferating rapidly should another antigen stimulation occur.

5.4 Outlook for the future

Allorestricted CTL have been shown to “engraft and retain their specific killing activity without causing GVHD” that is generally observed in patients who have been injected with allogeneic T cells (Gao et al., 1999). Allorestricted effector T cells have been shown to successfully inhibit growth of the Ewing tumour (Thiel et al., 2011) so of course the next step should be to test the central memory T cells in a murine model as well.
However, the generation of allorestricted T cells is time-consuming and labour-intensive therefore recent studies have begun to investigate the possibility of cloning T cell receptors making the generation of CTLs more economical and standarisable (Wälchli et al., 2011; Birkholz et al., 2009).

6. Summary

Allorestricted cytotoxic effector T lymphocytes have proven themselves to be effective tools in the treatment of Ewing tumours. Nonetheless, as effector T cells die in their hosts after short periods of time central memory T cells seem to be a necessary alternative. They rest in lymphatic tissue when they are not needed and can provide a continuous source of effector cells on antigen encounter. For the purpose of creating central memory cytotoxic T lymphocytes Interleukin-21 was used. It was assisted by Interleukin-15 and Interleukin-7 in order to enhance proliferation. The dose of IL-21 had been reported to be ideal at 30 ng/ml, but as the best concentrations of IL-15 and IL-7 were not known three different approaches were conducted with different cytokine uses. The general method to generate allorestricted cytotoxic T cells remained the same in all approaches. Firstly, $CD8^+$ cells were isolated from HLA-A*0201$^-$ PBMCs and primed with HLA-A*0201$^+$ peptide-loaded dendritic cells. For that purpose four different Ewing tumour antigens were used namely CHM1-319, STEAP1-86, GPR64-135 and EZH2-666. The cells were sorted for the highest of peptide-specific TCR expression and expanded by limiting dilution. The clones that had proliferated well were tested for their specificity by measuring their INFγ release in the presence of peptide-loaded T2 cells or tumour cell lines in an ELISpot assay. The clones with the highest specificities were expanded for further testing and staining with various flurochromes for analysis of the phenotype in flow cytometry.

It turned out that it was possible to generate a central memory phenotype with IL-21, which was used at antigen-specific stimulation of CTLs with DCs and every successive cell expansion. The best result was attained when 2 ng/ml IL-15 was added in the first expansion after the cell sorting followed by 10ng/ml IL-15 and 10 ng/ml IL-7 at the next cell expansion. The central memory T cells were specific for the tumour peptide with which they had been primed and recognised HLA-A*0201$^+$ Ewing tumour cell lines expressing that peptide. Furthermore it was possible to generate allorestricted central memory CTLs out of $CD8^+$ T cells isolated from blood of the umbilical cord. Those can be injected into mice that had been given a human immune system by transfer of human $CD34^+$ cells into new born mice and be tested for anti-tumour response *in vivo.*

7. References

Abudu A, Mangham DC, Reynolds GM, Pynsent PB, Tillman RM, Carter SR, Grime RJ: Overexpression of p53 protein in primary Ewing's sarcoma of bone: relationship to tumour stage, response and prognosis. *Br J Cancer* 79 (1999): 1185-89

Ahrens S, Hoffmann C, Jabar S, Braun-Munzinger G, Paulussen M, Dunst J, Rübe C, Winkelmann W, Heinecke A, Göbel U, Winkler K, Harms D, Treuner J, Jürgens H: Evaluation of prognostic factors in a tumour volume-adapted treatment strategy for localized Ewing sarcoma of bone: the CESS 86 experience. Cooperative Ewing Sarcoma Study. *Med Pediatr Oncol* 32 (1999): 186-95

Alves NL, Arosa FA, van Lier RA. IL-21 sustains CD28 expression on IL-15-activated human naive CD8+ T cells. *J Immunol* 175 (2005): 755- 62

Appay V, van Lier RA, Sallusto F, Roederer M: Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* 73 (2008): 975-83

Askin FB, Rosai J, Sibley RK, Dehner LP, McAlister WH. Malignant small cell tumor of the thoracopulmonary region in childhood: a distinctive clinicopathologic entity of uncertain histogenesis. *Cancer* 43 (1979): 2438-51

Bacci G, Ferrari S, Longhi A, Rimondini S, Versari M, Zanone A, Forni C. Prognostic significance of serum LDH in Ewing's sarcoma of bone. *Oncol Rep.* 6 (1999): 807-11

