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CD137-selektierte leukämiereaktive T-Zellen gesunder Spender zur adoptiven Immuntherapie stammzelltransplantierter Leukämiepatienten

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vorgelegt von Gregor R. Wenzel
aus Frankfurt am Main

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From the Department of Medicine III,
University Medical Center of the
Johannes Gutenberg University, Mainz

CD137-selected leukaemia-reactive T Cells from healthy donors for adoptive immunotherapy in stem-cell-transplanted leukaemia-patients

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List of Abbreviations

7-AAD	7-aminoactinomycin D
Ab	antibody
ABC	avidin-biotinylated-peroxidase-complex
AEC	3-amino-9-ethylcarbazole
Ag	antigen
AICD	activation induced cell death
AML	acute myeloid leukaemia
APC	allophycocyanin
APC	antigenpresenting cell
ASK-1	apoptosis signal-regulating kinase 1
B-LCL	B cell lymphoblastoid cell line
BC	buffy coat
Bcl-2	B cell lymphoma 2
BSA	bovine serum albumin
c-FLIP	cellular FLICE-inhibitory protein
CCR	CC chemokine receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CR	complete remission
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DLI	donor lymphocyte infusion
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB-X^{TM}	derivate of desthiobiotin
E/T ratio	effector/target ratio
EBV	Epstein-Barr virus
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
ERK	extracellular signal regulated kinase
F_C	constant fragment
FAB classification	French-American-British classification
FACS^{TM}	fluorescence activated cell sorting
FADD	fas-associated protein with death domain
FCS	fetal calf serum

FITC	fluorescein
FLICE	FADD-like IL-1β-converting enzyme
FLT3-ITD	Fms-like tyrosine kinase gene internal tandem duplication
FSC/SSC	forwardscatter/sidescatter
G-CSF	granulocyte-colony stimulating factor
GAM	goat-anti-mouse
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
gp100	glycoprotein 100
GVHD	graft-versus-host-disease
GVL	graft-versus-leukaemia
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HS	human serum
HSCT	haematopoietic stem cell transplantation
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
IL	interleukin
ILA	induced by lymphocyte activation
iTreg	adaptive/inducible regulatory T cell
IU	international unit
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
LCMV	lymphocytic choriomeningitis virus
MACS	magnetic cell separation
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MDS	myelodysplastic syndrome
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MLLC	mixed lymphocyte leukaemia culture
MLTC	mixed lymphocyte tumor culture
MRD	minimal residual disease
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	non-Hodgkin lymphoma
NK cells	natural killer cells
NOD	non-obese diabetic
NPM	nucleophosmin
nTreg	natural regulatory T cell
OT-I	OVA-specific TCR transgenic line
OVA	ovalbumin
p.i.	post infectionem
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	R-phycoerythrin

PGE_2	prostaglandin E_2
PHA	phytohaemagglutinin
PI3K/PKB	phosphoinositide 3-kinase/protein kinase B
PMA	phorbol myristate acetate
RCC	renal cell carcinoma
RING	really interesting new gene
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
SCID	severe combined immunodeficiency
SL	specific lysis
TAA	tumour-associated antigen
TAP	transporter associated with antigen processing
TCR	T cell receptor
TDLN	tumour draining lymph node
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFRSF	tumor necrosis factor receptor superfamily
TNFSF	tumor necrosis factor ligand superfamily
TRAF	TNF receptor-associated factors
Treg	regulatory T cell
WHO	World Health Organization
Z-VAD-FMK	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

1 Introduction

1.1 HSCT and DLI in Therapy of Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is an aggressive malignant neoplasm of the myeloid line of blood stem cells, that affects the bone marrow, the blood and potentially other organs. The diagnosis requires at least 20% of blasts in the bone marrow (World Health Organization (WHO) classification), which have to show a myeloid differentiation pattern. The most common symptom is fatigue in 50% of the patients while others complain about weight and appetite loss, fevers, frequent infections and bleeding. Typical clinical findings are fever, spleno- / hepatomegaly, lymphadenopathy and signs of infections, haemorrhages and organ infiltrations with leukaemic cells. Usually the patients have anaemia, thrombocytopenia and leukocytosis or leukopenia. In more than 95% of the cases leukaemic blasts can be found in the blood [1, 2].

After the initial diagnostics are completed, the treatment starts with the induction therapy. The aim is a complete remission (CR), which is crucial for the long-term outcome. For a CR the bone marrow cell content has to be at least 20% haematopoietic cells with less than 5% of blasts and no Auer's rods (needle or rod-shaped intracytoplasmic inclusion bodies, hallmark of AML [3]). Blood and other tissues except the bone marrow must not contain any blasts. The neutrophil count has to be at least 1000/µL and the platelet count a least 100,000/µL. Usually a combination of cytosine arabinoside, an anthracycline and etoposide is used for the induction therapy. In patients with high leukocyte counts (above 100×10^9/L), a cytoreduction by leukapheresis prior to the induction therapy should be considered [4]. The further management is summarized in figure 1.1.

The course of the disease without treatment would be lethal within a short time period, sometimes even within a few days, although rare cases of spontanous remission have been described [6]. The cure rate varies substantially depending on the patient's individual risk factors. Age, findings on the point of the initial diagnosis, cytogenetic and moleculargenetic anomalies are the most important prognostic factors. Dependent on the individual risk factors, haematopoietic stem cell transplantation (HSCT) can be necessary to have the chance of a permanent cure.

HSCT can be used to reconstitute the severely damaged hematopoiesis of a patient. Either autologous or allogenic blood stem cells, ideally from a fully human leukocyte

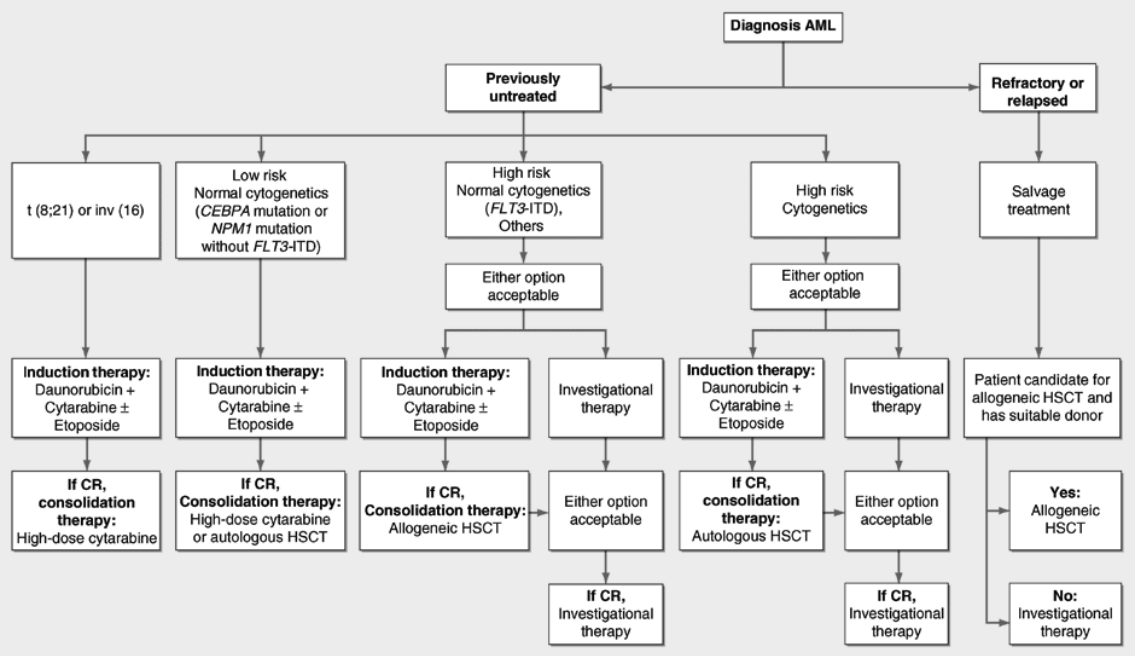


Figure 1.1: Basic management for AML. Allogenic HSCT is a standard treatment for some high-risk patients or those who do not respond sufficiently to the induction therapy. Modified from [5], reprint permission obtained.

antigen (HLA) - matched donor, can be used. Prior to the transplantation a preparative regimen is necessary to achieve sufficient immunosuppression to prevent rejection of the graft, to gain space in the bone marrow and to decrease the load of malignant cells. The preparative regimen consists of total body irradiation and/or high-dose chemotherapy [7]. Blood stem cells from HLA - matched family members or unrelated donors can be obtained by bone marrow puncture or by apheresis from periperial blood after mobilisation of stem cells with granulocyte-colony stimulating factor (G-CSF). Until the new haematopoietic system is engrafted and adequately functional, patients require extensive surveillance and prophylaxis for bacterial, viral and fungal infections.

Another threat for HSCT-patients is the graft-versus-host-disease (GVHD), a mainly T cell mediated autoimmune-like disease, that typically affects liver, bowel, skin and eyes. A classification distinguishes an acute form within the first 100 days after transplatation and a chronic form, whereat the latter one can also affect connective tissue and exocrine glands. Although GVHD can be life-threatening, it is associated with a decreased rate of malignant relapse [8]. This is usually explained by the graft-versus-leukaemia (GVL) effect, that is mainly T cell-mediated like GVHD and that is regarded as the major cause for the curative potential of HSCT. A donor lymphocyte infusion (DLI) can provide a GVL effect in patients with relapsed AML and could also be useful to prevent malignancy relapses, but it is associated with a significant risk for GVHD and bone marrow aplasia [9, 10].

Therefore various strategies to augment the GVL efficacy while decreasing the risk of GVHD are currently being investigated. A promising approach is the adoptive transfer of leukaemia-specific T cells for patients after allogenic HSCT.

1.2 Objective

Recently our lab developed protocols for the in-vitro generation of leukaemia-specific T cells from peripheral blood mononuclear cells (PBMC) of healthy, HLA-matched donors. Sufficient T cell numbers for adoptive transfer (up to 10^9) could be generated within six to ten weeks and showed biological significance in an adoptive-transfer experiments with non-obese diabetic / severe combined immunodeficiency (NOD/SCID) gamma-chain knock-out mice [11,12]. Numbers from 2×10^4 to over 1×10^{11} being required for a clinically relevant effect in adoptive immunotherapy (antiviral and antineoplastic) have been stated in the literature [13]. With our current protocol a median cell number of 2.6×10^6 T cells with an early differentiation phenotype (high to intermediate expression of CD45RO, CD45RA, CD27, and CXCR4, intermediate to low levels of CCR7 and CD62L) can be obtained within four weeks [12]. For clinical application a shorter culture period of about two to three weeks could further improve the phenotype of the generated T cells which should come with an improved persistence, proliferation potential and migration to their target tissues. The costs of the procedure would be more affordable, as for the translation into the clinical application ingredients and processes have to meet the good manufacturing practice (GMP) criteria.

Enrichment assays for antigen-specific T cells are an attractive approach for this, as the enrichment can shorten the culture period, increase the target-specificity and decrease alloreactivity. If the exact Ag of the leukaemia-specific T cells is unknown, enrichment via T cell activation markers is a possible strategy. The activation marker CD137, a member of the tumor necrosis factor α (TNF-α) receptor superfamily, is a promising candidate as it is reliably expressed on activated CD4 and CD8 T cells, the expression level allows exact discrimination of activated T cells from neighbouring non-specific T cells and it provides a potent costimulatory signal to the T cells. The costimulation has shown to improve the expansion and effector function of human T cells [14], the expansion of memory CD8 T cells [15] and to be possibly suitable for the generation of AML-reactive T cells [16].

The working program of this doctoral thesis comprised establishing a CD137 (CD137)-based enrichment protocol for the generation of leukaemia-specific T cells with reagents, that are available for GMP conditions. Therefore the CD137-expression kinetics in activated T cells was assessed, CD137-based stategies were compared to alternative methods, different CD137 enrichment techniques were compared to each other and the expression of the CD137 ligand (CD137L) was evaluated.

2 Literature Discussion

2.1 The Immunologic Mediators of GVHD and GVL

2.1.1 Donor Lymphocyte Infusion

The introduction of reduced intensity conditioning in allo-HSCT for leukaemia treatment has shown that the curative potential seems to derive from the immunological effect of the bone marrow graft. In case of high-risk constellations or chemorefractory leukaemias, the cytostatic therapy of the conditioning regimen facilitates the engraftment of the donor cells, but contributes little to the complete elemination of malignant cells [17]. The complete eradication of the recipient's bone marrow by myeloablative conditioning bears a remarkably higher risk of life-threatening side effects and a toxic, inflammatory cytokine-storm compared to the reduced intensity concept. After a successful myeloablative regimen and allogenic transplantation, the entire haematopoietic system is replaced by the engrafted donor-derived haematopoietic cells. In this state (full donor chimerism) the return of the host's haematopoietic system is usually due to a relapse. In contrast after a reduced intensity regimen the recipient's haematopoietic system is gradually replaced by the graft over a course of several months. Finally achiving full donor chimerism is regarded as an indicator for the elimination of the minimal residual disease and associated with a favourable prognosis. The toxicity of the conditioning can be dramatically reduced and a much broader group of patients – especially the elderly – has hence become eligible for allo-HSCT. However due to the widespread use of the reduced intensity approach, relapse has become the most common cause of treatment failure [18].

The idea of the existence of an immunological GVL effect has been derived from the clinical observations that GVHD is associated with reduced relapse rates and that withdrawal of immunosuppression can induce complete remissions in relapsed patients [18]. Further evidence comes from the observation that patients who achieve high lymphocyte counts rapidly after haematological reconstitution have the lowest relapse rates. This indicates a lymphocyte-dependent effect (T cell- or natural killer (NK) cell-mediated) [19]. Notably the GVL effect is not dependent on engraftment of the donor cells. Haploidentical donor lymphocytes can elicit anti-leukaemia responses (including CR) in non-transplanted high-risk AML patients without any engraftment. Possible reasons are mounting of host-derived

leukaemia-reactive T cells by breaking host tolerance, establishment of microchimerism or an initial GVL reaction prior to graft rejection [20, 21]. Acute and chronic GVHD are associated with lower relapse rates [17]. As these effects had been shown to be largely T cell mediated, the concept of the DLI has been introduced into clinical practice in 1990 [22]. Notably results from murine bone marrow chimeras that lack the major histocompatibility complex (MHC) on epithelial tissues suggest, that acute GVHD can occur MHC-independently, most likely mediated by stimulatory cytokines (also called GVHD-like reaction) [23]. This can also be seen in syngenic or autologous transplantations [24]. Nevertheless GVL responses do not coincide with GVHD targeted to Ag absent on leukemia cells which means that a GVL reponse requires direct interactions between donor T cells and the malignant cells and cannot be explained solely by paracrine (e.g. cytokine mediated) effects of the GVHD [25]. This treatment has been extraordinarily successful in the treatment of relapsed chronic myeloid leukaemia (CML) with durable CR rates up to 80%. For AML the DLI treatment is less effective with CR rates around 30% and likewise in other haematological malignacies. Furthermore in AML patients a DLI as a single agent is not able to induce a CR during a florid relapse [18]. The poor effect of the DLI in aggressive, rapidly proliferating malignancies is often attributed to outpacing of the engraftment process by malignant cells [17]. Many attempts to improve the efficacy of a DLI for these patients have been made, e.g. prophylactic DLI administration for high-risk AML, which improved the overall survival from 40% to 60% in a cohort of 77 patients [18, 24]. Furthermore DLI treatment has been regarded as a possibility to achieve full donor chimerism (see above) in patients who have undergone allo-HSCT with reduced intensity conditioning.

Acute and/or chronic GVHD is the most severe complication of DLI treatment and occurs in about 30% of the patients treated independently from the underlying malignancy. Although it can be life-threatening, moderate GVHD is a marker for the GVL effect and a reduced relapse risk [18]. Some publications state the strongest statistical associaion of the GVL effect with GVHD has been shown for chronic form [26] while others found that relapse rates were lower with the occurrence of both acute and chronic GVHD [17].

Several risk factors for developing GVHD have been identified including the cell dose, early DLI administration after HSCT, donor sex mismatch and higher patient age [18]. The decreasing risk of GVHD upon DLI administration with increasing time after HSCT is well described and most likely caused by decreasing inflammatory cytokine levels [27]. Particularly TNF-α leads to acute GVHD mediated by a a Th1/Tc1 response. Th2/Tc2 responses (like chronic GVHD) could be associated with a poor immunologic protection against malignacies, but chronic GVHD is a more Ag-specific response compared to the acute form and it is also associated with a GVL effect [26].

Most theories describe GVHD and GVL as two phenomena that are distinct and separable (however with considerable overlap and coincidence). In contrast another hypothesis states that GVL could be a mild form of a GVHD that only affects haematopoietic cells due to their particular susceptibility to graft-versus-host immune reactions. Amongst other hints the clinical and preclinical observation that the graft-reaction against the recipient bone marrow precedes and predicts GVHD suggests this theory. Furthermore bone marrow aplasia is the most common cause of death in patients who develop a GVHD as a complication of a blood transfusion (outside the context of HSCT). This also suggests that the haematopoietic cells could be the most susceptible tissue in the whole body. According to this concept not only the T cell specifictiy but also the overall vigorousness of the immune-reaction could contribute to the GVL specificity [25].

2.1.2 T Cell Based Approaches for Novel DLI Products

T cell based approaches to reduce this risk are dose escalating regimens, genetic engineering of donor T cells and selection of T cell subsets. Non-T cell based concepts like NK cell infusions, cytokine administration, immunosuppressants, cell mobilisation from the stem cell niche, growth factors, vaccination, dendritic cell manipulation, additional umbilical cord blood administration, donor mesenchymal stem cells and depletion of γδ T cells are reviewed in [26, 28] and will not be discussed in detail here. In clinical trials dose escalating regimens starting with a low T cell number for infusion and long time periods between the infusions have been successful to reduce GVHD without increasing the relapse rate. Transduction with suicide genes (herpes simplex virus thymidine kinase) allows pharmacological deletion (cells become sensitive to gancylovir) of donor T cells *in vivo* in case of severe GVHD. This approach has been feasible in a clinical setting, however with the limitation that the GVL effect is sacrificed in case of the pharmacological intervention. In terms of selection of T cell subsets CD8-depleted cell products are widely used. For CML CD8-depletion of the stem cell graft and DLI products have both shown to reduce the risk of GVHD without compromising the relapse rate [18]. CD8-depleted DLI can induce complete donor chimerism and enhance antiviral immunity [29,30]. Nevertheless in a phase I study with thoroughly CD8 (CD8)-depleted DLI in patients with various persistent haematological malignacies or mixed chimerism the administration of donor lymphocytes still caused severe GVHD in five of 28 patients including two lethal outcomes [31]. A likely conclusion could be that CD8 depletion as a straightforward approach is too unspecific to provide a separation of GVL and GVHD.