Bacci G, Ferrari S, Longhi A, Donati D, De Paolis M, Forni C, Versari M, Setola E, Briccoli A, Barbieri E: Therapy and survival after recurrence of Ewing's tumors: the Rizzoli experience in 195 patients treated with adjuvant and neoadjuvant chemotherapy from 1979 to 1997. *Ann Oncol*. 14 (2003): 1654-59

Bacci G, Longhi A, Briccoli A, Bertoni F, Versari M, Picci P: The role of surgical margins in treatment of Ewing's sarcoma family tumours: experience of a single institution with 512 patients treated with adjuvant and neoadjuvant chemotherapy. *Int J Radiat Oncol Biol Phys* 65 (2006): 766-72

Bacci G, Boriani S, Balladelli A, Barbieri E, Longhi A, Alberghini M, Scotlandi K, Forni C, Pollastri P, Vanel D, Mercuri M*:* Treatment of nonmetastatic Ewing's sarcoma family tumours of the spine and sacrum: the experience from a single institution*. Eur Spine J* 18 (2009): 1091-95

Bachmann MF, Wolint P, Schwarz K, Jäger P, Oxenius A: Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L*., J Immunol* 175 (2005): 4686-96

Baldwin TA, Hogquist KA, Jameson SC. The fourth way? Harnessing aggressive tendencies in the thymus. *J Immunol*. 173 (2004): 6515-20

Banchereau, J. and Steinman R. M.: Dendritic cells and the control of immunity. *Nature*. 392 (1998): 245-52

Birkholz K, Hofmann C, Hoyer S, Schulz B, Harrer T, Kämpgen E, Schuler G, Dörrie J, Schaft N. A fast and robust method to clone and functionally validate T-cell receptors. *J Immunol Methods* 346 (2009): 45-54

Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB: CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 3 (1995): 87-98

Burdach S*.* van Kaick B, Laws HJ, Ahrens S, Haase R, Körholz D, Pape H, Dunst J, Kahn T, Willers R, Engel B, Dirksen U, Kramm C, Nürnberger W, Heyll A, Ladenstein R, Gadner H, Jürgens H, Go el U: Allogeneic and autologous stem-cell transplantation in advanced Ewing tumours. An update after long-term follow-up from two centers of the European Intergroup study EICESS. Stem-Cell Transplant Programs at Dusseldorf University Medical Center, Germany and St. Anna Kinderspital, Vienna, Austria, *Ann Oncol* 11 (2000): 1451-62

Burdach S, Plehm, S., Unland, R., Borkhardt, A., Staege, MS., Müller-Tidow, C., Richter, GHS: Epigenetic maintenance of stemness and malignancy in peripheral neuroectodermal tumours by EZH2. *Cell Cycle* 8 (2009): 1991-96

Burdach S, Thiel U, Schoniger M, Haase R, Wawer A, Nathrath M, Kabisch H, Urban C, Laws HJ, Dirksen U, Steinborn M, Dunst J, Jurgens H: Total body MRI-governed involved compartment irradiation combined with high-dose chemotherapy and stem cell rescue improves long-term survival in Ewing tumour patients with multiple primary bone metastases. *Bone Marrow Transplant* 45 (2010): 483-89

Cao K Hollenbach J, Shi X, Shi W, Chopek M, Fernández-Viña MA*:* Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations, *Hum Immunol* 62 (2001): 1009 -30

Carey TE, Takahashi T, Resnick LA, Oettgen HF, Old LJ: Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proc Natl Acad Sci U S A* 73 (1976): 3278-82

Caux, C. Vanbervliet B, Massacrier C, Azuma M, Okumura K, Lanier LL, Banchereau J: B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. *J. Exp. Med.* 180 (1994): 1841–47

Chen LJ, Jia YX, Fan FF, Li XY, Expression of FLI-1 and analysis of prognostic factors in primitive neuroectodermal tumour*. Zhonghua Zhong Liu Za Zhi*. 32 (2010): 917-20

Cho BS, Lee SE, Song HH, Lee JH, Yahng SA, Eom KS, Kim YJ, Kim HJ, Lee S, Min CK, Cho SG, Kim DW, Lee JW, Min WS, Park CW., The graft-versus-tumour effect according to the type of graft-versus-host disease defined by NIH consensus criteria and the associated outcomes. *Biol Blood Marrow Transplant*. 18 (2012): 1136-43