A more sophisticated method is the depletion of alloreactive T cells prior to infusion. For HLA-mismatched patients at high risk of GVHD and low risk of leukaemia relapse

depletion of naive T cells is a promising strategy [32]. In a more general approach alloreactive T cells can be identified by activation markers like CD25, CD69, CD134 and CD137 after incubation of donor lymphocytes with recipient cells acting as antigenpresenting cells (APC). Depletion of activated T cells can significantly reduce alloreactivity while maintaining antiviral and antileukaemic reactivity *in vitro* [33]. In a phase I clinical trial using depletion of CD25 positive T cells efficient depletion of alloreactivity with preserved antiviral immunity, but a dissatisfying relapse rate were observed [34].

Potentially even more specific is the approach to generate leukaemia-reactive T cells *in vitro* for adoptive immunotherapy, which would ideally result in a complete separation of GVL and GVHD. The different possible Ag for adoptive T cell therapy are discussed below. Defined Ag can be targeted with genetically enigeneered T cells either in a HLA-dependent manner (T cell receptor (TCR) transfer) or independently from HLA with chimeric antigen receptors [35]. While these approaches can rapidly provide large cell numbers for adoptive immunotherapy, various security issues, that necessarily accompany genetic engineering, remain, e.g. insertion mutagenesis, emergence of replication-competent retroviruses from transduced cells [36]. The *in vitro* expansion of naturally occuring leukaemia-reactive T cells from either recipient or donor PBMC by stimulation with Ag-peptide loaded APC or artificial APC could generate T cells that are very similar to the physiologically occuring ones. However this approach relies on the knowledge of a suitable Ag for the individual patient. A major obstacle is that the diversity of HLA genes and the variety of peptides presented by malignant cells have made it difficult to find strong Ag that can be targeted in a majority of leukaemia patients.

Therefore the generation of T cells against unknown, but leukaemia-specific Ag would be an approach feasible for a large number of patients. Polyclonal T cells are thought to be favourable due to a broader specificity leaving less space for immune escape [25]. Another advantage of *in vitro* generated leukaemia-reactive T cells could be that no Ag presentation by professional APC in the host is required. Only CML shows T cell activation capabilities that are comparable to professional APC, while a GVL response against acute leukaemias is often thought to rely on crosspresentation of Ag by host APC. (AML blasts can be converted *in vitro* into a dendritic cell (DC) phenotype or differentiate spontanously [17,24]. Furthermore also unmanipulated AML blasts are capable of stimulating naive T cells under appropriate cell culture conditions [12]. Nevertheless for AML-derived DC an abnormal function has been found resulting in inhibition the induction of cytotoxic T lymphocyte (CTL), induction of T cell anergy and favouring the generation of Treg [19].) These professional APC are required if the tumour-reactive T cells originate from the naive compartment, as it is assumed for an unmanipulated DLI [27]. (Notably there are also results that suggest that GVHD-mediating alloreactivity originates from the naive

compartment, too. Murine models showed a strict association of the occurence of GVHD with adoptive transfer of naive T cells [26]. The similar case has been found for human T cells [32]. Furthermore murine experiments using naive T cell-depleted DLI showed a reduction in GVHD compared to unmanipulated DLI with retained GVL effects [26].) *In vitro* activated and adoptively transferred T cells should be independent from professional APC and capable of reacting to malignant cells directly.

2.1.3 Possible Antigens that Mediate a GVL Response

Different types of targets for GVL effect-mediating T cells have been identifed. These Ag seem to be presented by recipient APC, whereas donor APC seem to be of lesser importance [18]. There is evidence that there must be Ag that are restricted to the haemoatopoietic system or the malignancy, which mediate a GVL effect without GVHD. Most probably these Ag are minor antigens (see below) that are restricted to haematopoietic cells [17, 37].

- **Major histocompatibility complex antigens:** Differences in the HLA pattern between donor and recipient after non-fully-matched HSCT can lead to strong alloreactivity due to MHC Ag. These can cause GVL as well as GVHD. In the case of direct recognition a host HLA-molecule is the Ag for donor T cells wherein the peptide presented can even be irrelevant, e.g. a regular (non-mutated, non-polymorphic) self Ag. In contrast peptide fragments of host-MHC molecules on donor-APC can be the Ag for donor T cells (indirect recognition) [17].

- **Minor histocompatibility complex antigens** are MHC binding peptides originating from polymorphic genes that differ between the donor and recipient. The possible underlying mechanisms are single nucleotide polymorphisms, differential splicing, gene deletions, different types of mutations or amino acid substitution of endogenous proteins and subsequently differential intracellular transport and/or processing and altered MHC binding. Finally all mechanisms lead to MHC presentation of peptides on recipient cells that can be recognised by donor T cells, as these Ag are not presented in the immune system of the donor and no tolerance has developed. Minor Ag can be restricted to the hematopoietic system and these are expected to have an important role in GVL in several haematological malignancies. The minor Ag described up to now would be theoretically sufficient to generate specific T cells for approximately 25 to 40% of the stem cell transplanted leukaemia patients [17, 23, 38].

- **Tumor-associated antigens** are nonpolymorphic, non-minor self-Ag that arise from mutated or overexpressed genes in malignant cells. These Ag are targets for

tissue- or even tumour-specific immune responses. Examples are bcr-abl in CML for mutation, proteinase 3 and myeloperoxidase in AML for overexpression or prostate-specific antigen in prostate cancer for tissue-specific expression [17]. As there are several tumour-associated antigens (TAAs) that derive from mutations and therefore are neo-Ag these should lead to responses in syngenic transplantations (identical twins) which is rarely observed clinically [25]. This is a major reason that strong responses to TAA are rare which may be attributed to tumour-mediated immune escape [17]. Nevertheless case reports suggest that there are strong immune reactions against TAA that can lead to very good clinical results (vaccination against Wilms' tumour antigen 1 in AML induced CR [39]).

2.1.4 Minor Histocompatibility Antigens in GVL responses

Minor histocompatibility antigens are widely regarded as the most important targets for a T cell response against haematopoietic cells as a form of favourable GVL effect of a DLI. The rationale for this is the potential to eradicate the whole host haematopoiesis and with it the minimal residual disease (MRD)/leukaemic stem cell [19]. (For example in AML this may not apply to immunologically privileged sites, (e.g, the central nervous system) where late AML relapses have been observed frequently [24].) Antibodies against stem cell Ag have been found in some patients sera subsequently to successful DLI treatment [40].

Minor Ag are often regarded as favourable targets (compared to TAA) as during the evolution of the malignacy there is a constant selection pressure mediated by responses to TAA but not to minor Ag. So the probability that a malignancy expresses strongly immunogenic minor Ag is thought to be much higher than for TAA [25]. Ideal minor Ag would be derived from proteins that are exclusively expressed in haematopoietice cell lines and are also crucial for cell function. This would leave little potential for leukaemia cells to evade T cell responses by losing expression of the Ag [23]. (Immune evasion by mutation has been found in AML for NK cell and CTL mediated immune responses [41].) However at least in some cases just long-term control of the malignancy is provided by the graft (markers of MRD remain at stable low levels despite haematological CR) [24].

For humans the minor antigens HA-1 and HA-2 are well described as they are both resticted to the haematopoietic system and presented on HLA-A2 molecules. Treatment of three allo-HSCT patients for relapsed HA-1- positive and/or HA-2-positive malignancies with DLI from HA-1-negative and/or HA-2-negative donors induced CR and tetramer-detectable levels of HA-1-specific and HA-2-specific CD8 cells in all patients. In two of the patients the DLI caused GVHD that required immunosuppressive treatment which lead to molecular relapse and disappearance of HA-1-specific T cells in one patient [42].

T cells specific for HA-1 and a few other, partly haematopoiesis-restricted minor Ag have been found in the blood of stem-cell-transplanted patients. These T cells showed cytolytic activity against the original malignancy *in vitro*. Furthermore a retrospective analysis showed that recipients expressing HA-1 transplanted with a HA-1-negative graft had a lower relapse rate than HA-1-compatible pairs. No higher rate of GVHD was observed in the HA-1-incompatible pairs [23]. A pilot study recently showed that adoptive transfer of *in vitro* generated HA-1-specific CD8 T cells for leukaemia treatment is feasible, however without clinical responses [43].

Warren et al. generated CTL against unknown minor Ag from posttransplantation PBMC and administered these to the patients in escalating doses. In 4 out of 6 patients a morphologic CR was achieved, however with transient T cell persistence and relapses in all patients. Flow cytometry of the CTL showed a differentiated effector memory phenotype (see below). Strikingly pulmonary damage including one very severe case due to the DLI was observed in 3 out of 7 patients. Pulmonary expression of the antigen was take into account as a likely reason [44].

Ususally minor antigens of widespread expression are regarded as important targets for GVHD-mediating T cells. Results for different minor antigens showed higher rates of GVHD in minor antigen-mismatched donor-recipient pairs or higher numbers of minor antigen-specific T cells in the blood of patients who developed GVHD compared to clinically unaffected ones. However there are Ag that show strong expression in the haematopoietic system and low expression levels in some peripherial tissues. These are also regarded as potential targets for adoptive immuotherapy as there is evidence that these could be safe to use. For example leukaemia-reactive T cells against a minor Ag (UTY, encoded on the Y chromosome, relevant for male patients recieveing a graft from a female donor) do not lyse also Ag-expressing fibroblasts [23].

2.2 Adoptive T Cell Transfer for Malignancies

One of the key advantages for the use of T cells for cancer treatment is the potential to clear even the last malignant cell from the patients body in a very selective way compared to chemotherapy or radiation. This is of even greater importance in primarily systemic malignacies like leukaemias where no options for local (e.g. surgical) treatment are available. Although hematologic neoplasms usually originate from the bone marrow or the lymphatic system, local manifestations like chloromas or central nervous system affection can hamper the effect of cytostatic treatment. As T cells can infiltrate in to Ag-expressing tumour deposits, this key point for solid tumours can also apply for hematologic malignacies [45].

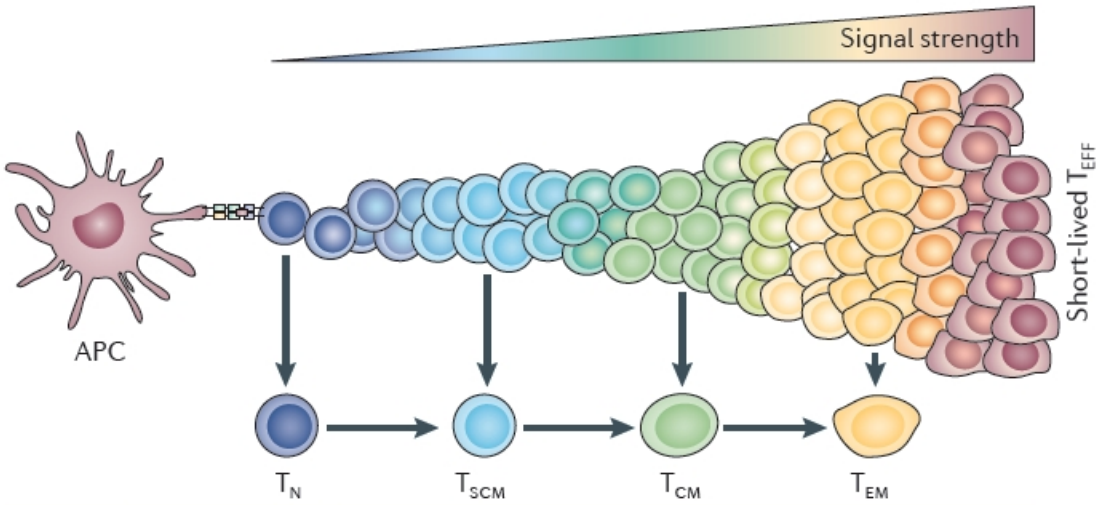
A striking advantage of immunotherapy over pharmacological approaches is the potential

to mount long-term immunological memory and control of the malignancy. CD8 memory T cells are thought of as T cells that home to lymphoid tissues, survive without Ag or under chronic antigenic stimulation, expand clonally on reactivation and generate effector T cells. Adoptive transfer of a single naive Ag-specific T cell can lead to development of both effector and memory cells demonstrating that the fate of naive T cells is undetermined and that multiple subsets can derive from one progenitor cell [46]. A subdivision into central and effector memory cells is thought to depend particularly on the expression of the lymphoid tissue homing receptor CCR7 [47]. Three major models of memory-formation have been discussed in the literature: One linear differentiation model states that priming of naive T cells induces effector cells, of which a few may survive and become effector memory cells and eventually long-lived central memory cells. According to an asymmetric division model the first cell division after priming of a naive T cell results in separate effector and memory lineages [48, 49].

Concerning the *in vitro* generation of T cells for adoptive transfer another linear model is often favoured (figure 2.1). According to this hypothesis first T cells of central-memory phenotype form out of naive T cells. Later on they aquire an effector-memory and finally an effector phenotype. The early central-memory phenotype is mostly favoured for adoptive immunotherapy [50, 51]. Recently memory T cells of naive-like phenotype (CD45RA, CD62L, CCR7, CD27, CD28, CD127) have been described, that could represent an intemediate stage between naive and central memory T cells. According to the respective hypothesis this subset could be regarded as a memory stem cell with high proliferative capacity, long lifespan, self-renewal and multipotency [49, 52]. In peripherial blood chemotherapy-resistant memory T cells can been found which could be direct progeny of memory stem cells [53, 54].

Effector-memory T cells are often considered to have a more limited lifespan and less proliferative potential compared to central-memory ones. A major point in the *in vitro* generation and expansion is the idea that sustained stimulatory signals drive T cells to a short-lived effectors, while more transient (but sufficient) signals lead to generation of memory cells [55]. Favourable results for CD8 T cell memory formation have been described for various cytokines (e.g. common γ-chain cytokines IL-7, IL-15) and costimulatory molecules [56].

The majority of the approaches for adoptive T cell treatment focuses on *ex vivo* manipulated autologous T cells. As in the majority of the cases the only fully HLA-matched T cells are available from the patient, this approach could be widely applicable to several immunogenic malignacies. Various techniques can be applied *ex vivo* to break the tumour-tolerance. There is direct evidence that tumour-reactive T cells can influence the course of a malignant disease, e.g. for melanoma-specific T cells [57] or B cell-specific chimeric antigen receptor T cells [58].



		T_N	T_{SCM}	T_{CM}	T_{EM}
Phenotype	CCR7	+++	+++	++	–
	CD62L	+++	+++	++	–
	CD27	+++	+++	++	+/–
	CD28	++	+++	+++	+/–
	CD45RA	+	+	–	+/–
	CD45RO	–	–	+	+
	CD122	–	++	+++	+++
	CD95	–	++	+++	+++
	KLRG1	–	–	+/–	++
Function	IFNγ	–	+	++	+++
	IL-2	–	++	+++	+/–
	Cytoxicity	–	+/–	+	+++
	Telomere	+++	+++	++	+
	Self-renewal	+	+++	++	+

Figure 2.1: Differentiation processes that have to be taken into account in the *in vitro* generation of T cells for adotive transfer. An important mechanism is the early downregulation of lymphoid tissue homing-receptors and costimulatory molecules during progressive differentiation, which could be responsible for a poor *in vivo* activation of T cells. Modified from [49], reprint permission obtained.

A major limitation in *ex vivo* T cell generation is the long culture period needed to generate sufficiently high cell numbers. (Also application of probably unnaturally high cytokine concentrations *in vitro* during T cell expansion is thought to contribute to failing *in vivo* persistence after adoptive transfer [23].) During this culture period with repeated Ag-specific restimulations T cells tend to differentiate into the effector-memory phenotype. In murine models this phenotye has only shown short-term efficacy without formation of a long-term memory. Limited effects in clinical studies have been attributed to this differentiation of T cells during *ex vivo* culturing [45]. Initially the high cytolytic activity and the strong interferon-γ (IFN-γ) release of effector and effector-memory T cells seemed to suggest these subtypes as optimal candidates for tumour-immunotherapy. However further results showed that central memory T cells with the ability to home to seconday lymphoid tissues are superior in conferring protective immunity against viral or bacterial challenges. The surface molecules CD62L, CCR7 and CXCR4 are usually regarded as most important homing markers [23, 59]. For example a retrospective analysis of adoptive cell transfer for melanoma treatment revealed that the *in vitro* IFN-γ release and cytolytic activity did not predict the clinical reponse *in vivo* [51].

Klebanoff et al. demonstrated in a murine melanoma model that central memory cells were not only superior to effector memory cells concerning shrinkage of established tumour but also in terms of long-term survival [59]. Another experiment series in the same model demonstrated a progressive gain of *in vitro* CTL effector function after repeated stimulation (up to 3 times) that came along with a decreased efficacy of *in vivo* tumour control and *in vitro* IL-2 secretion [51].

Furthermore the presence of CD4 T cells with corresponding specificity seems to be required for a successful CD8 T cell memory. In a clinical study adoptive transfer of cytomegalovirus (CMV) specific CD8 T cells in patients after allogenic HSCT was performed and the CMV-specific responses were followed up for 12 weeks. Patients who showed a recovery of CD4 T cell responses for CMV also had sustained CD8 T cell responses. In contrast decreasing CD8 T cell reactivity was found in patients without recovery of CD4 T cell responses for CMV [60]. Also for tumour immunotherapy adoptive transfer of CD4 and CD8 T cells is regrarded as most rewarding, as it facilitates a secondary expansion of the CTL after re-encounter with Ag and the memory formation [45]. Furthermore recently effector functions of CD4 T cells have been shown even in humanized tumour mouse models. Purified CD4 were capable of eradicating an established leukaemia *in vivo* without CD8 T cells [61].