Cotterill SJ, Ahrens S, Paulussen M, Jürgens HF, Voûte PA, Gadner H, Craft AW*:* Prognostic factors in Ewing's tumour of bone: analysis of 975 patients from the European Intergroup Cooperative Ewing's Sarcoma Study Group*. J Clin Oncol* 18 (2000): 3108-14

Cyster, J. G*.:* Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs, *J Exp Med* 89 (1999): 447-50

Delaplace M, Mélard P, Perrinaud A, Goré C, Vergier B, Machet L.: Primitive cutaneous Ewing's sarcoma: a diagnostic and therapeutic dilemma*. Ann Dermatol Venereol* 138 (2011): 395-98

Delattre O, Zucman J, Melot T, Garau XS, Zucker JM, Lenoir GM, Ambros PF, Sheer D, Turc-Carel C, Triche TJ*,:* The Ewing family of tumours--a subgroup of small-round-cell tumours defined by specific chimeric transcripts, *N Engl J Med* 331 (1994): 294-99

Draube A, Klein-González N, Mattheus S, Brillant C, Hellmich M, Engert A, von Bergwelt-Baildon M: Dendritic cell based tumour vaccination in prostate and renal cell cancer: a systematic review and meta-analysis. *PLoS One* 6 (2011): e18801

Dutoit V, Guillaume P, Romero P, Cerottini JC, Valmori D: Functional analysis of HLA-A*0201/Melan-A peptide multimer+ CD8+ T cells isolated from an HLA-A*0201- donor: exploring tumour antigen allorestricted recognition. *Cancer Immun* 2 (2002): 7

Esiashvili N, Goodman M, Marcus RB Jr: Changes in incidence and survival of Ewing sarcoma patients over the past 3 decades: Surveillance Epidemiology and End Results data. *J Pediatr Hematol Oncol* 30 (2008)*:* 425-30

Farber DL, Ahmadzadeh M: Dissecting the complexity of the memory T cell response. *Immunol Res* 25 (2002): 247-59

Ferrari S, Sundby Hall K, Luksch R, Tienghi A, Wiebe T, Fagioli F, Alvegard TA, Brach Del Prever A, Tamburini A, Alberghini M, Gandola L, Mercuri M, Capanna R, Mapelli S, Prete A, Carli M, Picci P, Barbieri E, Bacci G, Smeland S.*:* Nonmetastatic Ewing family tumours: high-dose chemotherapy with stem cell rescue in poor responder patients. Results of the Italian Sarcoma Group/Scandinavian Sarcoma Group III protocol*. Ann Oncol.* 22 (2011): 1221-27

Felix NJ, Allen PM: Specificity of T-cell alloreactivity, *Nat Rev Immunol* 7 (2007): 942-53

Findley HW Jr, Cooper MD, Kim TH, Alvarado C, Ragab AH: Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. *Blood*. 60 (1982): 1305-09

Gao, L., T. H. Yang, S. Tourdot, E. Sadovnikova, R. Hasserjian, and H. J. Stauss.. Allo-major histocompatibility complex-restricted cytotoxic T lymphocytes engraft in bone marrow transplant recipients without causing graft-versus-host disease. *Blood* 94 (1999): 2999-06

Gervais A, Eymard JC, Toulmonde E, Bernard J. Selected allogeneic dendritic cells markedly enhance human tumour antigen-specific T cell response in vitro*., Cancer Immunol Immunother* 58 (2009): 1831-41

Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, and Parks WP: In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors *J Natl Cancer Inst* 51 (1973): 1417-23

Goldrath AW, Sivakumar PV, Glaccum M, Kennedy MK, Bevan MJ, Benoist C, Mathis D, and Butz EA: Cytokine Requirements for Acute and Basal Homeostatic Proliferation of Naive and Memory $CD8^+$ T Cells. *J Exp Med.* 195 (2002): 1515–22

Grier HE, Krailo MD, Tarbell NJ, Link MP, Fryer CJ, Pritchard DJ, Gebhardt MC, Dickman PS, Perlman EJ, Meyers PA, Donaldson SS, Moore S, Rausen AR, Vietti TJ, Miser JS. Addition of Ifosfamide and Etoposide to Standard Chemotherapy for Ewing's Sarcoma and Primitive Neuroectodermal Tumour of Bone*, N Engl J Med* 348 (2003): 694-01

Grunewald TG, Diebold I, Esposito I, Plehm S, Hauer K, Thiel U, da Silva-Buttkus P, Neff F, Unland R, Müller-Tidow C, Zobywalski C, Lohrig K, Lewandrowski U, Sickmann A, Prazeres da Costa O, Görlach A, Cossarizza A, Butt E, Richter GH, Burdach S: STEAP1 is associated with the invasive and oxidative stress phenotype of Ewing tumors. *Mol Cancer Res*. 10 (2012): 52-65

Habib T, Senadheera S, Weinberg K, Kaushansky K: The common gamma chain (gamma c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3. *Biochemistry*. 41 (2002): 8725-31

Hendriks J, Xiao Y, and Borst J: CD27 Promotes Survival of Activated T Cells and Complements CD28 in Generation and Establishment of the Effector T Cell Pool. *J Exp Med* 198 (2003): 1369–80

Hinrichs CS, Spolski R, Paulos CM, Gattinoni L, Kerstann KW, Palmer DC, Klebanoff CA: Rosenberg SA, Leonard WJ, Restifo NP: IL-2 and IL-21 confer opposing differentiation programs to CD8+T cells for adoptive immunotherapy, *Blood* 111 (2008): 5326 -33

Hoffmann C, Ahrens S, Dunst J, Hillmann A, Winkelmann W, Craft A, Göbel U, Rübe C, Voute PA, Harms D, Jürgens H*:* Pelvic Ewing sarcoma: a retrospective analysis of 241 cases. *Cancer* 85 (1999): 869-77

Hubert RS, Vivanco I, Chen E, Rastegar S, Leong K, Mitchell SC, Madraswala R, Zhou Y, Kuo J, Raitano AB, Jakobovits A, Saffran DC, Afar DE, STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumours, *Proc Natl Acad Sci U S A* 96 (1999): 14523-28

Jawad MU, Cheung MC, Min ES, Schneiderbauer MM, Koniaris LG, Scully SP: Ewing sarcoma demonstrates racial disparities in incidence-related and sex-related differences in outcome: an analysis of 1631 cases from the SEER database, 1973-2005. *Cancer* 115 (2009): 3526-36

Jeon IS, Davis JN, Braun BS, Sublett JE, Roussel MF, Denny CT, Shapiro DN : A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. Oncogene. 10 (1995)*:* 1229-34

Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, Naji A, Caton AJ: Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2 (2001): 301-06

Juergens C, Weston C, Lewis I, Whelan J, Paulussen M, Oberlin O, Michon J, Zoubek A, Juergens H, Craft A*:* Safety assessment of intensive induction with vincristine, ifosfamide, doxorubicin, and etoposide (VIDE) in the treatment of Ewing tumours in the EURO-E.W.I.N.G. 99 clinical trial, *Pediatric Blood Cancer* 47 (2006): 22-9

June CH, Adoptive T cell therapy for cancer in the clinic, *J Clin Invest* 117 (2007): 1466 -76

Kasaian MT, Whitters MJ, Carter LL, Lowe LD, Jussif JM, Deng B, Johnson KA, Witek JS, Senices M, Konz RF, Wurster AL, Donaldson DD, Collins M, Young DA, Grusby MJ: IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity*. 16 (2002): 559-69

Kim HP, Imbert J, Leonard WJ: Both integrated and differential regulation of components of the IL-2/IL-2 receptor system*. Cytokine Growth Factor Rev.* 17 (2006): 349-66

Kim SY, Tsokos M, Helman LJ*:* Dilemmas associated with congenital ewing sarcoma family tumours. *J Pediatr Hematol Oncol* 30 (2008): 4-7

Kirchhoff C, Osterhoff C, Samalecos A: HE6/GPR64 adhesion receptor co-localizes with apical and subapical F-actin scaffold in male excurrent duct epithelia. Reproduction 136 (2008): 235-45

Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA, Restifo NP: Central memory self/tumour-reactive CD8+ T cells confer superior antitumour immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A* 102 (2005): 9571-76

Klinger P, Surmann-Schmitt C, Brem M, Swoboda B, Distler JH, Carl HD, von der Mark K, Hennig FF, Gelse K: Chondromodulin 1 stabilizes the chondrocyte phenotype and inhibits endochondral ossification of porcine cartilage repair tissue., *Arthritis Rheum* 63 (2011): 2721-31