In patients who have undergone allogenic HSCT approaches using allogenic donor-derived HLA-matched T cells is possible. These can be regarded as a modified DLI, which has been shown to be effective in enhancing the GVL effect. Major advantags in

the allogenic approaches are the availability of larger quantities of T cells even before the haematologic reconstitution of the patient after the HSCT and a generally better condition of the T cells from a healthy donor who does not suffer from a malignancy and a chemotherapy-compromised hematopoietic system. Furthermore healthy donors have a superior thymic function and a wider T cell repertoire [25].

2.3 CD137 in General

2.3.1 Structure and Signaling of CD137

The murine CD137 was first described in 1989 and named 4-1BB. In 1993 the human homologue was found in T cells and named "induced by lymphocyte activation (ILA)". It was later classified in the cluster of differentiation (CD) system and identified as a member (TNFRSF9) of the tumor necrosis factor receptor superfamily (TNFRSF). The human CD137 gene is located on 1p36 in a region that contains other genes encoding for members of the TNFRSF. The similarity in localisation and function of these genes (e.g. OX40/CD134 on CD4 T cells) has lead to the hypothesis, that these genes derive from gene-duplication events [62]. CD137 is a fully sequenced type I transmembrane protein, contains 255 amino acids and has a molecular weight of 27.9 kDa [63]. The majority of the data on signaling and function for CD137 in T cells are available for the murine homologue, which has 60% amino acid identity to the human protein. Besides the strictly activation-dependent expression in T cells, CD137 is also expressed in monocytes, DC, follicular DC, regulatory T cells (Treg), activated eosinophils, B cells NK cells and other tissues like chondrocytes, neurons, microglia and endothelial cells [64, 65]. In this complex picture especially the effect of CD137 on DC is regarded as a major difficulty in interpreting various, earlier found results, as CD137 increases the secretion of DC-cytokines, upregulates costimulatory molecules and therefore indirectly enhances T cell function [66].

The CD137 signaling pathways are summarized in figure 2.2. The aggregation of a CD137-trimer has been shown to be necessary for the signal transduction [67, 68]. The intracellular domain of the human CD137 binds the members TRAF1, TRAF2 and TRAF3 of the TNF-α receptor-associated factor (TRAF) family. The RING finger domain of TRAF2 is responsible for the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which upregulates the expression of the antiapoptotic Bcl-2 family members Bfl-1 and Bcl-X_L [69]. (Bcl-X exists as a smaller pro-apoptotic Bcl-X_S splice-variant or as a generally more abundant anti-apoptotic form Bcl-X_L, which is larger in size [70].) By interference with the intrinsic pathway of apoptosis on the mitochondrial level, Bcl-X_L reduces apoptosis [71].

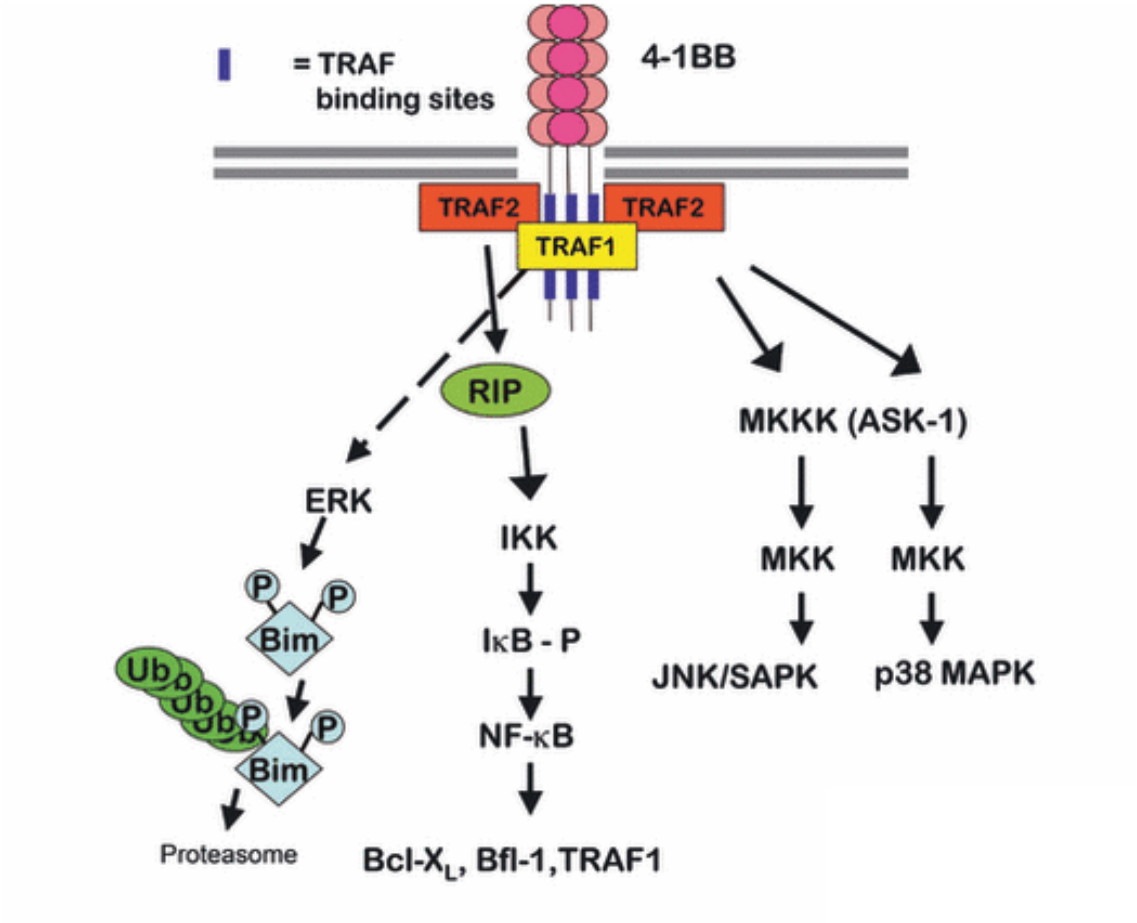


Figure 2.2: Signaling pathways of CD137. Adapted from [72], reprint permission obtained.

Furthermore, TRAF2 activates apoptosis signal-regulating kinase 1 (ASK-1) (systematic name: mitogen-activated protein kinase kinase kinase (MAPKKK) 5) which leads to interleukin-2 (IL-2) and IFN-γ secretion [73]. This is mediated by p38 MAPK, which is also activated by CD3 signaling. c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is also activated downstream of ASK-1, but the influence on IL-2 secretion is not clear in a setting with simultanous TCR activtion [74].

While TRAF2 activates multiple signal cascades, TRAF1 improves the signal transduction by stabilizing, promoting the release from lipid rafts and reducing the degradation of TRAF2. It also enhances the ubiquitinyaltion and degradation of the proapoptotic molecule Bim through the extracellular signal regulated kinase (ERK) pathway. As TRAF1 expression is low in resting T cells and rises upon activation, the antiapoptotic effects are particularly important in activated T cells.

Overall the CD137 signal alone mediates survival while in combination with a TCR

signal proliferation and development of effector functions are increasd. The pattern of signal cascades activated is different from other costimulatory pathways like CD27 and CD28 [72]. However there is an overlap in terms of the second messenger molecule activated during signaling. This leads to the hypothesis that the effect of costimulatory molecules of the TNF receptor-associated factors (TRAF) family is mainly quantitative rather than specific for a particular pathway or function [62]. The role of TRAF3 and the phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) pathway are still not entirely clear [72]. Some findings on signaling for T cell survival have shown, that CD137 and CD28, although belonging to different protein superfamilies, share common downstream pathways. The anti-apoptotic protein c-$FLIP_{short}$ inhibits Fas-mediated activation induced cell death (AICD), reduces caspase-8 activation and increases proliferation and cytokine secretion in T cells. The expression is regulated via the PI3K/PKB pathway, which is activated downstream of both CD28 and CD137 [71].

2.3.2 CD137 Ligand

The ligand of CD137, a type II transmembrane glycoprotein of 254 amino acids termed CD137L, tumor necrosis factor ligand superfamily member 9 (TNFSF9) or 4-1BBL is expressed on monocytes/macrophages, DC and B cells. Monocytes express it constitutively while the expression level on DC is usually low, but rises under inflammatory conditions (IL-1, CD40 ligand, lipopolysaccharides, double stranded ribonucleic acid (RNA)). Usually CD137L is not found on T cells. Like other members of the tumor necrosis factor ligand superfamily (TNFSF) and the TNFRSF, CD137 and CD137L show bidirectional signaling, which is a rare phenomenon. The so-called reverse signal transduction of CD137L in monocytes leads to activation, migration, prolonged survival and growth. The effects in DC comprise higher expression of CD80, CD86 and MHC class II, increased IL-12 production, enhanced migration and capability of T cell stimulation [64]. In contrast CD137L signaling in human T cells has also shown reverse effects, i.e. decreased proliferation and apoptosis. This effect can be observed upon stimulation with immobilized CD137 and it is independent of Fas/Fas ligand interaction [75].

There are three splice variants of the CD137L messenger ribonucleic acid (mRNA) that lack the transmembrane domain and encode for a soluble form of CD137L. The so-called sCD137 is a natural inhibitor of the CD137/CD137L interaction and is secreted by activated lymphocytes. *In vitro* studies with human PBMC have shown that sCD137L antagonizes the stimulatory effect of CD137 activation. In combination with the secretion by activated lymphocytes, this could be a negative feedback mechanism. In contrast stimulatoy effects of non-aggregated sCD137L [76] and a correlation with AICD have

been observed. Furthermore, the receptor-shedding of CD137L by metalloproteases has been described as a possible mechanism for the release of sCD137L into the serum [76]. This could also be a possible explanation for the weak correlation of sCD137L serum levels with the expression of the membrane-bound counterpart in several malignacies (see below) [64].

Elevated serum levels of sCD137L have been described in several hematological malignant diseases (AML, non-Hodgkin lymphoma (NHL), chronic lymphocytic leukaemia (CLL), myelodysplastic syndrome (MDS)) and autoimmune diseases. Comparably high serum levels have been found for AML and higher levels are associated with poor prognosis subtypes and shorter progression-free survival [64, 77]. Shedding of CD137L molecules by AML blasts has been found experimentally and is discussed as a mechanism of immune escape [19]. The impairment of the CD137/CD137L interaction by the soluble ligand could possibly lead to an impaired immune reaction and thus to a more rapid progression of the disease. This correlation is also observed for MDS but not for NHL. About 20% of circulating AML blasts express CD137L on the cell surface but the serum levels of sCD137L do not correlate with the expression of membrane-bound CD137L in individual patients. If DC are generated from the blood of AML patients, they also express CD137 ligand (CD137L) in about 35% of the cells [64, 77].

2.3.3 Costimulation via CD137 in T Cells

Most data on functional effects of CD137 in CD8 T cells suggest a potent costimulatory effect, that includes a favorable cytokine pattern for adoptive transfer, inhibition of AICD, increased proliferation and *in vivo* persistence.

Knock-out mice studies indicate a role of CD137 for the generation of CD8 T cells as fewer Ag-specific CD8 cells and fewer memory T cells were seen in these mice [78–80]. Accordingly tumor-models showed increased CD8 T cell responses and sometimes increased cell numbers of Ag-specific T cells upon different methods of CD137-stimulation (Ab or tumor cells transfected with CD137L) [81–83].

The natural role of TNF receptor (TNFR) family members seems to be an additional costimulation subesquently to the initial CD28-B7 costimulatory signal. Influenza-infected mice showed an impaired initial T cell response, when CD28 was knockeed out and impaired late response upon CD137 knock-out. Interestingly the lack of an initial CD28 stimulus could be reversed with a stimulatory CD137 Ab [84]. As these results come from mouse models it must be considerd, that CD137 has at least *in vitro* slower kinetics in murine than in human T cells. Therefore both costimulatory pathways could be active simulatanously in human T cells [65]. At least in mice blocking of CD137 does not affect

the initial proliferation of T cells but the expansion to high numbers later on, which is due to apoptosis that occurred in T cells that had already several cell divisions [85]. However there are multiple results to show that CD137-costimulation is independent from CD28-B7 [14, 67, 68] and sufficient to activate T cells without previous CD28-B7 signal [86,87]. Nevertheless in naive T cells CD137 cosimulation may rely on previous CD28 activation. This may be necessary for CD137 expression on the T cells and a subsequent costimulatory signal, but does not contradict the results that show independence of the two molecules in terms of the signal transduction [88].

2.3.3.1 Cytokine Secretion and T Cell Subpopulations

In terms of a most effective cytokine secretion pattern for adoptively transferred CD8 T cells, usually the Tc1 type is regarded as most important, as Tc1 cells are regarded as more effective in cell killing [62] and therefore most effective for antitumor responses [26, 89, 90]. Tc1 CD8 T cells predominantly secrete IFN-γ, furthermore IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and kill by either perforin- or Fas-mediated mechanisms [91]. For the generation of type 1 T cells IL-12 is crucial. With the differentiation towards Tc1 the costimulatory molecule CD28 gets downregulated. At birth nearly all CD8 T cells are positive for CD28 and these are believed to be naive T cells. The fequency of CD28 negative T cells increases with age and is found in chronic infection and malignacies. These T cells often express also NK cell markers, which have shown to mediate stimulatory and inhibitory signals. These T cells are regarded as likely mediators of tumour immunity [89]. Tc2 CD8 T cells preferentially secrete IL-4, IL-5, IL-10 and IL-13 and mediate cytotoxicity via the perforin pathway [91]. These T cells ususally do not downregulate CD28 due to IL-4 secretion [89]. However it needs to be considered that T cells that achieve maximal effector function *in vitro* are already too differentiated to be efficient after adoptive transfer, despite IFN-γ secretion is a surrogate marker for cytotoxicity *in vitro* and the *in vivo* IFN-γ production mediates the cytotoxic effect [51, 59]. Own observations from our lab show that in some CTL IFN-γ secretion and cytotoxicity are less strictly correlated than stated in the literature.

Some publications show *in vivo* antitumor reactivity after adoptive transfer only for the Tc1 type and a correlation with IFN-γ while data on Tc2 cells are controversial [91–93]. Costimulation via CD137 could shift the cytokine pattern towards type 1 and increase the effector function of CD8 T cells. This applies particularly to human T cells, while the results on cytokine patterns in murine models are less clear [65, 94].

Wen et al. simulated either purified, human CD4 or CD8 T cells or total T cells by APC carrying either anti-CD3 or CD137L or both molecules on the surface. For high

cytokine production, secretion and proliferation of CD8 T cells, CD4 cells were necessary, but not vice versa. IFN-γ was produced by both subsets, but the vast majority of IL-2 was produced by CD4 cells [14]. (As these experiments were carried out without additional cytokines, this does not necessarily contradict results that show that pure CD8 T cell cultures can be costimulated via CD137 in the absence of CD4 T cells.)

Additional stimulation with CD137L on human monocytes compared to unmodified monocytes was shown to lead to higher intrcellular IL-2, IFN-γ, TNF-α, perforin and granzyme A in virus specific CD8 T cells. At low peptide-concentrations rapid cytokine production could be acieved with CD137L-monocytes, while unmodified monocytes could not induce any response. Nevertheless there were no significant differences in comparison to B7.1-costimulation in terms of phenotype and cytotoxicity. The virus-specific T cells in this setting were regarded as central and effector memory phenotype and both subsets could be expanded by CD137L-stimulation [95].

In an adoptive transfer mouse-model with tumor draining lymph node cells additional CD137-stimulation shifted the T cell phenotype towards type 1 (CD4 and CD8; IFN-γ, GM-CSF and IL-10 release tested). For stimulation immobilised Ab were used. In comparison to CD3/CD28 stimulated T cells, the T cells with additional CD137-stimulation caused superior regression of metastatic tumor nodules and prolonged survival after adoptive transfer. The tumor regression could be further enhanced by *in vivo* IL-10 neutralisation and reversed by IFN-γ neutralisation [96]. A murine GVHD model showed also markedly enhanced IFN-γ production by CD8 T cells upon Ab-stimulation of CD137 [97]. Another murine model showed impaired differentiation of naive CD8 T cells towards IFN-γ secreting Tc1 (intracellular and reduced secretion) when CD137 was blocked during priming *in vivo* [85].

Only few data are available on the role of CD137 in the costimulation of human, naive T cells. One experiment series used umbilical cord blood T cells of which 91% expessed a CD45RA+CD27+CD8+ phenotype (regarded as naive). Nearly all CD8 T cells (99.8%) were found to be negative for CD137. Upon unspecific stimulation of the TCR (OKT3 Ab) the umbilical cord blood CD8 T cells showed a peak expression of 50% CD137+ cells after 3 days, which was slower and a lower percentage compared to resting, Ag-experienced adult CD8 T cells (75% CD137+ cells after 12 h). The priming of naive umbilical cord blood mononuclear cells against an influenza-peptide was tested with and without an additional CD137 signal on day 1 after peptide-stimulation. CD137-agonists (CD137L more than anti-CD137 Ab) increased the frequency tetramer-positive cells when adherent monocytes were used as APC. For DC as APC possible adverse effects on T cell expansion were found . With monocyte-stimulation intracellular IFN-γ and improved cytotoxicity were also increased in CD137L-stimulated cells after 14 days. Similar results were obtained

for the priming of Epstein-Barr virus (EBV) specific T cells [98]. In accordance with these results also T cell isolation experiments (see also T cell selection section) showed a CD137 expression on naive T cells upon Ag-specific stimulation that was sufficient to isolate the CD137+ cells [99]. Contrastingly a murine *in vivo* model using systemic administration of CD137 Ab found a dimishment of naive T cells and an expansion of the effector-memory compartment (see below for details) [100].

Bertram et al. analysed *in vivo* T cell memory formation, which revealed a role for CD137 in a murine influenza model. CD137L-deficient mice show normal T cell expansion, but a reduced Ag-specific CTL number in a later stage and significantly weaker T cell expansion upon a second infection challenge compared to wild-type mice. The second response was comparable to a primary response, which suggests an impaired memory formation [101]. This result was further supported by adoptive transfer experiments showing that *in vitro* generated memory T cells survived better in wild-type than in CD137L-deficient mice. carboxyfluorescein succinimidyl ester (CFSE) staining of the Ag-specific cells showed that the proliferation of the T cells is similar in wild-type and knock-out mice, which suggests that the difference in cell numbers is due to impaired survival in CD137L-deficient hosts [102].