Koscielniak *E.* Gross-Wieltsch U, Treuner J, Winkler P, Klingebiel T, Lang P, Bader P, Niethammer D, Handgretinger R: Graft-versus-Ewing sarcoma effect and long-term remission induced by haploidentical stem-cell transplantation in a patient with relapse of metastatic disease, *J Clin Oncol* 23 (2005): 242-4

Krasin MJ, Rodriguez-Galindo C, Billups CA, Davidoff AM, Neel MD, Merchant TE, Kun LE*:* Definitive irradiation in multidisciplinary management of localized Ewing sarcoma family of tumours in pediatric patients: outcome and prognostic factors. *Int J Radiat Oncol Biol Phys* 60 (2004): 830-8

Krönig H, Hofer K, Conrad H, Guilaume P, Müller J, Schiemann M, Lennerz V, Cosma A, Peschel C, Busch DH, Romero P, Bernhard H.: Allorestricted T lymphocytes with a high avidity T-cell receptor towards NY-ESO-1 have potent anti-tumour activity, *Int J Cancer* 125 (2009): 649-55

Kuchen S, Robbins R, Sims GP, Sheng C, Phillips TM, Lipsky PE, Ettinger R: Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration. *J Immuno*l. 179 (2007): 5886-96

Kuttesch JF Jr, Wexler LH, Marcus RB, Fairclough D, Weaver-McClure L, White M, Mao L, Delaney TF, Pratt CB, Horowitz ME, Kun LE., Second malignancies after Ewing's sarcoma: radiation dose-dependency of secondary sarcomas*., J Clin Oncol* 14 (1996): 2818-25

La TH, Meyers PA, Wexler LH, Alektiar KM, Healey JH, Laquaglia MP, Boland PJ, Wolden SL, Radiation therapy for Ewing's sarcoma: results from Memorial Sloan- Kettering in the modern era*. Int J Radiat Oncol Biol Phys* 64 (2006): 544-50

Lahl M, Fisher VL, Laschinger K: Ewing's sarcoma family of tumours: an overview from diagnosis to survivorship, *Clin J Oncol Nurs* 12 (2008): 89-97

Lambolez F, Kronenberg M, Cheroutre H. Thymic differentiation of TCR alpha beta(+) CD8 alpha alpha(+) IELs. *Immunol Rev*. 215 (2007): 178-88

Lang A, Nikolich-Zugich J: Functional CD8 T cell memory responding to persistent latent infection is maintained for life*., J Immunol* 187 (2011): 3759-68

Leonhartsberger N, Ramoner R, Falkensammer C, Rahm A, Gander H, Höltl L, Thurnher M.: Quality of life during dendritic cell vaccination against metastatic renal cell carcinoma.*,Cancer Immunol Immunother.* 61 (2012): 1407-13

Li Y, Bleakley M, Yee C: IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response*. J Immunol* 175 (2005): 2261-9

Li Y, Li X, Fan G, Fukushi JI, Matsumoto Y, Iwamoto Y, Zhu Y:Impairment of p53 acetylation by EWS-Fli1 chimeric protein in Ewing Family Tumours*. Cancer Lett.* 320 (2012): 14-22

Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA: Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation*., J Exp Med* 173 (1991): 721-30

Llombart-Bosch A, Pellín A, Carda C, Noguera R, Navarro S, Peydró-Olaya A: Soft tissue Ewing sarcoma--peripheral primitive neuroectodermal tumor with atypical clear cell pattern shows a new type of EWS-FEV fusion transcript. *Diagn Mol Pathol*. 9 (2000): 137-44

Lozzio, C.B.; Lozzio, B.B.: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome", *Blood* 45 (1975): 321–34

Lucas KG, Schwartz C, Kaplan J., Allogeneic stem cell transplantation in a patient with relapsed Ewing sarcoma. *Pediatr Blood Cancer* 51 (2008): 142-4

Mackensen A, Meidenbauer N, Vogl S, Laumer M, Berger J, Andreesen R. Phase I study of adoptive T-cell therapy using antigen-specific CD8+ T cells for the treatment of patients with metastatic melanoma. *J Clin Oncol*. 24 (2006): 5060-9

Markowicz S, Nowecki ZI, Rutkowski P, Lipkowski AW, Biernacka M, Jakubowska- Mucka A, Switaj T, Misicka A, Skurzak H, Polowniak-Pracka H, Walewski J.,:Adjuvant vaccination with melanoma antigen-pulsed dendritic cells in stage III melanoma patients., *Med Oncol.* 29 (2012): 2966-77