Also Ag-independent effects of CD137-stimulation on the memory T cell compartment have been reported. Systemic administration of an agonistic CD137 Ab in mice lead to expansion of memory T cells ($CD44^{hi}$) due to proliferation with no effect on the naive compartment. Overexpression of CD137L selectively on APC caused an accumulation of effector memory T cells in spleen an lymph nodes and subsequent diminshment of the naive compartment. Knock out experiments (adoptive transfer of CD137 knock out T cells in wild-type mice and vice versa) showed that CD137 on T cells was directly responsible for the proliferation while CD137 on other cell types was irrelevant. This effect was not due to a general impairmant of T cells from CD137 knock out mice as the T cell development in these mice has been shown to be normal and the T cells responded to unspecific stimulation. The effect of the CD137 Ab did not require Ag administration and was independent from MHC, IFN-γ and IL-15 as confirmed with respective knock out mice [100].

Besides CD137 also other TNFRSF members seem to be involved in the development of CD8 T cell memory. For adoptively transferred Ag-specific CD8 T cells TRAF1 (TRAF1) deficiency impaired the formation of memory cells more than the absence of a CD137 signal. This supports the hypothesis that at least one other TNFRSF member is involved in the signaling [72].

Eventually various regulatory T cell (Treg) subsets seem to be influenced by CD137. Tumour-infiltrating Tregs have been shown to be associated with a poor prognosis in several tumour types (e.g. heptaocellular carcinoma, breast cancer) [45]. In contrast to this murine

donor Treg in the setting of allo-HSCT inhibit alloreactive T cells that cause GVHD while sparing the GVL effect *in vivo*. Similar results have been shown for human T cells *in vito* [26]. Tregs comprise of the nTreg (CD4+,CD25+, FoxP3+), iTreg (CD4+,FoxP3+) and the CD8+ Treg. The generation of nTreg and iTreg (in the thymus and peripheral lymphoid organs) requires CD28 dependent costimulation and IL-2, which suggests that further costimulatory molecules could be involved. CD137 increases cell division, anti-apoptoic proteins and survival in nTregs in a similar fashion to effector T cells, but there are no clear data on FoxP3 expression and the functionality of these Treg. Interestingly for CD4+,FoxP3+ Treg a reduced capability to suppress effector T cells after CD137 activation has been shown. This was either mediated by direct engagement of receptors on the Treg, which inhibits the suppressive function or by triggering signals on the responder T cell population, which makes the responder cells resistant to Treg suppression. Finally some autoimmunity-models suggested that CD137 could be involved in the generation of CD8+ Treg [103].

2.3.3.2 Survival of T Cells

Many reports show increased antiapoptotic protein levels in T cells after CD137 signals and subsequently less apoptotic cells in cell cultures [65, 68, 69, 104]. This is usually attributed to the increased expression of Bcl-xL in human T cells (and additionally Bcl-2 in murine ones). Upregulation of both proteins does not increase T cell proliferation but confers a higher T cells yield in cultures by AICD inhibition [90].

In cell cultures of murine tumour draining lymph node (TDLN) cells stimulated with either immobilised CD3 and CD28 Ab alone or an additional CD137 Ab less apoptotic cells were detected with annexin V / propidium iodide staining with anti-CD137. (Anti-apoptotic proteins see below.) For the *in vivo* persistence (tracked with CFSE) no significant advantage was found for an additional CD137 Ab, however the proliferation (measured by CFSE decrease per cell) was improved [90].

In *ex vivo* human CD8 T cell expansion by artificial APC generated from K562 cells (details see below) an additional CD137 signal increased the Bcl-X_L and IL-2 expression by the T cells upon restimulation, resulting in a relatively stable number of viable T cells in annexin V / propidium iodide staining. In contrast to that in cultures stimulated by artificial APC without CD137L or CD3/CD28 beads an increasing percentage of apoptotic cells was found within the first three weeks [105].

However accessory cells like APC could be necessary for the antipoptotic effect. Purified human CD8 T cells were stimulated with OKT3, anti-CD137/CD137L and with or without APC (monocytes or DC). Only when APC were present, CD137-stimulation lead

to an increased percentage of Bcl-X_L positive cells in flow cytometry analysis. In absence of APC the same signal decreased the percentage of Bcl-X_L positive cells substantially, indicating a pro-apoptotic effect [98].

2.3.3.3 Proliferation and Effector Functions

Numerous studies show an enhancement of T cell proliferation by a CD137 signal in addition to a TCR or anti-CD3-mediated signal in very different experimental setups [14, 66, 71, 72, 90, 95, 98, 104, 106]. CFSE staining has revealed that in human T cells cell divison starts approximately 72 h following CD3 and CD137-stimulation [14]. Murine CD8 and CD4 T cells responded to either CD137L or an agonistic anti-CD137 Ab in a ^{3}H-thymidine incorporation assay and showed cell division in a CFSE staining. However in this murine setting the effect of CD3 + CD137-stimulation was remarkably weaker than CD3 + CD28 [104].

As most of the results were obtained with CD137-stimulation by Ab and CD137L (soluble and on APC) a murine model was established to assess the role of CD137 in *in vivo* CTL generation with ovalbumin (OVA)-specific T cells from OVA-specific TCR transgenic line (OT-I) mice. Blocking of CD137 by an constant fragment (F_C) fusion protein led to reduced expansion of naive CD8 T cells resulting in a fewer number of Ag-specific CTL due to apoptosis of T cells. Nevertheless functional CTL could be generated when CD137 was blocked (reduced IFN-γ but nearly normal cytotoxicity) [85].

In a human model for virus-specific CTL the costimulatory effect of B7.1 and CD137L on macrophages were compared. There were favorable results in terms of cytokines (see above), initial prolifertion rate and effector function. However in some donors a drop of tetramer-positive T cells was detected after one week for the CD137L-costimulation. This was attibuted to a loss of proliferation of CD137L costimulated T cells due to exhaustion [95].

Results on the influence of CD137 on the cytolytic response of human CTL showed an inprovement by adding an anti-CD137 signal compared to the anti-CD3 signal alone. The improvment was dependent on the presence of the CD137 signal during the initial stimulation, but not during the killing [14].

2.3.3.4 Timing Effects

Murine infection models (lymphocytic choriomeningitis virus (LCMV) and influenza) *in vivo* showed that the timing between TCR signal and CD137 activation could be crucial for the response in CD4 and CD8 T cells. Mice that had been given a CD137 Ab before or shortly after the infection showed a collapse of antiviral CD8 immunity (reduced numbers

of tetramer-positive CD8 T cells, decreased CTL function in a chromium-release-assay and absence of IFN-γ secreting T cells)on day 8 post infectionem (p.i.) and increased mortality. (until day 2 post p.i. for LCMV, until day 1 p.i. for influenza.)

In contrast to this the CD137-Ab led to a polyclonal CD8 T cells activation of non-Ag-specific T cells due to elevated TNF-α levels. Further experiments showed that the immunosuppressive effect was not due to blocking the CD137/CD137L interaction by the Ab and that the effect required CD137 on CD8 T cells, but not on DC. The CD8 T cell function was decreased by TNF-α and IL-10 in a Fas-dependent manner.

In conformity with other results [107] for stimulatory effects for CD137-Ab administration at a later point in time p.i. lead to increased survival of the mice. Remarkably this effect occured from day 3 p.i. on for LCMV and from day 1 p.i. on for influenza. The contrary effects were indepentent from the infectious agent, the class of the Ag and the mode of delivery, which indicates a relatively robust effect, that might not only apply to a very specific experimental setup [108]. Following experiments showed that the CD137-mediated suppression of CD8 T cells only occurs if DC are present. [109]

2.3.4 CD137 as an Activation Marker in T Cells

Usually the molecule is only expressed for a comparably short time period, which makes it a favorable candidate as an activation marker for selection of recently activated T cells. For the human CD137 most publications describe a peak of expression around 24 h [33,99,110] to 48 h [14] after activation or even earlier (10 to 20 h) for Ag-experienced, human adult CD8 T cells [15,98,111]. In contrast to this the murine homologue peaks much later *in vitro* (e.g. 96 h [104]) and is detectable up to 5 days after stimulation [65]. On the other hand the *in vivo* kinetics in a murine model showed again expression of CD137 from 12 to 24 h after activation [112].

At least for murine T cells the CD137 expression has been shown to be infuenced by the cell density in the cell culture plate. Optimal expression was achieved with 1×10^6 /mL and less CD137 expression was observed when using lower cell densities. IL-2, accessory cells or anti-CD28 thogether with immobilised anti-CD3 increased the expression of CD137 at suboptimal cell densities [113]. Other experiments have confirmed that despite the fact that CD137 is not dependent on CD28, it is increased by CD28-stimulation [114].

The upregulation of CD137 is usually regarded as highly specific for T cells that have recently been activated via the TCR (either by APC or by anti-CD3) [115,116]. Reagents that cross-link the TCR or CD3 [114] and mitogens (phytohaemagglutinin (PHA), calcium ionophore and phorbol myristate acetate (PMA) [98]) can induce 4-1BB on both CD4 and CD8 cells. Only high doses of IL-15 (100 ng/mL) have been reported to induce

CD137 expression unspecifically without TCR activation in murine memory T cells [102]. Comparable results have been obtained for human bone marrow CD8 memory T cells [72]. IL-1α, IL-2, IL-4, IL-7, IFN-γ and anti-CD28 have been shown not to induce CD137 in a TCR-independent manner [102, 113, 116]. Therefore CD137 can be regarded as a marker for target-specific T cells in T cell cultures stimulated with clonal target cells and as a Ag-specific marker in those cultures stimulated with a single Ag.

CD137 has been found on CD8 and CD4 T cells including Th1 and Th2 cells, which contradicts some early reports which did not show the expression in CD4 T cells. However the upregulation seems to be more rapid and to higher expression levels in CD8 T cells [65]. Persistent expression has been shown in the context of chronic immune activation like graft-rejection [117] and recurrent infection models [118].

2.4 CD137 for T Cell Selection

Given the characteristics of CD137 as an activation marker for a broad range of CD8 and CD4 T cells, it has ideal properties to select specific T cells with unknown Ag. As discussed above only few Ag for a selective GVL response are characterised in detail. Often known Ag are not applicable in an individual patient due to either the rarity of the Ag or to an unsuitable HLA profile. Techniques allowing the selection of T cells that respond to defined target cells but to an unknown Ag could circumvent this obstacle. Many cell selection techniques based on cytokine production have been developed for this purpose. Intracellular cytokine staining is usually incompatible with the selection of viable cells for adoptive transfer and detection of secreted cytokines requires a complex multistep procedure with high cell loss. Furthermore these assays often do only detect a small subset of T cells because of heterogenous cytokine profiles and miss naive T cells that are known to secrete only low quantities of cytokines [99, 119].

An antibody-detectable surface marker on activated T cells of different subsets could provide a means to select target-specific T cells if the marker is upregulated uniformly upon activation and not (or at low levels) expressed on resting T cells. Among known activation markers (CD25, CD69, CD71, CD95, CD134, CD137, CD154) the most favourable characteristics have been shown for CD137. More than 90% of T cells expressing CD137 are also positive for the activation markers CD25 and CD69 which indicates that CD137-selection identifies a broad repertoire of T cell subpopulations. Very good correlations of CD137 expression with IFN-γ and TNF-α production, CD107a (degranulation marker) and tetramer binding have been found for human PBMC. Furthermore the costimulatory capabilities make this molecule an ideal marker for the selection of T cells that must be expanded after the isolation procedure [33, 99, 119].

Stimulation of either human purified virus-specific (CMV and EBV) T cells or PBMC confirmed that upon stimulation with a defined viral Ag only tetramer-positive T cells specific for the stimulator Ag upregulate CD137 in seropositive donors. A CD137 enrichment assay using bead-based cell sorting upon T cell stimulation with viral Ag can be used to select tetramer-positive and IFN-γ producing T cells. Compared to a selection method based on IFN-γ secretion this assay is not only more straightforward to use but also provides a higher cell yield and specific CD8 and CD4 T cells with a better *in vitro* proliferative capacity [119]. Furthermore the CD137 selection is capable of selecting all CMV-tetramer positive CTL from seropositve donor T cells, which is indicated by a CD137 negative fraction depleted of tetramer-positive CTL after the isolation procedure. Compared to this a IFN-γ secretion assay left teramer-detectable CTL behind in the negative fraction after enrichment [99]. Another experiment series comparing different isolation methods for CMV- and EBV-specific T cells confirmed a proliferation rate advantage for CD137 isolated T cells and a purity comparable to tetramer-based selection [111].

Zandvliet et al. extended these findings for numerous viral MHC class I binding peptides and viral protein spanning 15-mer peptide pools for CMV, EBV, human adenovirus and influenza. Human PBMC were stimulated directly *ex vivo* with peptides and repetetivly stained for CD137 expression. On both CD4 and CD8 T cells the maximum of CD137 could be detected after 1 to 2 days. For CD8 T cells the Ag-specific upregulation of CD137 could be confirmed by multimer-staining. For three class I peptides and two class II peptides a CD137 enrichment assay was compared to an IFN-γ secretion assay. Both methods resulted in efficient enrichment of virus-specific T cells with significantly higher frequencies of specific CD8 T cells (over 90%) than specific CD4 T cells (10 to 60%). Also the simultanous isolation of CD4 and CD8 T cells with multiple specificties upon stimulation with viral protein spanning peptide pools was possible with both enrichment assays. Multimer-staining and intracellular IFN-γ staining after Ag-specific stimulation confirmed that T cells for each specificity in the pool were present in the culture [120].

Some publications also state that enrichment of low-frequency naive T cells by CD137 selection is feasible. In a naive starting population of T cells from hepatitis C negative donors tetramer-binding T cells specific for a hepatitic C virus peptide could be detected in a frequency below 1% after one week of peptide-loaded DC-stimulation. Further restimulation did not result in an enrichment of peptide-specific CTL but by using CD137 enrichment a mean frequency of 16% teramer-positive T cells could be achieved. Also naive T cells could be isolated directly *ex vivo* using the CD137-enrichment after stimulation with the melanoma-associated peptide Melan-A. Further propagation under Th1/Tc1 conditions lead to IFN-γ, TNF-α and IL-2 producing T cells. Approximately 25% of these T cells did not produce IFN-γ, but the other cytokines, which indicates that these T cells might be

missed by a IFN-γ-based isolation method. The Melan-A-specific T cells lysed Ag-positive melanoma cell lines *in vitro* [99].

In further experiments focussing on the enrichment of Ag-specific T cells from the naive pool CD45RO depleted human PBMC were used as responder cells and stimulated with autologues DC loaded with CMV or minor histocompatibility Ag peptides. All donors were seronegative for CMV or negative for the respective minor Ag. The CD45RO depletion step reduced the percentage of non-naive T cells and Tregs, which are mostly positive for CD45RO, considerably. Stimulation and restimulation with autologous APC in the presence of IL-7 did not yield detectable tetramer-positive T cells when unmodified PBMC were used as responder cells. In contrast with CD45RO depleted PBMC tetramer positive T cells for different CMV peptides and minor Ag were found after 21 days of culture and a CD137 enrichment step. The role of the Treg depletion was evaluated by adding back purified Treg after the CD45RO depletion which resulted in reduced percentages of tetramer-positive T cells. The generation of CMV-specific T cells was possible in over 95% of the experiments if a sufficiently large starting population of CD45RO depleted PBMC (over 25×10^6) was used. The T cells showed cytotoxicity against target cells that were exogenously loaded with peptide or presented endogenously processed Ag. T cells specific for minor Ag could only be generated in 50 to 80% of the attempts depending on the Ag. The frequencies of tetramer-positive T cells achieved with CD137 enrichment were usually below 10%. To obtain large numbers of purified tetramer-positive T cells further culturing and one or two rounds of tetramer-enrichment were necessary [121].

Also the isolation of polyspecific T cells with unknown Ag by CD137 isolation is possible. In a selective allodepletion approach aiming at the reduction of the GVHD risk of DLI by depleting T cells that respond to mismatched HLA molecules the CD137-selection was used to identify all T cells responing to the mismatched HLA molecules regardless of the peptide presented. Tumour/leukaemia-reactive T cells were first generated by stimulating CD8 T cells with HLA-mismatched (single allele mismatch or haploidentical) tumour/leukaemia cells. After a restimulation with K562 (see also Material and Methods section) cells transfected with the mismatched HLA molecules a depletion of CD137 positive T cells was performed which reduced the alloreactivity against the respective HLA to approx 10%. The antitumour-reactivity was not impaired in a chromium release assay. Restimulations of the depleted cell fraction with the mismatched HLA did not bring back the alloreactivity to previous levels. Also recipient fibroblasts (carrying all mismatched HLA and endogenously processed peptides, serving as possible target cells of GVHD) were used as stimulator cells with similar efficacy. The percentage of virus-specific T cells (CMV and EBV) was only slightly reduced by the CD137-depletion. In contrast to other selective allodepletion approaches that target alloreactive T cells directly *ex vivo* this

method could provide higher frequencies of tumour-reactive T cells due to the previous stimulations with malignant cells [33].

2.5 CD137 for T Cell Activation and Expansion

Generation of sufficient T cell numbers for adoptive immunotherapy often requires an *in vitro* expansion step. The use of stimulator cells is often thought to be problematic as either DC have to be generated in large numbers first or in case of biological artificial APC GMP conditions are hard to meet for the final product. Acellular synthetic stimulators in the form of beads could fulfill the GMP requirements while remaining flexible and modular for the respective purpose. Less quality controls during the manufacturing process are required and the reproducibility is remarkably better compared to biological APC. Liposomal materials, latex beads, magnetic beads and polymers have been used as carriers for Ab, costimulatory molecules, releasable cytokines and adhesion molecules. A comparably low TCR signal strength (low density of anti-CD3 or MHC) can be used in a defined system like a synthetic stimulator to expand only high-avidity T cells. Currently established are either non Ag-specific expansion beads (coated with Ab against CD3 and CD28) or artificial-APC beads (coated with peptide-loaded MHC and an Ab against CD28) [122].

Various studies show that additional CD137 signaling can substantially increase *ex vivo* and *in vitro* expansion of Ag- or tumor-specific T cells. Compared to other costimulatory molecules of the TNFRSF CD137 seems to be exceptionally powerful in stimulating CD8 T cells. A study with human functionally impaired human immunodeficiency virus (HIV)-specific CD8 T cells CD137 stimulation expands a population of Ag-specific CD8 T cells of effector phenotype from the functionally impaired starting population. Other TNFRSF members were not at all or not equally effective [123].

CD137L expressing monocytes are able to activate virus-specific T cells in a similar and equally effective fashion to B7.1 expressing monocytes. In contrast to experiments showing a synergistic effect of CD137 and B7 for polyclonal activation [105, 106], this in experiments no synergy was observed for Ag-specific T cells [95]. On the other hand blocking of a minimal B7-costimulation in a human setting has been shown to reduce the effect of CD137-stimulation, which nevertheless remains detectabele in complete absence of the B7 signal [14].

In a murine model tumor draining lymph node T cells were stimulated with immobilised CD3 and CD28 Ab *ex vivo*. Crosslinked CD137 Ab and Bcl-2 family inhibitors were added to determine the effect of CD137 in proliferation and apoptosis inhibition. The additional CD137 simulation led to an increased expression of Bcl-2 and Bcl-X_L, which eventually decreased the percentage of apoptotic cells during stimulation and a better

proliferation of the cells after adoptive transfer. The effect of CD137 on AICD inhibition could be reversed by Bcl-2 family protein inhibitors [90].

In an experiment series using anti-CD3 Ab and non-HLA matched CD80, CD86 and/or CD137L expressing lung cancer cells for the stimulation of human T cells, the expansion of T cells was investigated in detail. Double costimulation with CD80 and CD137L expanded T cells more effectively than either ligand alone. Notably prolonged (more than 14 days) proliferation could also be achieved with single CD137-costimulation but not with CD80. Using double costimulation a higher proportion of cells having undergone multiple divisions were detected in a CFSE staining on day 14 compared to either ligand alone. Cultures stimulated with anti-CD3 alone or with just additional CD80/CD86-costimulation showed decreasing cell counts after the first restimulation. Still after 3 weeks of the decline in cell ounts to 1/3 of the initial number a newly added CD137 signal was able to reverse this trend and expand the T cells to higher cell numbers than the initial one. No functional data were shown for these T cells [106]. As a possible explanation a rapid CD28 downregulation on T cells upon CD80 and CD86-costimulation was found. Single CD137-costimulation caused less CD28 loss and could restore CD28 expression on T cells, that had become CD28 negative. The reexpression of CD28 occured only when the T cells were restimulated with CD137L expressing lung cancer cells, but not with cells expressing both costimulatory ligands. Unfortunately no functional or proliferation data were published for the CD28 reexpressing T cells [124].

Maus et al. analysed the potential for *ex vivo* human CD8 T cell expansion of artificial APC generated from K562 cells compard to CD3/CD28 Ab-coated beads. The K562 cells were transfected with a low-affinity F_C receptor and loaded with CD3 and CD28 Ab. These APC were compared to ones, that were transfected additionally to express human CD137L. All three approaches showed equal potential to expand CD8 T cells for the first week. In long term culture (up to 25 days) the bead-stimulated and the CD3/CD28 APC-stimulated cultures reached a plateau, while the CD3/CD28/CD137L APC-stimulated culture continued to proliferate. After three weeks the cell number was aprox. 10-fold higher for CD3/CD28 APC and aprox. 100-fold higher for CD3/CD28/CD137L APC compared to the bead-stimulated culture. To test the influence of the unspecific stimulation on long term culture of Ag-specific T cells, influenza-peptide tetramer-positive and tetramer-negative human T cells were stimulated separately with anti-CD3/anti-CD28/CD137L APC over 70 days. The specificity was preserved when the T cells were repeatedly tested in a chromium-release assay, but the frequency of tetramer-positive T cells in the respective branch declined from 60% (day 17) to 20% (day 60) [105].

In melanoma adoptive immunotherapy has been extraordinarily successful: For clinical studies tuomur-reactive T cells were expanded *in vitro* either by stimulation with melanoma

cells or peptide-stimulation (Melan-A, gp100). These T cells were administered together with IL-2 (high and low dose approaches) in patients with therapy-refractory metastasized melanoma. Variable responses were observed including considerable tumour regressions of up to 20 months duration without serious side effects [57, 125]. Also stimulation with artificial APC in the format of magnetic beads has been evaluated in a humanized mouse model. Melan-A specific CTL were generated by bead-stimulation (coated with peptide-loaded HLA-A2 and anti-CD28) and injected into severe combined immunodeficiency (SCID) mice together with Melan-A positive melanoma cells. The CTL generated with artificial APC were equally efficient as DC generated CTL in terms of tumour control (reduced growth), persisted in the blood for two weeks and could be detected in tumour sites on day 3 by bioluminescence. Furthermore the growth of established melanomas could be slowed down by the bead-stimulated CTL [126].

Also CD137 Ab coating on MHC-coated beads (polystyrene particles) was tested for the generation of melanoma-specific T cells. Melan-A specific T cells were expanded from purified CD8 T cells from healthy donors using beads coated with HLA-A*2 loaded with Melan-A peptide and additional costimulatory Ab (CD28, CD137) in different ratios. A ratio of 1:3 CD28:CD137 was found to be most effective leading to over 70% tetramer-positive T cells after four rounds of stimulation compared to 15% for CD28 alone. However the T cells generated showed a relatively differentiated effector-memory phenotype (CCR7neg, CD45RAneg) and concordantly strong responses in a chromium-release assay and intracellular IFN-γ staining [122].

For the expansion of polyclonal, leukaemia-specific T cells for clinical application magnetic beads based on Ag-independent TCR-stimulation with GMP requirements could be useful. Magenetic beads could be particularly favourable as they have considerable handling advantages, e.g. in the removal of beads prior to infusion. Magnetic beads coated with CD3 and CD28 Ab are well established for experimental and clinical applications [127, 128]. Given the previous results on the favourable effects for CD137-stimulation using artificial APC, magnetic beads that are available for clinical application were tested for the expansion of tumour-specific CTL. New magnetic beads coated with CD3,CD28 and CD137 Ab were compared to established CD3/CD28 beads, target-specific stimulation with renal cell carcinoma (RCC) cells and simple cytokine-stimulation.

3 Materials and Methods

3.1 Materials

A list of the manufacturers (Mfr.) can be found at the end of the "materials" section. Unless otherwise stated, the German branch of the manufacturing companies is indicated.

3.1.1 Devices

Device	**Model**	**Mfr.**
autoclave	VX-150	Sys
cell centrifuge	Megafuge 1.0R	Hae
cell irradiation device	Gammacell 2000	Moo
cell sorter	FACS Aria	BD
cryobank	Espace 331 Gaz	AL
dispenser	Ceramus classic 0.4 - 2 mL	HL
drying cabinet	BE 500	Mem
flow cytometer	FACS Canto II	BD
fridge / freezer	+4 °C, −20 °C	Bo
	+4 °C, −20 °C	Lie
	Herafreeze −80 °C	Hae
gamma-counter	Wizard 2	Per
ice machine	UBE 50/35	Zie
incubator	9040-0007	Bin
	Heracell 240	Hae
magnets	miniMACS, midiMACS	Mil
	MPC-L	Dyn
microscope	Axiovert 25	Zei
	Axio Imager M1	Zei
milligram scale	EW150-3W	Ker

Item	Model	Mfr.
liquid nitrogen tank	XL-180	TW
pipettes	Research 0.5 - 20 µL	Epp
	Pipetman 2 – 20 µL	Gil
	Pipetman 20 – 200 µL	Gil
	Pipetman 100 – 1000 µL	Gil
	m100 5 – 100 µL	Bih
	m300 30 – 300 µL	Bih
	25 – 200 µL	Dun
	ErgoOne 10 – 100 µL	Sta
	ErgoOne 30 – 300 µL	Sta
pipettor	Pipetboy acu	Int
sterile bench	NU-440-601E	Nu
	Herasafe HS18	Hae
shaker	Rotamax 120	Hei
	Minishaker MS2	IKA
water bath		GFL
water deionization machine	Purelab Classic	Elg

3.1.2 Plastic Material

Item	Model	Mfr.
cell culture flask	Cellstar 50 mL, 25 cm^2 with fiter top	Gr
	Cellstar 250 mL, 75 cm^2 with or w/o fiter top	Gr
cell culture plates	96 well, V-/U-/flat bottom, coated	Gr
	48w/24w/6w, flat bootom, coated	Gr
	24w/12w, flat bottom, uncoated (suspension)	Gr
cryo-box	Mr. Frosty	Nal
cryo-tube	Cryo-S	Gr
ELISPOT-plate	Multiscreen MSIPS4510	Mp
FACS-tubes	Falcon 5 mL, polystyrene	BD
magnetic columns	MS/LS/LD column	Mil

Item	Model	Mfr.
nylon mesh filter	Preseparation filter 30 µm	Mil
	Cell Strainer 100 µm	BD
petri dish	35/60/95 mL, polystyrene	Gr
pipette tips	TipOne 10 µL, 200 µL, 1000 µL	Sta
single-use pipette	Cellstar 1/2/5/10/25 and 50 mL, polystyrene	Gr
sterile filter	Stericup Express Plus 0.22 µm	Mp
	Stericup Durapore 0.45 µm	Mp
tube	0.6 mL polystyrene, "cytotox - tube"	Gr
	Cellstar 15 mL/50 mL, polypropylene	Gr
tubes with frit	Leucosep, polypropylene	Gr

3.1.3 Glass Material

Item	Model	Manufacturer
cover slip	Menzel-Gläser	Men
counting chamber	Fuchs-Rosenthal	Mar

3.1.4 Chemicals

Substance	Manufacturer
3-amino-9-ethylcarbazole tablets (AEC)	Sig
acetic acid	Rot
albumin, bovine BSA	Sig
albumin, human	Beh
^{51}chromium (sodium chromate)	AB
N,N - dimethyl formamid	Rot
dimethyl sulfoxide (DMSO)	Rot
DNase I	Roc
Dulbecco's phosphate-buffered saline solution (PBS)	Gib
Dulbecco's PBS dry chemical "Instamed"	Bic
ethylenediaminetetraacetic acid (EDTA)	Sig
ethanol >99%	Rot
ethanol 70%	Ned

Substance	Manufacturer
FACS Clean	BD
FACS Flow Sheath Fluid	BD
FACS Rinse Solution	BD
FACS Shutdown Solution	BD
formaldehyde 37%	Mer
hydrogen peroxide	Sig
lymphocyte separation medium LSM 1077	PAA
phytohaemagglutinin (PHA)	Mur
synthetic peptide “CMV-NLV” NLVPMVATV	PSL
synthetic peptide “MelanA” ELAGIGILTV	IHB
Triton X-100	Sig
trypan blue	Mer
tween 20	App
Z-VAD-FMK General Caspase Inhibitor	RD

3.1.5 Buffers and Solutions

Item	Ingredients
acetate buffer (ELISPOT)	985.3 mL H_2O + 14.7 mL acetic acid 99% + 2.88 g sodium acetate
AEC solution	20 mg 3-amino-9-ethylcarbazole (1 tablet) 2.5 mL N,N - dimethyl formamid 47.5 mL acetate buffer filter with 0.45 µm filter
FACS-buffer	500 mL PBS + 0.5 g BSA
FACS-fixative	PBS + 1% Vol formaldehyde
MACS-buffer	500 mL PBS + 2.5 g BSA + 2 mmol EDTA
PBS	H_2O + 137 mmol NaCl + 2.7 mmol KCl + 12 mmol phosphate (10 L H_2O + 95.5 g PBS dry chemical)
PBS + 0.05% Tween	10 L PBS + 5 mL tween 20

Item	Ingredients
sort-buffer	PBS + 1% Vol. human serum + 2 mmol EDTA
trypan blue stock solution	1 L H_2O + 2 g trypan blue diluted 1:4 with 125 mmol saline for use

3.1.6 Substances and Media for Cell Culture

Substance	Manufacturer
AIM-V medium	Gib
fetal calf serum (FCS)	PAA
heparin-natrium 25000	rat
RPMI 1640 + 1% L-glutamine medium	Gib
penicillin/streptomycin (PenStrep)	Gib
trypsin-EDTA	Gib

Human serum (HS) refers to mixed sera of 10 to 20 young, healthy blood donors (donation after informed consent), which was passed through a sterile filter, heat inactivated for 30 min at 56 °C and stored frozen at −80 °C.

Medium	Ingredients
DC-medium	AIM-V + 1% HS
freezing medium	AIM-V + 8% human albumin + 10 IU/mL heparin + 10% DMSO (DMSO added prior to use) 0.1 mg/mL DNase I (for leukapheresis)
medium A (for T cells)	AIM-V + 10% HS
medium B	RPMI 1640 + 10% FCS + 1% PenStep

3.1.7 Cytokines

All cytokines used were human and recombinantly produced.

Cytokine	Mfr.
granulocyte-macrophage colony-stimulating factor (GM-CSF, Leukine^{TM})	Bay
interferon gamma (IFN-γ)	RD
interleukin 2 (IL-2, Proleukin^{TM})	Nov

Cytokine	Mfr.
interleukin 4 (IL-4)	Mil
interleukin 6 (IL-6)	Mil
interleukin 7 (IL-7)	RD
interleukin 12 (IL-12)	RD
interleukin 15 (IL-15)	RD
interleukin 21 (IL-21)	bm
prostaglandin E_2 (PGE_2)	Sig
tumor necrosis factor alpha (TNF-α)	Pro

3.1.8 Kits, Manufactured Articles

Item	Mfr.
anti-biotin microbeads	Mil
CD8 microbeads human	Mil
CD8 naive T cell isolation kit: biotin Ab cocktail anti-biotin microbeads CD8 microbeads	Mil
CD137 microbead kit: CD137-PE Ab anti-PE beads	Mil
Dynabeads CD137 (clone CD137: 4B4-1 or BBK-4, "low" or "high" Ab-density, respectively)	Dyn
Dynabeads^{TM}T cell expander CD3/CD28	Dyn
Dynabeads^{TM}T cell expander CD3/CD28/CD137, (clone CD137: 4B4-1)	Dyn
FlowComp CD137 Kit: DSB-X^{TM} biotinylated CD137 Ab (BBK-4) FlowComp Dynabeads FlowComp release buffer	Dyn
IFN-γ Secretion Assay (PE): IFN-γ catch reagent IFN-γ detection Ab anti-PE beads	Mil
Vectastain Elite Kit	Vec

3.1.9 Antibodies

Antigen	Conjugation	Mfr.	Volume (in µL)
biotin	APC	Mil	10
CD3	FITC	BC	3 (PBMC), 2 (T cells)
	PE	BC	3 (PBMC), 4 (T cells)
	APC	BC	2 (PBMC), 3 (T cells)
	V450 Horiz.	BD	2 (1:5 diluted)
CD4	FITC	BC	2
	PE	BC	2
	APC	BC	1
CD8	FITC	BC	2 (PBMC), 5 (T cells)
	PE	BC	2 (PBMC), 3 (T cells)
	APC	BC	1 (PBMC), 2 (T cells)
	PerCP	BD	3 (PBMC), 10 (T cells)
	V450 Horiz.	BD	2 (1:10 diluted)
CD16	FITC	BC	5
CD27	FITC	BD	20
CD28	PE	BC	10
CD33	PE	BC	5
CD45RA	APC	Mil	5
CD45RO	PE	BC	5
CD56	PE	BC	5
CD57	APC	Mil	5
CD62L	PE	BC	10
CD127	FITC	eB	20
CD137	PE,APC	BD, Mil	4
	biotin	Mil	*
CD184 (CXCR4)	APC	BD	10
CD197 (CCR7)	FITC	RD	5
goat-anti-mouse	PE	BC	50 (1:100)
IFN-γ	none/biotin	Mt	**

Antigen	Conjugation	Mfr.	Volume (in µL)
IgG1 mouse	FITC, PE, APC, PerCP, PC5, V450 Horizon	BC, BD	***
TCR vβ 8	PE	Imt	5
TCR vβ 13.2	PE	BC	5

The column "volume" specifies the volume of Ab in µL used for 1×10^5 cells.
* Used for Miltenyi CD137 biotin enrichment.
** Used for ELISPOT.
*** For negative controls the volume of Ab was the same as the highest volume of another Ab that was used in this panel and conjugated to this dye.

3.1.10 Fluorescent Dyes for Flow Cytometry

Substance	Conj.	Mfr.	Vol. (in µL)
7-AAD	-	BD	5
Annexin V	FITC, PE	BD	5
Tetramer HLA-A2 MelanA (ELAGIGLTV)	PE	BC	2

3.1.11 Abbreviations of Manufacturers

Abbreviation	Manufacturer
AB	Amersham Biosciences, GE Healthcare Europe, Freiburg
AL	Air Liquide, Düsseldorf
App	AppliChem, Darmstadt
Bay	Bayer, Leverkusen
BC	Beckman Coulter, Karlsruhe
BD	BD Pharmigen/Biosciences, Heidelberg
Beh	CSL Behring, Marburg
Bic	Biochrom KG, Berlin
Bih	Biohit, Rosbach v.d.H.
Bin	Binder, Tuttlingen
bm	biomol, Hamburg
Bo	Robert Bosch Hausgeräte, München
Dun	Dunn Labortechnik, Asbach
Dyn	Invitrogen Dynal, Oslo, Norway

Abbreviation	Manufacturer
eB	eBioscience, Frankfurt am Main
Elg	Elga LabWater, Celle
Epp	Eppendorf, Hamburg
GFL	GFL, Burgwedel
Gib	Invitrogen Gibco, Karlsruhe
Gil	Gilson, Limburg
Gr	Greiner, Frickenhausen
Hae	Haereus, Hanau
Hei	Heidolph Instruments, Schwabach
IHB	IHB-LUMC, Leiden, Netherlands
IKA	IKA, Staufen
Imt	Immunotech, Marseille, France
Int	Integra Biosciences, Fernwald
HL	Hirschmann Laborgeräte, Herrenberg
Ker	Kern, Balingen
Lie	Liebherr-Hausgeräte, Ochsenhausen
Mar	Marienfeld, Lauda Königshofen
Mem	Memmert, Schwabach
Men	Menzel, Braunschweig
Mer	Merck, Darmstadt
Mil	Miltenyi Biotec, Bergisch-Gladbach
Moo	Moolsgard Medical, Gansloe, Denmark
Mp	Millipore, Eschborn
Mt	Mabtech, Hamburg
Mur	Murex Biotech, Kent, UK
Nal	Nalgene Nunc, Wiesbaden
Ned	Nedalco, Heilbronn
Nov	Novartis, Nürnberg
Nu	Nuaire, Plymouth, USA
PAA	PAA, Pasching, Austria
Per	Perkin-Elmer, Rodgau

Abbreviation	Manufacturer
Pro	PromoCell, Heidelberg
PSL	Peptide Specialty Laboratories, Heidelberg
rat	ratiopharm, Ulm
RD	R&D Systems, Wiesbaden-Nordenstadt
Roc	Roche Applied Science, Mannheim
Rot	Carl Roth AG, Karlsruhe
Sig	Sigma Aldrich, Steinheim
Sta	Starlab, Ahrensburg
Sys	Systec, Wettenberg
TW	Taylor-Wharton, Husum
Vec	Vector Laboratories, Burlingame, USA
Zei	Zeiss, Göttingen
Zie	Ziegra, Isernhagen

3.2 Methods

3.2.1 General

3.2.1.1 Preface

Cenrifugation means a 5 min centrifugation at 1500 rpm (470 g) at room temperature (RT) using the built-in brake to slow down the centrifuge more quickly, unless otherwise stated. Incubation in the incubator refers to 37 °C, 5% CO_2 and 100% relative air humidity, unless otherwise stated.