McCaughtry TM, Hogquist KA: Central tolerance: what have we learned from mice? Semin Immunopathol. 30 (2008): 399-409

McGargill MA, Derbinski JM, Hogquist KA: Receptor editing in developing T cells. *Nat Immunol* 1 (2000): 336-41

Merkenschlager M, Beverley PC: Evidence for differential expression of CD45 isoforms by precursors for memory-dependent and independent cytotoxic responses: human CD8 memory CTLp selectively express CD45RO (UCHL1). *Int Immunol* 1 (1989): 450-9

Merlo A, Turrini R, Dolcetti R, Zanovello P, Amadori A, Rosato A.: Adoptive cell therapy against EBV-related malignancies: a survey of clinical results., *Expert Opin Biol Ther* 8 (2008): 1265-94

Merlo A, Turrini R, Dolcetti R, Zanovello P, Rosato A: Immunotherapy for EBV-associated malignancies., Int J Hematol 93 (2011): 281-93

Mey U, Hitz F, Lohri A, Pederiva S, Taverna C, Tzankov A, Meier O, Yeow K, Renner C., Diagnosis and treatment of diffuse large B-cell lymphoma*. Swiss Med Wkly* 142 (2012): 0

Meyer-Wentrup F, Burdach S, Efficacy of dendritic cell generation for clinical use: recovery and purity of monocytes and mature dendritic cells after immunomagnetic sorting or adherence selection of CD14+ starting populations, *J Hematother Stem Cell Res* 12 (2003): 289-99

Middleton D, Menchaca L, Rood H, Komerofsky R: New allele frequency database: *http://www.allelefrequencies.net*, *Tissue Antigens* 61 (2003): 403-7

Nguyen H, Weng NP: IL-21 preferentially enhances IL-15-mediated homeostatic proliferation of human CD28+ CD8 memory T cells throughout the adult age span. J Leukoc Biol. 87 (2010): 43-9

Osterhoff C, Ivell R, Kirchhoff C. Cloning of a human epididymis-specific mRNA, HE6, encoding a novel member of the seven transmembrane-domain receptor superfamily. *DNA Cell Biol* 16 (1997): 379-89

Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D.: Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 408 (2000): 57-63

Paulussen M, Ahrens S, Burdach S, Craft A, Dockhorn-Dworniczak B, Dunst J, Fröhlich B, Winkelmann W, Zoubek A, Jürgens H: Primary metastatic (stage IV) Ewing tumour: survival analysis of 171 patients from the EICESS studies. European Intergroup Cooperative Ewing Sarcoma Studies. *Ann Oncol.* 9 (1998): 275-81

Paulussen M, Fröhlich B, Jürgens H.: Ewing tumour: incidence, prognosis and treatment options. *Paediatr Drugs*. 3 (2001): 899-913

Perret R, Ronchese F: Memory T cells in cancer immunotherapy: which CD8 T-cell population provides the best protection against tumours?, *Tissue Antigens* 72 (2008): 187-94

Pieper S, Ranft A, Braun-Munzinger G, Jurgens H, Paulussen M, Dirksen U: Ewing's tumors over the age of 40: a retrospective analysis of 47 patients treated according to the International Clinical Trials EICESS 92 and EURO-E.W.I.N.G. 99. *Onkologie* 31 (2008): 657-63

Pirson Stefan, Generation of autologous and allorestricted cytotoxic T cell clones directed against Ewing Tumour antigens, PH.D. thesis 2009

Pittet MJ, Speiser DE, Valmori D, Cerottini JC and Romero P: Cutting Edge: Cytolytic Effector Function in Human Circulating CD8 + T Cells Closely Correlates with CD56 Surface Expression. *J Immunol* 164 (2000): 1148- 1152

Raney RB, Asmar L, Newton WA Jr, Bagwell C, Breneman JC, Crist W, Gehan EA, Webber B, Wharam M, Wiener ES, Anderson JR, Maurer HM.*:* Ewing's sarcoma of soft tissues in childhood: a report from the Intergroup Rhabdomyosarcoma Study, 1972 to 1991. *J Clin Oncol* 15 (1997)**:** 574-82

Rescigno, M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P: Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria, *Nat Immunol* 2 (2001) : 361-67