Medium A is AIM-V medium supplied with 10%Vol human serum and was ususally used for T cell cultures. Medium B is Roswell Park Memorial Institute (RPMI) 1640 medium supplied with 10%Vol FCS and 1% PenStrep and was ususally used for immortal cell lines. DC-medium is AIM-V supplied with 1%Vol human serum and was ususally used for generation of dendritic cells.

3.2.1.2 Cell Counting

Trypan blue is a diazo dye which is excluded by vital cells due to their intact cell membrane. On a phase contrasted image these cells appear bright while dead cells with trypan blue in their cell plasm exhibit a darkish-blue colour. Prior to counting the cell suspension

was diluted 1:2 with trypan blue solution and then filled into a Fuchs-Rosenthal counting chamber. Usually only viable cells were counted; in some leukaemias there were "borderline"-cells, that were only counted if these cells were known to survive overnight in this particular leukaemia.

3.2.1.3 Freezing and Thawing

For freezing all cell types except leukaemias were centrifuged and resuspended in 1 mL freezing medium supplemented with 10% dimethyl sulfoxide (DMSO) per cryo-tube. 1 mL cell suspension was pipetted in each cryo-tube (max. 100×10^6 cells per tube) and the tubes were put in 4 °C precooled, isopropanol-filled cryo boxes. These were put immediately into a −80 °C freezer and the cryo-tubes were transferred into a liquid nitrogen bank within a few days.

For thawing the cryo-tubes were taken out of the nitrogen bank and the frozen cells were washed with cell culture medium without serum. The freezing medium containing 10% DMSO was diluted with 7 mL cell culture medium and the cells were centrifuged immediately to minimize the cytotoxic effect of DMSO at RT.

3.2.1.4 Irradiation

The cells were exposed to a gamma radiation source under radiation protection precautions for a defined time period equivalent to a defined absorbed radiation dose. The dose chosen should preserve the intended task (presenting Ag, feeder cell effect) of the cells for a few days, but the cells were not intended to survive for more than 7 days (see table 3.1). Some AML were irradiated with an increased dose because the cells showed remarkable viability and signs of monocytoid differentiation (shape, plastic adherence) upon irradiation with the standard dose.

Function	**Cell name**	**Dose**
target cells (malignant)	RCC 1257	100 Gy
	AML (in general)	35 Gy
	MZ653-AML, MZ574-AML	60 to 70 Gy
peptide presentation	mature DC	25 Gy
feeder cells	PBMC, PBMC subsets	25 to 35 Gy

Table 3.1: Absorbed radiation doses for different cell types

3.2.1.5 Density Gradient Centrifugation

Whole blood or buffy coats were diluted 1:2 with phosphate-buffered saline (PBS) and pipetted as an unmixed layer on top of 15 mL sucrose-epichlorohydrin-copolymer solution (Ficoll-Paque^{TM}) in a 50 mL tube. The tubes were centrifuged for 20 min at 2000 rpm (836 g) at RT without brake. The PBMC formed a horizontal ring that could be harvested with a pipette and transferred into another 50 mL tube. Afterwads the cells were washed two times with 4 °C RPMI 1640 (first washing step 1800 rpm, 10 min).

3.2.2 Cell Culture

All cell cultures were handeled in a sterile manner and kept in an incubator at 37 °C, 5% CO_2 and 100% relative air humidity.

3.2.2.1 Immortal suspension cell lines

The following cell lines were cultured in Medium B (see Materials) in cell culture flasks. The cell concentration was set to 4×10^5 /mL twice a week. The cell culture flask was renewed every other week.

- EBV infected B cell lines (referred to as B cell lymphoblastoid cell line (B-LCL)) of leukaemia patients and healthy donors. The cell lines were created *in vitro* according to standard methods.
- The K562 cell line has been derived from a CML in over 175 serial passages [129]. The cells show an extremely low HLA expression on their surface [130], so that they can serve as a NK cell target and as a well-defined target in functional assays when transfected with HLA molecules [33, 131]. Although the cells express almost no HLA, which prevents alloreactivity, they do express T cell interaction molecules CD54(intercellular adhesion molecule 1 (ICAM-1)) and CD58 [105].
- T2 cells (kindly provided by E. Märker-Hermann, Wiesbaden) derive from a T-B lymphoblast hybrid, are HLA-A2-positive and have deficient transporter associated with antigen processing (TAP) genes. This hampers the loading of HLA class I molecules with endogenous peptides, which appear "empty" on the cell surface [132, 133]. Therefore these cells can be exogenously pulsed with synthetic peptides.

3.2.2.2 Adherent Cell Lines

The MZ-RCC 1257 cell line has been generated from tumour tissue of a clear RCC patient (encoded as MZ1257). The cell line was kindly provided by Dr. H. Gabbert, Mainz. It

proliferates rapidly *in vitro* with an amplification factor of two to four per week and was shown to be immunogenic [134]. Serological HLA typing showed the class I profile A *02/*03, B *07/*44 and Cw *05/*07. The adherent RCC cells were cultured as monolayers in medium B in cell culture flasks. To harvest the cells the medium was removed and the cell layer was rinsed with PBS followed by a 5 min incubation period with Typsin – ethylene-diamine-tetra-acetic acid (EDTA) in the incubator. The cell layer was then detached from the bottom of the flask by gentle bashes. The cells were washed out with RPMI 1640 medium and centrifuged to remove the Trypsin. For the next passage the cells were seeded at a concentration of $5{\times}10^5$ cells/ml in a cell culture flask.

3.2.2.3 Leukaemias

Leukaemia blasts were regularly obtained from AML patients, who underwent therapeutic leukapheresis and occasionally from peripherial blood or bone marrow aspirate. The samples were taken after initial diagnosis before application of any cytostatic therapy. White blood cells were isolated by density gradient centrifugation (q. v.) and frozen in freezing medium supplied with 0.1 mg/mL DNase. Prior to use the cells were thawed, seeded in a petri dish at a concentration of 3×10^6 /mL and rested over night in medium A in the incubator. Details of the leukaemia blasts used can be found in table 3.2.

3.2.2.4 Mixed Lymphocyte Tumor Culture

Immunogenic tumor cells (e. g. RCC) can be incubated with HLA class I identical (high resolution typed) CD8 T cells to generate polyclonal, Ag-specific cytotoxic T lymphocytes [136, 137]. To expand the T cells usually periodic restimulations with tumor cells are used. In general the T cell lines can reach an age of 80 to 120 days before the reactivity to their Ag recedes and the growth-factor drops below 1.

In this project T cells from a mixed lymphocyte tumor culture (MLTC) with RCC 1257 were used to investigate the possibilities of Ag-independent bead-stimulation. The cell lines were maintained in AIM-V medium supplied with 5% human serum and 100 IU/mL IL-2 at a concentration of 0.5×10^6 /mL and restimulated weekly with irradiated RCC in a ratio of 1:10 (tumor cells : T cells).

Splitting was performed in a similar manner to mixed lymphocyte leukaemia cultures, but after splitting the wells were filled up with cytokine-supplied medium to keep the cytokine concentration as constant as possible.

Patient Code	Age	FAB	Karyo	RGA	HLA class I
MZ 201-AML	73	M5b	46,XX	FLT3-ITD neg	A *0101/*0201 B *0801/*5601 Cw *0102/*0701
MZ 369-AML	29	M4	46,XX	FLT3-ITD neg	A *0101/*2402 B *1801/*3801 Cw *0702/*1203
MZ 418-AML	48	M5b	n/a	n/a	A *02/*03 B *35/*44 Cw *04/*05
MZ 529-AML	35	M1	46,XX	FLT3-ITD pos	A *0301/*1101 B *1501/*3501 Cw *0304/*0401
MZ 561-AML	75	M5	n/a	n/a	A *24/*32 B *40/*44 Cw *02/*05
MZ 574-AML	42	M4eo	46,XY inv(16)	FLT3 D835 point mutation	A *0101/*0201 B *0702/*1501 Cw *0304/*0701
MZ 580-AML	54	M4	46,XY	FLT3-ITD pos NPM1 mut.	A *0101 B *5701 Cw *0602
MZ 653-AML	74	M5	46,XX	n/a	A *0101/*0301 B *0801/*1302 Cw *0602/*0701
MZ 667-AML	53	M1	46,XX	FLT3-ITD pos NPM1 mut.	A *2301/*3301 B *1402/*4901 Cw *0701/*0802
MZ 911-AML	75	M5	46,XX	FLT3-ITD pos NPM1 wt	A *02/*24 B *15/*51 Cw *03/*04
MZ 964-AML	48	M4	complex aberrant	n/a	A *03/*32 B *35 Cw *04
MZ 987-AML	55	M4	46,XY	FLT3-ITD pos NPM1 wt	A *0201 B *1501/*1517 Cw *0304/*0701

Table 3.2: Details of the leukaemia blasts used for T cell stimulation and as target cells in functional assays. The patient column states the code used for anonymised naming of the leukaemias. The age of the patients at initial diagnosis, the subtype according to the FAB classification and the karyotype can be found in the respective column. Unless otherwise specified the karyotypes were normal. Recurrent genetic abnormalities (RGA) that are relevant to the WHO classification [135] are specified in the RGA column (wt:wild type, mut:mutated). High-resolution genomic HLA typing data (4 digits) are shown if available, otherwise serological data (2 digits) were used.

3.2.2.5 Mixed Lymphocyte Leukaemia Culture

To generate polyclonal, leukaemia-specific T cells, whole CD8 T cells or naive CD8 T cells isolated from PBMC were incubated with HLA class I identical (high resolution typed) leukaemia blasts at a ratio of 1:1 [11, 12, 32]. Usually a 24-well plate was used with 2 mL of medium A with cytokines per well and a T cells concentration of 0.5×10^6 /mL. If there were less than $1{\times}10^6$ T cells, smaller wells were used and the number of T cells per well was downscaled according to the ground area of the wells. The cytokine concentration was 5 ng/mL IL-7, 1 ng/mL IL-12, 5 ng/mL IL-15 during the first 7 days. Afterwards IL-12 was replaced by 50 IU/mL IL-2. In some experiments also 10 ng/mL IL-21 were added over the whole time. There were also a few approaches without cytokines or with only 2 ng/mL IL-7.

As single-HLA-mismatched or suballel-HLA-mismatched patient-donor combinations can be used in hematopoetic stem cell translantation, several experiments were also performed in these systems.

If the medium turned bright yellow or the bottom of the wells was completely covered with T cells, the culture was split. Ususally the cells were resuspended and half of the cell suspension in a well was transferred to a new well (1:2 split). The wells were filled up to the previous volume with medium A without cytokines. Cultures were split at least two days prior to a functional test or the next restimulation because previous observations suggested that recent manipulation of the T cells might cause background IFN-γ release. If the medium was extremly bright yellow on a day before a functional test, half of the medium volume was replaced by fresh medium A without resuspending the cells.

3.2.2.6 Generation of mature Dendritic Cells out of PBMC

Mature, myeloid dendritic cells (mDC) can be generated within seven days [138] out of monocytes, which can be isolated from PBMC by plastic adherence. As they can be loaded exogenously with synthetic peptides and they are able to induce activation of naive T cells, mDC are optimal to generate peptide-specific T cells against tumor-associated peptides.

Day 0: 2 to $3{\times}10^7$ PBMC per well were plated in 6-well plates in 3 mL of DC-medium and put into the incubator for 60 to 90 min. Subsequently the wells were rinsed three times with 37 °C PBS and non-adherent cells were harvested and frozen. Finally 3 mL of DC-medium per well were plated and the adherent monocytes were incubated for 24 h in the incubator.

Day 1: Cytokines were added to the medium as follows (final concentration in brackets): GM-CSF (800 IU/mL), interleukin (IL)-4 (1000 IU/mL)

Day 3: 1 mL of medium per well was aspirated, centrifuged and the pellet was resuspended in the pervious volume of DC-medium supplemented with GM-CSF (1600 IU/mL) and IL-4 (1000 IU/mL). 1 mL thereof was added to each well and the plates were put back in the icubator.

Day 5: To harvest the immature DC, the plates were put on ice and the cells were washed out with 4 °C PBS. For maturation the DC were plated in 12 or 24-well-suspension-plates (no surface coating) at 2 to 3×10^5 DC in 1 mL DC-medium per well (refers to 24w-plate, double volume and double cell count for 12w-plate). The medium was supplemented with cytokines as follows: GM-CSF (800 IU/mL), IL-4 (500 IU/mL), IL-1β (10 ng/mL), TNF-α (10 ng/mL), IL-6 (1000 IU/mL), prostaglandin E_2 (PGE_2) (1 µg/mL). The plates were then put in the incubator for 48 h.

Day 7: To harvest the mature DC the plates were put on ice and the cells were washed out with 4 °C PBS. The cells were either frozen or pulsed with a peptide and used for T cell stimulation instantly.

3.2.2.7 Stimulation of T Cells with autologous, peptide-pulsed APC

T cells specific for tumor-associated peptides can be generated by stimulating naive CD8 T cells with autologous, exogenously peptide-pulsed, mature DC. For further restimulations autologous peptide-pulsed PBMC can be used.

Initial Stimulation. Mature DC were incubated with the peptide at a concentration of 10 µg/mL in 0.5 mL medium A for 4 h in the incubator. Meanwhile naive CD8 T cells were isolated from autologous PBMC. During the last 30 min of the peptide-incubation the DC and non-naive PBMC (feeder cells) were irradiated with 25 Gy. The cells were plated at a ratio of 1:10:10 (dendritic cells : T cells : feeder cells) with a T cell concentration about 1×10^6 /mL in medium A supplemented with 10 ng/mL IL-21. On day 3 after stimulation IL-7 and IL-15 were added to a final concentration of 5 ng/mL each.

Restimulation. Autologous PBMC were loaded with peptide in a similar fashion to DC on day 0. Irradiated (25 Gy) PBMC and T cells were plated at a ratio of 1:2 to 1:3 (PBMC : T cells) with a T cell concentration about 1×10^6 /mL in medium A without cytokines.

24 h later cytokines were added to final concentrations as follows: IL-2 (25 IU/mL), IL-7 (5 ng/mL), IL-15 (5 ng/mL), IL-21 (10 ng/mL)

3.2.3 Magnetic Cell Separation

For immunomagnetic cell separation surface markers of cells are first labeled with antibodys which are conjugated to magnetic beads. Inside a magnetic field the bead-labeled cells are retained while the non-labeled cells can be rinsed. After the magnetic field is removed, the bead-labeled cells can be handled like a regular cell suspension again and the cell mixture has been separated into a surface-marker-positive and negative population.

In terms of technical details the following points are of particular importance:

- **Separation method:** In column-based methods the cells are passed through a 30 µm mesh and over a column, which contains ferromagnetic material. The column is placed in a special magnet while the cells run through the column. Afterwads the column is removed from the magnet and the bead-labeled cells are flushed out. In tube-based methods the tube is put in a magnetic rack, the labeled cells gather at the wall of the tube and the non-labeled cells can be pipetted out of the tube. After removing the tube from the magnetic rack, the labeled cells have remained in the tube.

- **Antibody-bead binding:** There are Ab-coated beads commercially available, in which the Ab is directly bound to the bead. If the Ab is directed against the desired surface marker, these beads can be used. Furthermore, there are anti-fluorochrome Ab-coated beads, which can be used in combination with fluorescence-dye-conjugated Ab. Finally there are streptavidin- and anti-biotin-Ab-coated beads, that fit biotinylated Ab. With special modified-biotin/nitrated-streptavidin-combinations, the binding between the Ab and the bead can be released with biotin-containing buffer after the separation procedure.

- **Separation type:** Positive-selection-kits are focussed on high purity of the bead-labeled fraction. Contrasingly depletion-kits are designed to eleminate a certain population as thoroughly as possible.

- **Bead size:** Depending on the manufacturer, the bead size varies from the order of 10^{-8} (Milteny) to 10^{-6} m (Dynal). Beads in the order of µm can cause receptor-crosslinking and subsequent downstream-signaling in the labeled cells.

3.2.3.1 CD8 / Naive CD8 Isolation

CD8 positive T cells were isolated from PBMC with Miltenyi CD8 Microbeads (Ab directly bound to bead) in columns for positive selection. Afterwards the purity was checked in flow cytometry, especially the percentage of NK cells (CD16-pos., CD56-pos., CD3-neg., mostly CD8-pos.).

The isolation using the Miltenyi naive CD8 isolation kit starts with the depletion of differentiated T cells and NK cells from PBMC by labeling the surface markers CD45RO, CD56, CD57 and CD244 with biotinylated Ab. Then anti-biotin beads are added and the cells are passed through a depletion column. Afterwards CD8 Microbeads were added to the non-labeled cells and CD8-positive cells were selected using a positive-selection column. In some experiments the non-naive PBMC were flushed out of the depletion column and used as feeder cells (irradiated with 25 to 30 Gy). The purity was assessed in flow cytometry including stainings for a typical naive T cell phenotype (CD45RA/CD45RO co-staining, CD27, CD28, CCR7, CD62L, CXCR4, CD127), contaminating CD4 cells and NK cells.

3.2.3.2 CD137 Enrichment using Miltenyi Kits

Two column-based isolation methods with Miltnyi materials were compared. One method uses biotinylated Ab and anti-biotin beads, the other one R-phycoerythrin (PE)-conjugated anti-CD137 Ab and anti-PE beads. The CD137 enrichment was performed in the period of 10 to 18 hours after restimulation for leukaemia-stimulated T cells as a CD137-kinetics had shown the maximum expression of CD137 and the best discrimination between positive and negative cells to be in that period. For peptide-stimulated T cells the isolation procedure was performed 24 h post restimulation as recommended in the literature [99,119]. Both methods were tested at RT and cooled (4 °C/ice-cooled) for all steps of the whole procedure.

Labeling. The restimulated T cells were washed in magnetic cell separation (MACS)-buffer and labeled with 2 µL anti-CD137-biotin per 1×10^6 cells in a total volume of 10 µL per 1×10^6 cells (filled up with MACS-buffer) or 1 µL anti-CD137-PE per 1×10^6 cells in a total volume of 5 µL per 1×10^6 cells, respectively. The cells were incubated for 10 min at the respective temperature and then washed with MACS-buffer. Subsequently the cells were labeled with 2 µL anti-biotin beads per 1×10^6 cells in a total volume of 10 µL per 1×10^6 cells (filled up with MACS-buffer) for 15 min. After washing up to 100×10^6 cells were resuspended in 0.5 mL MACS-buffer.

Isolation. The labeled cells were passed through a MS-column (max. 1×10^7 labeled cells, max. 2×10^8 total cells) with a preseparation filter (30 µm mesh). The tube, the filter and the column were rinsed with MACS-buffer one after another to minimize the cell loss. The column was flushed out with 1 mL medium A, so CD137-positive cells in the column could be used for culture without another centrifugation step.

Further Steps. To check the purity in flow cytometry, the biotin-isolated cells were stained with anti-biotin-APC Ab or goat-anti-mouse (GAM) and further Ab (e.g. CD8). The PE-isolated cells were only stained for other markers than CD137 as CD137 had already been labeled during the isolation procedure with PE-Ab. The CD137-positive and -negative fraction and an unseparated control group were plated in medium A with cytokines as described in the T cell culture section ususally at a cell concentration of 0.5×10^6 /mL.

In some experiments irradiated leukaemia blasts (irradiated simulatanously to the blasts used for restimulation, referred to as AML d2) were added in a ratio of 1:1 to improve the comparability with the unseparated fraction. The rationale was that remaining leukaemia blasts should be in the CD137-negative fraction after isolation and that these could have a stimulatory effect of the CD137-negative cells masking the advantage of the positive fraction. Furthermore the effect of adding feeder cells after the isolation procedure was investigated. Irradiated PBMC were added in a ratio of 1:9 (T cells : feeder cells) with a T cell concentration of 2.5×10^5 /mL.

3.2.3.3 CD137 Enrichment using Dynal Kits

The Dynal cell isolation methods were tube-based and the size of the beads used was in the order of T cells. Both isolation methods required to know the percentage of CD137-positive cells in the culture, which was determined in flow cytometry prior to the enrichment procedure. The procedure prior and after the enrichment was identical to the Miltenyi-methods. All steps were performed in MACS-buffer, unless otherwise stated.

Indirect Method. The method with Dynal materials referred to as "indirect" used DSB-X^{TM} biotinylated CD137-Ab and beads coated with a nitrated streptavidin, which permitted the possibility to release the beads after the isolation procedure and to analyze the cells in flow cytometry.

The T cells were incubated with biotinylated CD137-Ab (final concentration 1 µg per 1×10^6 expected CD137-positive cells) 20 min at 4 °C. The streptavidin-beads were washed as described in the bead-stimulation section but finally resuspended in the initial volume of buffer. Subsequently, the T cells were washed, resuspended in 20 µL buffer per 1×10^6 cells

and 75 µL of washed beads were added. During the next incubation at RT, cells and beads were kept in suspension by an apparature that provided simultanous rotation and tilting as demanded in the manufacturer's protocol. Then 0.5 mL buffer were added, the tube was placed in a magnetic rack and the non-bead-bound cells (CD137-negative) were pipetted into another tube. The tube was taken out of the magnetic rack, the remaining, bead-bound cells were resuspended in 1 mL buffer and the tube was placed in the magnetic rack again. Non-bead-bound cells were discarded and the CD137-positive fraction remained in the tube. After the tube had been removed from the magnetic rack, the cells were resuspended in 1 mL "Release Buffer" and incubated at RT on the rotation-tilting machine for 20 min. The suspension was then vigorously pipetted up and down ten times and the tube was put in the magnetic rack. The released cells were transferred into another tube, which again was put in the magnetic rack. The final CD137-positive cell fraction was taken out of this tube, centrifuged and resuspended in medium A.

Direct Method. The "direct" method used direclty Ab-coated beads (two variants with lower and higher Ab density), that remained in the cell culture after the isolation. Due to the large bead size, the cells of the CD137-positive fraction could not be analyzed in flow cytometry.

The manufacturer recommended to use 4 beads per CD137-positive cell. The required amount of beads was calculated (stock: 40×10^6 beads/mL) and washed as described for the indirect method. The T cells were resuspened in 10 µL per 1×10^6 cells and the calculated amount of beads were added. After 20 min incubation on the rotation-tilting machine at RT, 0.5 mL of buffer were added, the suspension was gently mixed and the tube was put into the magnetic rack. The non-bead-bound CD137-negative fraction was pipetted into another tube and the remaining CD137-positive fraction was resuspended in medium A.

After five days the beads were removed from the cell culture by resuspending cells and beads and tranferring them into a tube. The tube was put into the magnetic rack and the cells were pipetted out, which should be possible due to the downregulated CD137-expression at that time.

3.2.4 Bead-Stimulation

Antibody-coated beads can be used for polyclonal T cell stimulation as they may provide a T cell receptor signal via anti-CD3 and B7-costimulation via anti-CD28 [139]. Furthermore new beads, that feature an additional, stimulatory anti-CD137 Ab, were compared to the standard beads [116]. For stimulation the T cells were coincubated with beads at a defined

bead:cell ratio. The beads were clearly visible under the microscope and could be counted in a counting chamber like cells.

3.2.4.1 Washing Beads

The desired amount of beads (with pipetting excess) was pipetted into 3 mL of Dynal buffer in a 15 mL tube and mixed. The tube was placed in a magnetic rack and the beads gathered as a brown spot on the wall of the tube. The buffer was removed without touching the beads and the tube was taken out of the rack. Finally, the beads were resuspended in medium A at the concentration required for the stimulation and added to the cell culture.

3.2.4.2 Stimulation of PBMC

Thawed PBMC were plated at a concentration of 1×10^6 /mL in AIM-V supplied with 5% human serum and 100 IU/mL IL-2 (final concentration). Beads were added in a ratio of 1:1 (beads : T cells). CD137 expression was assessed for PBMC stimulated with both kinds of beads and IL-2 alone after 0, 6, 12, 24, 48 and 72 h.

3.2.4.3 Stimulation of T Cell Lines/Clones

T cells were plated at a concentration of 5×10^5 /mL in medium A supplemented with 100 IU/mL IL-2 (final concentration). Irradiated stimulator cells were added in a ratio of 1:10 (stimulator cells : T cells) for RCC and 1:1 for AML. Both types of beads were usually used in a ratio of 1:5 (beads : T cells). In some experiments lower bead concentrations were prepared in dilution series and compared to the standard ratio. Bead-stimulation was always compared to stimulator cells (positive control) and IL-2 alone (negative control). In some experiments also simultanous stimulation with beads and stimulator cells was investigated.

To keep the cytokine concentration as constant as possible, medium A supplemented with 100 IU/mL IL-2 was used to refill evaporated medium and to fill up splitted wells. The cell-stimulated T cells were restimulated weekly similarly to the previous restimulations.

3.2.5 Flow Cytometry

3.2.5.1 General Aspects

Flow cytometry (alternative name FACS^{TM}, trademark of BD Biosciences) facilitates analysis of fluorescent-dye-labeled molecular structures for high cell numbers (up to the order of 10^7). Most frequently surface markes are stained with specific, directly fluorochrome-conjugated Ab.

For the measurement suspended cells are brought from the specimen tube into a sheath fluid by positive pressure and are thereby singularized. In the measuring chamber ("flow cell") the cells arrive as single cells where they encounter laser beams of different wave lenghts. The fluorescent dyes bound to the cells can be excited by laserlight within their excitation spectrum and send out light of their emission spectrum. The emmitted light is separated into multiple frequency bands ("channels") by interference filters and the light intensity in the different channels is measured by photomultipliers.

In adequately equiped devices ("cell sorter") the specimen stream can be fractionized after signal processing into electrostatically charged fluid droplets, which are subsequently deflected in an electric field and collected in different tubes. This provides to separate cells by fluorescence intensity and with it by expression-strength of defined surface markers into subpopulations. Furthermore, it is possible to sort cells by multiple surface markers simultanously.

3.2.5.2 FACS-Stainings

Standard FACS-Staining. Per FACS-tube 10^5 cells in 1 mL FACS-buffer were used. (If the volume of cell culture medium was < 200 µL per tube, the medium was diluted ad 1 mL with FACS-buffer without prior centrifugation.) The tubes were centrifuged, decanted and the cells were resuspended. The optimal volume of Ab (determined by titration, if not available manufacturer's recommendations) was added to the remaining rest of buffer in the tubes. After a 15 min incubation (4 °C, dark) the cells were washed with 1 mL FACS-buffer per tube. Subsequently the cells were resuspended in 200 µL FACS-fixative and stored in the fridge (4 °C, dark) until measurement within seven days.

Non-directly fluorochrome-conjugated Ab. Biotinylated Ab bound to a surface structure can be stained with a secondary, fluorochrome-conjugated anti-biotin Ab. Another possibility is a secondary, fluorochrome-conjugated Ab directed against the F_C of a particular species (e. g. GAM).

FACS-Staining Using Tetramers. MHC multimers provide direct identification of antigen-specific (peptide and HLA molecule) CD8 T cells by linking the TCR with a fluorochrome. Tetramers consist of four biotinylated and peptide-loaded MHC class I molecules that are subsequently bound by a fluorochrome-coupled streptavidin molecule in a tetrahedral fashion. In pentamers five MHC molecules are multimerized by a self-assembling coiled coil domain providing a very high avidity of the complex [140].

According to the manufacturer's recommendations the tetramer was added together with the other Ab (if no decreased binding together with the Ab-clones described). The incubation was adjusted to 30 min at RT.

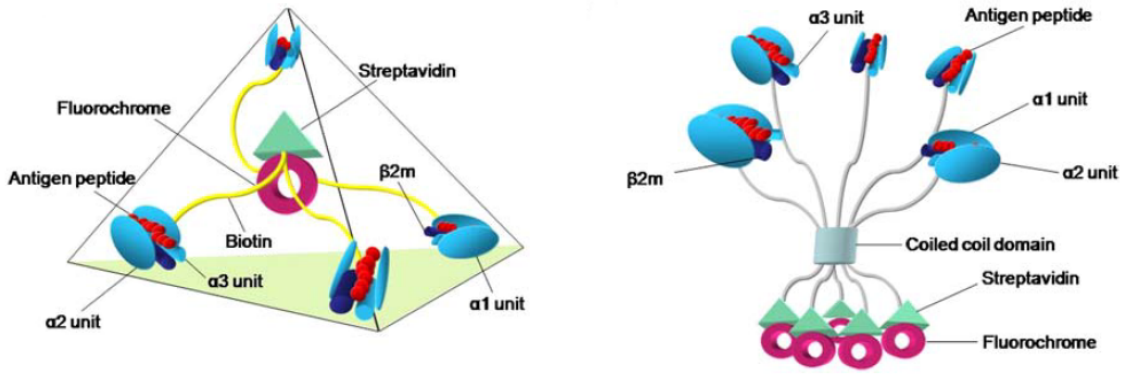


Figure 3.1: The molecular structure of MHC multimers. Left: tetramer complex, right: pentamer complex. Modified from [140], reprint permission obtained.

CFSE-Staining. The fluorescent dye CFSE permeates the cell membrane, is bound to intracellular proteins and is therefore retained intracellularly. It can be used to distinguish cell populations of similar phenotype in mixed cell cultures or transferred cells in animals. As the dye is distributed equally between daughter cells upon cell division, it can be used to determine the number of cell divisions [141].

CFSE lyophylisate was stored at −20 °C and a stock solution was prepared by dissolving the lyophylisate in DMSO directly prior to use. 2 µL of 5 mmol CFSE stock solution per mL of 37 °C FACS buffer were added to a final concentration of 10 µmol. Lower concentrations of CFSE were also assessed in some experiments. For the staining procedure the cells were resuspended at a concentration of 1×10^6 /mL in FACS buffer supplemented with CFSE and put in the incubator for 15 min. Subsequently the cells were centrifuged and resuspended in 37 °C medium A. After 30 min in the incubator the cells were centrifuged again and plated.

Cell Viability. In addition to a standard FACS-staining, the cells were also stained with 7-AAD and/or annexin V. This can yield detailed information on the cell viability.

Like the primary substance actinomycin D, 7-AAD binds deoxyribonucleic acid (DNA) and hence can be used to label cells that have lost their membrane integrity due to cell death [142]. By introduction of the amino group, the molecule becomes a fluorescent dye, whose fluorescence increases substantially upon DNA-binding [143]. The calcium-dependent protein annexin V binds phosphatidylserine, a phospholipid that appears on the cell surface during early stage apoptosis [144]. A 7-AAD positive control was generated by incubating the cells in FACS-buffer with 30% DMSO for 15 min.

At first a standard FACS-staining with incubation at RT was performed until the second washing step. Instead of FACS-fixative, 100 µL of a calcium containing buffer

("annexin-binding-buffer") were used to resuspend the cells. Prior to measuring 5 µL 7-AAD and/or annexin V per tube were added, followed by 15 min incubation (RT, dark). Afterwards 100 µL of annexin-binding-buffer were added and the specimen was measured instantly. To ensure comparability with non-vital-dye-containing specimens, these were not fixed and measured immediately, too.

3.2.5.3 Measurement and Evaluation.

Every measurement included unstained and/or cells stained with a fluorochrome-conjugated Ab against an irrelevant Ag of another species ("isotype") as a negative control. If there was overlapping of emmission spectra within a relevent frequency band ("channel"), electronic compensation was used. Therefore single-color stainings of a strong Ag expressed by a high percentage of cells were prepared, usually CD3 for PBMC and CD8 for isolated CD8 T cells. If possible, the automatic compensation of the FACS Diva software was used, but often in 5-fold and vital stainings manual compensation was necessary. The command variable in manual compensation was the median of the frequency distribution of the fluorescence intensities measured. In both compensation methods cell debris was excluded in the forwardscatter/sidescatter (FSC/SSC)-plot (see below).

All data were aquired on a FACS Canto II device run with FACS Diva software. Usually the Expo 32 software from Beckman Coulter was used for evaluation of measured data. At first for the evaluation the events forming a dot-cloud with the light-scatter properties of the desired cell type (usually the "lymphocyte-cloud") were selected in the FSC/SSC-plot. If there were clearly discrete populations, the gates to descriminate different cell populations by means of fluorescence intensity were chosen that way that discrete cell populations were optimally separated (cut-off on local minimum of the histogram). If there were no obvious distinct populations, the cut-off for "positive" events was chosen that way that $\geq$98% of the events of the negative control were in the "negative" range.

3.2.5.4 Cell Sorting

As in a regular FACS-staining, an unstained control and single-stained controls were prepared. Instead of FACS-buffer, sort-buffer was used for all steps. The cells for sorting were stained in a 50 mL-tube using about 50 to 60 µL of Ab per 1×10^8 cells at RT. After staining the cells for sorting were washed with 30 mL sort-buffer and passed through prewetted nylon-mesh. Finally, the concentration was set to $1-2\times10^7$ /mL in a 15 mL-tube. Sorting was performed on a FACS Aria sorter at RT and the sorted cells were collected in sterile FACS-tubes containing 0.5 mL medium A. After sorting the cells were recounted manually and plated again in medium A.

3.2.6 Functional Testing

3.2.6.1 Interferon-γ-ELISPOT

A modified enzyme linked immunosorbent assay (ELISA), the enzyme-linked immunosorbent spot (ELISPOT) provides quantification of cyokine secreting cells in a cell culture. First a primary Ab against a certain cytokine is fixed on a hydrophobic polyvinylidene fluoride membrane. Then cytokine secreting cells are plated together with stimulator cells and incubated. The secreted cytokine-molecules are captured by the primary antibody. After the cells have been washed off, a biotinylated, secondary Ab direced against another epitope of the cytokine-molecule is added. Then a streptavidin-enzyme complex binds to the biotinylated antibodys. Finally an uncoloured substrate is converted into a dye by the enzyme and forms coloured spots on the membrane.

Prewetting, Coating. The membranes were pretreated with 20 µL 35% ethanol for a few seconds, then washed three times with 150 µL PBS and kept moist throughout the whole procedure. Then, 60 µL per well of the primary antibody against IFN-γ in a concentration of 10 µg/mL in PBS were plated and the plate was stored for 24 h to 7 days at 4 °C.

Blocking, Plating. Prior to plating the plates were washed three times with 150 µL PBS and unspecific protein binding was blocked with 100 µL medium A per well (incubation min. 1 h in the incubator). Effector and target cells were counted and set to the desired concentration. Usually 2,000 to 20,000 effector cells and 50,000 target cells (100,000 for some leukaemias) were plated in a final volume of 100 µL medium A per well. For positive controls PHA was added to T cells in a final concentration of 1 µg/mL. The plates were incubated for 20 h in the incubator.

Detection. After incubation the plates were washed six times with PBS + 0.05% Tween and 60 µL of biotinylated, secondary Ab were plated in a concentration of 2 µg/mL. Then the plate was put in the incubator for at least 2 h. 30 min prior to use, a avidin-biotinylated-peroxidase-complex (ABC) (Vectastain kit) was prepared in PBS + 0.1 % Tween. After the incubation period the plate was again washed six times with PBS + 0.05% Tween and 100 µL of ABC were plated followed by 1 h incubation at RT. Subsequently the plates were washed three times with PBS + 0.05% Tween and three times with PBS. 10 mL 3-amino-9-ethylcarbazole-sulution (substrate) were mixed with 5 µL H_2O_2 (catalyst) and 100 µL of the mixture per well were plated. When spots were clearly visible (usually 7 to 10 min), the plate was rinsed ten times with tap water and dried in the dark.