Richter GH, Plehm S, Fasan A, Rössler S, Unland R, Bennani-Baiti IM, Hotfilder M, Löwel D, von Luettichau I, Mossbrugger I, Quintanilla-Martinez L, Kovar H, Staege MS, Müller-Tidow C, Burdach S: EZH2 is a mediator of EWS/FLI1 driven tumor growth and metastasis blocking endothelial and neuro-ectodermal differentiation. *Proc Natl Acad Sci U S A*. 106 (2009): 5324-9

Richter GH, Fasan A, Hauer K, Grunewald TG, Berns C, Rössler S, Naumann I, Staege MS, Fulda S, Esposito I, Burdach S: G-Protein coupled receptor 64 promotes invasiveness and metastasis in Ewing sarcomas through PGF and MMP1. *J Pathol*. 230 (2013): 70-81

Robbins PF, Dudley ME, Wunderlich J, El-Gamil M, Li YF, Zhou J, Huang J, Powell DJ Jr, Rosenberg SA.: Cutting edge: persistence of transferred lymphocyte clonotypes correlates with cancer regression in patients receiving cell transfer therapy., *J Immunol* 173 (2004): 7125-30

Roberts DJ, Franklin NA, Kingeter LM, Yagita H, Tutt AL , Glennie MJ and Bullock TNJ: Control of established melanoma by CD27 stimulation is associated with enhanced effector function and persistence, and reduced PD-1 expression, of tumour infiltrating $CD8^+$ T cells*. J Immunother* 33 (2010): 769-79

Rodríguez-Galindo C, Liu T, Krasin MJ, Wu J, Billups CA, Daw NC, Spunt SL, Rao BN, Santana VM, Navid F*:* Analysis of prognostic factors in ewing sarcoma family of tumours: review of St. Jude Children's Research Hospital studies*. Cancer* 110 (2007): 375-84

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A.: Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401 (1999): 708-12

Salter RD, Howell DN, Cresswell P. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics*. 21 (1985): 235-46

Sanchez-Velasco P, Karadsheh NS, Garcia-Martin A, Ruiz de Alegria C, Leyva-Cobian F, Molecular analysis of HLA allelic frequencies and haplotypes in Jordanians and comparison with other related populations, *Hum Immunol* 62 (2001): 901-9

Schluns KS, Williams K, Ma A, Zheng XX, Lefrançois L*: Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. J Immunol* 168 (2002): 4827-31

Shamberger RC, Laquaglia MP, Krailo MD, Miser JS, Pritchard DJ, Gebhardt MC, Healey JH, Tarbell NJ, Fryer CJ, Meyers PA, Grier HE*:* Ewing sarcoma of the rib: results of an intergroup study with analysis of outcome by timing of resection*. J Thorac Cardiovasc Surg* 119 (2000): 1154-61

Shukunami C, Takimoto A, Miura S, Nishizaki Y, Hiraki Y. Chondromodulin-I and tenomodulin are differentially expressed in the avascular mesenchyme during mouse and chick development. *Cell Tissue Res*. 332 (2008): 111-22

Staege MS, Hutter C, Neumann I, Foja S, Hattenhorst UE, Hansen G, Afar D, Burdach SE. DNA microarrays reveal relationship of Ewing family tumors to both endothelial and fetal neural crest-derived cells and define novel targets. *Cancer Res*. 64 (2004): 8213-21

Suvà ML, Riggi N, Stehle JC, Baumer K, Tercier S, Joseph JM, Suvà D, Clément V, Provero P, Cironi L, Osterheld MC, Guillou L, Stamenkovic I.*:* Identification of cancer stem cells in Ewing's sarcoma*. Cancer Res* 69 (2009) *:* 1776-81

Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, and Surh CD*:* IL-7 is critical for homeostatic proliferation and survival of naïve T cells. *Proc Natl Acad Sci U S A* 98 (2001): 8732-37

Tedder TF, Steeber DA, Chen A, Engel P: The selectins: vascular adhesion molecules*. FASEB J*. 9 (1995): 866-73

Thiel U, Pirson S, Müller-Spahn C, Conrad H, Busch DH, Bernhard H, Burdach S, Richter GH.: Specific recognition and inhibition of Ewing tumour growth by antigen-specific allorestricted cytotoxic T cells*.* Br J Cancer 104 (2011): 948-56

Thulesen S, Nissen MH, Ødum N, Röpke C: Induction of cytotoxic CD8+CD56+ T cells from human thymocytes by interleukin-15*. J Interferon Cytokine Res.* 21 (2001): 905-11

Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P, Delattre O. Mesenchymal stem cell features of Ewing tumours. *Cancer Cell* 11 (2007) : 421-9

Topp MS, Riddell SR, i Akatsuka Y, Jensen MC, Blattman JN, and Greenberg PD: Restoration of CD28 Expression in $CD28^-$ $CD8^+$ Memory Effector T Cells Reconstitutes Antigen-induced IL-2 Production*. J Exp Med.* 198 (2003) : 947-55

Urano F, Umezawa A, Yabe H, Hong W, Yoshida K, Fujinaga K, Hata J : Molecular analysis of Ewing's sarcoma: another fusion gene, EWS-E1AF, available for diagnosis. *Jpn J Cancer Res*. 89 (1998): 703-11

Van den Berg H, Dirksen U, Ranft A, Jürgens H: Ewing tumours in infants. *Pediatr Blood Cancer* 50 (2008): 761-4

Vincent K, Roy DC, Perreault C: Next-generation leukemia immunotherapy. *Blood* 118 (2011): 2951-9

Wälchli S, Løset GÅ, Kumari S, Johansen JN, Yang W, Sandlie I, Olweus J.: A practical approach to T-cell receptor cloning and expression. *PLoS One* 6 (2011): e27930

Weidner N, Tjoe J, Immunohistochemical profile of monoclonal antibody O13: antibody that recognizes glycoprotein p30/32MIC2 and is useful in diagnosing Ewing's sarcoma and peripheral neuroepithelioma, *Am J Surg Pathol* 18 (1994): 486-94

Whang-Peng J, Triche TJ, Knutsen T, Miser J, Douglass EC, Israel MA. Chromosome translocation in peripheral neuroepithelioma. *N Engl J Med* 311 (1984): 584-585

Whang-Peng J, Triche TJ, Knutsen T, Miser J, Kao-Shan S, Tsai S, Israel MA: Cytogenetic characterization of selected small round cell tumors of childhood. *Cancer Genet Cytogenet.* 21 (1986):185-208

Wölfl M, Merker K, Morbach H, Van Gool SW, Eyrich M, Greenberg PD, Schlegel PG: Primed tumour-reactive multifunctional CD62L+ human CD8+ T cells for immunotherapy., *Cancer Immunol Immunother* 60 (2011): 173-86

Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD: Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumour effect of transferred T cells. *Proc Natl Acad Sci U S A* 99 (2002): 16168-73

Yoshioka M, Yuasa S, Matsumura K, Kimura K, Shiomi T, Kimura N, Shukunami C, Okada Y, Mukai M, Shin H., Yozu R, Sata M, Ogawa S, Hiraki Y, Fukuda K.: Chondromodulin-I maintains cardiac valvular function by preventing angiogenesis. Nat Med. 12 (2006): 1151-59

Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, Pise-Masison CA, Radonovich MF, Brady JN, Restifo NP, Berzofsky JA, Leonard WJ: Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function., *J Exp Med* 201 (2005): 139-48

Zhou J, Nagarkatti P, Zhong Y, Nagarkatti M: Characterization of T-cell memory phenotype after in vitro expansion of tumour-infiltrating lymphocytes from melanoma patients. *Anticancer Res* 31 (2011): 4099-109

Zhou LJ, Tedder TF: Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J Immunol.* 154 (1995): 3821-35

8. Acknowledgements

I would to like to thank Prof. Stefan Burdach for giving me the opportunity to work at the well-equipped laboratory for Funktionelle Genomik und Transplantationsbiologie of Klinik und Poliklinik für Kinder- und Jugendmedizin Kinderklinik München Schwabing.

I particularly want to express my gratitude to Dr. Günther Richter to whom I am greatly indebted for his continuous guidance and support throughout the course of my research period. Without his instruction and advice this doctoral thesis would not have been possible.

I would also like to thank Dr. Uwe Thiel who showed me my way around the laboratory. He always patiently answered all of my questions and frequently provided me with constructive criticism and useful suggestions. I could not have done it without him.

I wish to thank Dr. Thomas Grünewald and Colette Zobywalski for the many tips they gave me regarding the technical executions. In addition, I am obliged to the whole team including Stephanie Plehm and Kristina Hauer for creating such a friendly atmosphere for me to work in.

I also want to thank my mother and sister who have always supported me in everything