Evaluation. The dry membranes were stripped from the plates with an adhesive foil and photographed on a reflected-light microscope. For picture analysis and automated spot counting, the Zeiss KS Elispot Software was used [145].

3.2.6.2 ^{51}Chromium-Release-Assay

The chromium isotope ^{51}Cr is a gamma emitter with a half-life of 27.7 days and can be used in the soluble form of $NaCrO_4$. The assay starts with an incubation of the target cells with $NaCrO_4$, in which chromium is taken up and stored inside the cells. Afterwards the chromium-labeled target cells are incubated with CTL. If the target cells are lysed, the chromium is released and distributes in the medium due to diffusion. Finally the activity of the supernatant is measured in a gamma counter. All steps including radioactive chromium were performed under radiation protection precautions.

Labeling. 10^6 target cells (5×10^5 cells when targets labeled poorly) were incubated for 90 min with an amount of ^{51}Cr equivalent to 100 µCi in 50 µL FCS in the incubator. The cells were resuspended every 30 min. After washing the cells four times with medium B, they were counted in medium A and set to a concentration of 1000 cells per 80 µL (1500 cells when targets labeled poorly).

Plating. Effector cells were counted and plated in 80 µL medium A per well in a 96well V-bottom plate. A dilution series with five 1:3-dilution-steps of each effector cell was prepared. With the equal number of target cells added (usually 1000/well, 1500/well when targets labeled poorly), the usual range of effector/target ratios was 90:1 ... 1:1, occationally 60:1 ... 0.74:1 or 30:1 ... 0.3:1. Each combination of effector cell, target cell and effector/target ratio (E/T RATIO) was prepared twice.

Six wells of target cells without effector cells were plated to determine the spontanous relaease of radiochromium (min control). For maximum lysis six wells of target cells with 1% Triton X 100 were prepared (max control).

Incubation, Harvesting, Measuring. The 96w plates were put in the incubator for usually 5 h. Afterwards the plates were centrifuged at 900rpm (282 g) for 5 min without brake. 80 µL of supernatant were transfered into cytotox-tubes without touching the cell pellet. The activity in counts per minute for each tube was measured in a gamma counter. The specific lysis for a preparation x was calculated as follows (A is the activity of a particular specimen):

$$\textit{specific lysis}(x) = \frac{A(x) - A(min)}{A(max) - A(min)}$$

3.2.6.3 Interferon-γ-Secretion-Assay

In cytokine secretion assays an affinity matrix for the particular cytokine is set up around the cytokine-secreting cells of interest. When the cytokine is released from the cells, it gets caught in the affinity matrix and can be labeled with a fluorochrome-conjugated Ab. Afterwards the cells can be detected in flow cytometry or isolated with magentic anti-fluorochrome beads [146].

In this project the IFN-γ secretion assay was compared to the CD137 isolation methods as an established control method. Therefore, both procedures were performed in the period of 10 to 18 hours after restimulation, even though the IFN-γ secetion assay can be done earlier.

The technical procedure was carried out as recommended by the manufacturer. In particular several steps had to be performed unter strictly cool conditions to prevent IFN-γ secretion at the wrong time and cross-contamination in between the T cells, which required centrifuagion steps at 4 °C in a cooled centrifuge. The T cells were washed out after restimulation with AML blasts and the affiniy matrix was set up by incubating the cells 5 min on ice with IFN-γ catch reagent (dimer of anti-CD45 and anti-IFN-γ).

Afterwards, the T cells were warmed up by adding 10 mL per 10^6 cells of 37 °C medium A. During this 45 min IFN-γ secretion period in the incubtor, the cells were resuspended every 5 minutes to avoid cross-contamination. At the end of the incubation the cells were cooled down rapidly by putting the tubes on ice and adding 4 °C MACS-buffer. All following steps were performed at 4 °C or on ice. After centrifugation the secreted IFN-γ in the affinity matrix was labeled with anti-IFN-γ-PE Ab.

If the cells should be separated, after another centrifugation step anti-PE beads were added for 15 min. After washing the cells were passed through a positive-selection column and the non-labeled cells were regarded as IFN-γ negative. The labeled cells were flushed out and passed through a second positive-selection column. The non-retained cells were discarded and the eluate of the second column was regarded as IFN-γ positive. The purity was assessed in flow cytometry, usually with a CD8/IFN-γ/CD137 co-staining.

4 Results

4.1 Stimulation and Expansion of RCC and Leukaemia-Reactive T Cells Using CD3/CD28/CD137 Ab Coated Beads

Aiming at the production of leukaemia-reactive T cell products for clinical use compliant with GMP requirements, the possibility to expand specific T cells by Ag-independent bead-stimulation was investigated. Due to the results published in the literature as described in the "T cell expansion" section, various Dynabeads^{TM} products for T cell stimulation and expansion were compared to the established Ag-dependent stimulation with neoplastic cells. Previous results were achieved by D. Teschner (published in [116]) using a well-known robust *in vitro* model for HLA-matched tumour-reactive CTL. From two different healty unrelated donors (named BC I and BC V) tumour-reactive CTL were generated in a mixed lymphocyte tumor culture against the RCC1257 cell line (see "Methods" section). After several rounds of weekly stimulation with RCC the CTL were restimulated either fully Ag-independently with magnetic beads or simultanously with beads and RCC as described above. At a bead:cell ratio of 1:5 the stimulation with CD3/CD28/CD137 beads were equally efficient as RCC cells resulting in growth factors of approx. 12 per 14 days while CD3/CD28 beads were significantly ($p=0.03$) less efficient. Functional testing in an ELISPOT assay showed equivalent IFN-γ release for all stimulation methods. To investigate the Ag-specific functionality further testing in chromium release assays was performed.

4.1.1 Bead-Stimulation of RCC-Specific CTL at Different Points in Time of Long-Term Culture

In five independent experiments two RCC-reactive CTL-lines were stimulated for two weeks with either RCC, CD3/CD28 or CD3/CD28/CD137 beads in various bead:cell ratios (beads:cells 1:5, 1:50, furthermore 1:10, 1:100 in one experiment). Cell counting was performed on days 3, 7, 10 and 14 of culture; in RCC stimulated conditions the CTL were restimulated on day 7; CTL in bead-stimulated conditions were restimulated only once within 14 day according to a previously established protocol. When thawed the CTL line BC I was at day 21 of long-term culture, BC V was at day 21+4 or day

56 respectively. The rationale for trying lower bead concentrations than in the previous experiments were interim results from AML-reactive CTL showing adverse effects on proliferation for higher concentrations of beads. In contrast for BC I better proliferation was observed in all experiments (3 of 3) for the highest concentration of beads compared to lower concentrations. As demonstrated before by D. Teschner the CD3/CD28/CD137 beads (1:5) were approximately equally efficient as RCC-stimulation while the CD3/CD28 beads led to less proliferation (figure 4.1 A). The more aged CTL BC V (day 56) only showed moderate proliferation upon RCC-stimulation, but no expansion could be achieved with any kind of bead-stimulation in 4 of 4 experiments. Representative data are shown in figure 4.1 B. For a less aged aliquot of BC V (day 21+4) a moderate expansion (growth factor 4 in two weeks) could be achieved with both kinds of beads in a 1:5 ratio (growth factor for RCC-stimulation : 5, IL-2 only : 3).

4.1.2 Functional Testing of Bead-Expanded RCC-Specific CTL

Three independent experiments with functional testing in a chromium release assay were performed. In terms of cytotoxic effector function for BC I marked differences between RCC stimulated CTL (65 - 80% maximal specific lysis (SL)) and the control group (IL-2 only, 20 - 30% maximal SL) were found thoughout all experiments. The SL of any bead-stimulated CTL ranged inbetween the curves of the two reference CTL. However the values for SL were without exception more than one standard deviation below the values obtained with RCC-stimulation for all effector:target ratios. Figure 4.1 C shows a representative experiment. An advantage of the CD3/CD28/CD137 beads found in another experiment could not be reproduced. The better SL of cells stimulated with higher bead-concentrations compared to lower concentrations was found in 2 of 3 experiments. The experiments using BC V can not be regarded as undoubtedly evaluable as only moderate to poor proliferation was seen in the RCC control group and no efficient bead-stimulation was possible. Nevertheless a difference of more than one standard deviations was seen between RCC and no specific stimulation in all experiments, even despite high SL values for the IL-2 only-CTL (up to 55% maximal SL). As observed for BC I the specific lysis of the bead-stimulated CTL was inbetween the two reference CTL with a tendency of better specific lysis for CD3/CD28 stimulated CTL in all experiments as seen in figure 4.1 D.

4.1.3 Bead-Stimulation of AML-Specific CTL Clones

Leukaemia-reactive cell clones were generated as described in [11, 12] with or without IL-21 and restimulated for 14 days with CD3/CD28 or CD3/CD28/CD137 beads, respectively. AML restimulation and medium with cytokines only served as positive

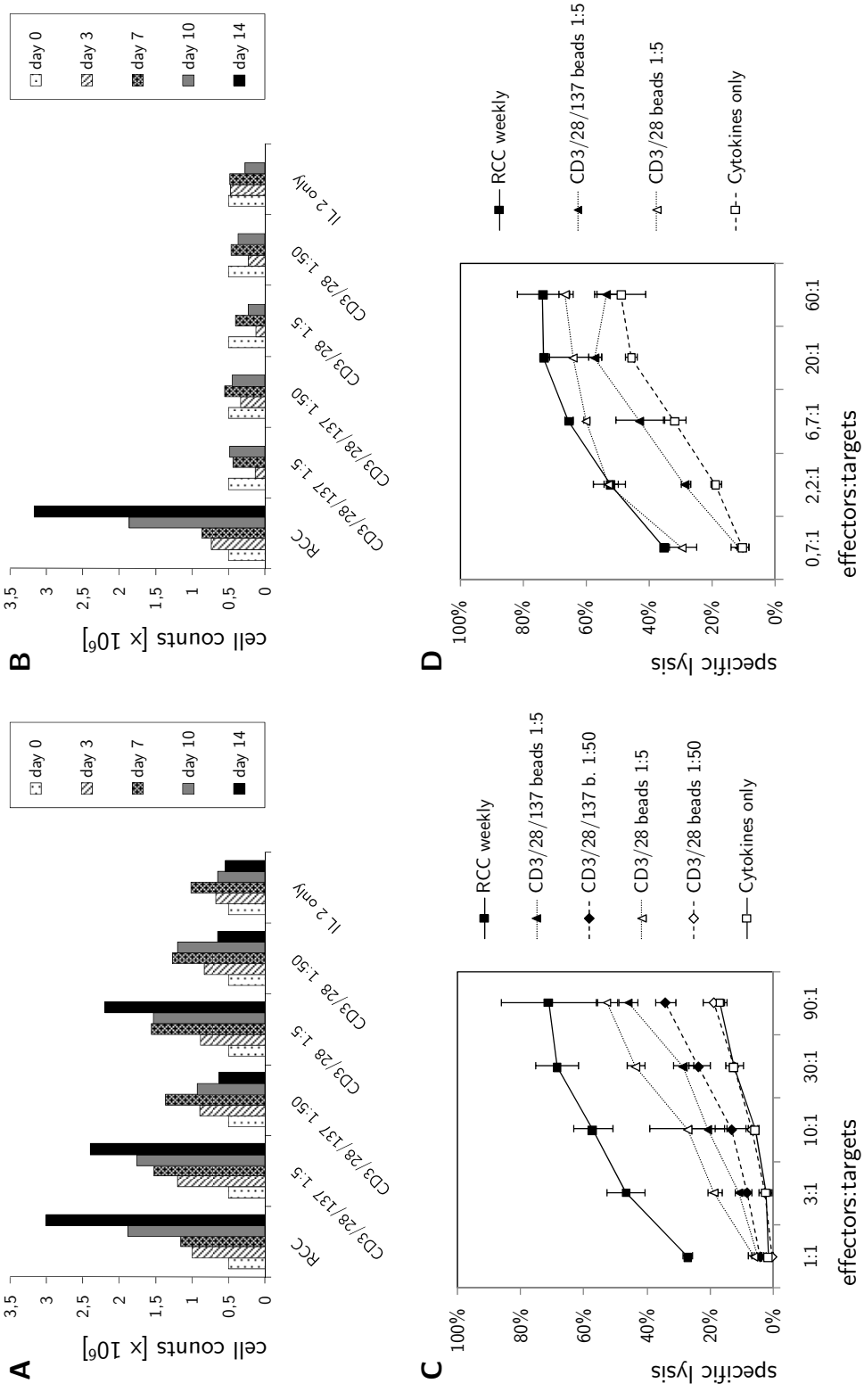


Figure 4.1: Bead-stimulation of RCC-reactive CTL lines (see "Methods" section) and subsequent functional testing in a chromium release assay on day 12 after bead-stimulation. A: A less aged aliquot of BC I (day 21) could be equally expanded by RCC and bead-stimulation. B: In contrast an aged aliquot of BC V (day 56) could only be expanded by RCC-stimulation, but not by using beads. C: The corresponding functional test for the BC I stimulation in panel A shows a reduced cytotoxicity for bead-stimulated CTL. The specific ranking of the bead-stimulated conditions could not be reproduced. D: The corresponding chromium relaese assay for the BC V stimulation in panel B shows a less impaired functionality compared to panel C. For clarity purposes the 1:50 bead-stimulation conditions are not shown here. Note the different effector:target ratios in the panels C and D. Due to the lacking CTL expansion less T cells were available for BC V.

and negative controls. In six independent experiments CTL clones from three different patient-donor-combinations were used.

1. Clone 5H11: MZ580-AML/donor 931, d56 of long-term culture, slowly proliferating (growth factor 3 to 4 per 14 days) described in [12]

2. Clone 1C6: MZ201-AML/donor 332, d63 of long-term culture, restimulated with MZ 418-AML, moderately proliferating (growth factor approx. 38 per 14 days), described in [11]

3. Clone 8F11: MZ653-AML/donor 069, d42 of long-term culture, very rapidly proliferating (growth factor approx. 800 to 1140 per 14 days), described in [12]

For the 5H11 clone beads were used in a 1:5 bead:cell ratio with 250 IU/mL IL-2 and 5 ng/mL IL-7 and IL-15 as described in the "mixed lymphocyte leukaemia culture (MLLC)" section. No T cell expansion could be achieved with both kinds of beads. During the first 3 days the CD3/CD28/CD137 beads caused a drop in cell number with small and wrinkled morphology of the T cells. Subsequently a recovery to the initial cell number within 14 days was found. Exposure to CD3/CD28 beads led to a rapid decline in cell numbers, i.e. over 10-fold within 7 days and to zero within 14 days, even so when AML blasts were added to the culture in the concentration of the positive control. Remarkably this drop in cell number was considerably faster than in the negative control which showed a growth factor of 0.5 within 14 days. Figure 4.2 A shows an example.

Subsequently several titration steps for both types of beads were tested for the 1C6 clone starting at 1:5 to 1:200 beads:cells. The medium was supplemented with 100 IU/mL IL-2. Only for CD3/CD28/CD137 beads at the lowest bead concentration a very minor expansion of a growth factor of 3 in 14 days could be observed (equivalent to the negative control, positive control: growth factor 38 within 14 days). All other bead-exposed CTL ranged below the negative control as seen in figure 4.2 B. Furthermore prolonged observation for one more week (21 days in total) showed no major change in the trends.

The very rapidly proliferating clone 8F11 was tested in 3 independent experiments with different cytokine combinations and concentrations as well as with bead ratios of 1:5 to 1:100 beads:cells. The combination of 250 IU/mL IL-2 and 5 ng/mL IL-7, IL-15 led to considerable T cell expansion in the negative control with a growth factor of 120 in 14 days. 250 IU/mL IL-2 alone decreased the growth factor of the negative control to 36 per 14 days and further reduction to 100 IU/mL IL-2 alone led to growth factor of 4.5 per 14 days. The growth factor of the positive control was very high at 360 to 1140 per 14 days (extrapolation, approx. 10 divisions per cell in 14 days). In one experiment moderate expansion, however inadequate for this CTL, could be found for low bead concentrations:

CD3/CD28/CD137 beads at 1:50 and 1:100 and CD3/CD28 beads at 1:100 led to expansions within 14 days that were 17- to 20-fold lower than the positive control. All other higher bead concentrations in this experiment led to no expansion, remarkably the decline in cell number was faster for higher bead concentrations. As observed for 5H11 CD3/CD28 beads at 1:5 and 1:10 caused a drop of the cell number to zero within 14 days.

Ag-presentation in form of regular AML blast-stimulation (1:1 AML:CTL) in addition to bead-stimulation (1:5 beads:CTL) could reverse the adverse effects of the beads on the proliferation of the 8F11 clone. For CD3/CD28/CD137 beads the proliferation was equivalent to the positive control and only slight inhibition of proliferation was observed for CD3/CD28 beads. Figure 4.2 C shows the respective experiment. The pan-caspase inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) has been reported to inhibit AICD and apoptosis in tumour-reactive T cells efficiently [147, 148]. The 8F11 clone was used to test whether the inhibitory effects of the bead-stimulation could be reversed by caspase blocking. The caspase-inhibitor Z-VAD-FMK was used in a final concentration of 5 µmol/L according to the manufacturers recommendations. Adding Z-VAD-FMK to the positive and negative controls showed a slight, but constant inhibition of proliferation by pan-caspase blocking. Concerning the bead-stimulation adding Z-VAD-FMK did not lead to any expansion of 1:5 bead-stimulated CTL over 14 days. See also figure 4.2 D.

4.1.4 CD137L on Leukaemia Blasts

As some AML blasts are known to express CD137 [77], staining for CD137L was included in phenotypical analysis of blasts from two patients to confirm these findings for our leukaemia bank. The MZ561-AML and MZ653-AML blasts showed over 90% CD137L expression and less than 6% CD137 expression on day 1 after thawing (data not shown).

4.2 Selection of Leukaemia-Reactive T Cells from Bulk Cultures by CD137 Isolation

4.2.1 Expression Kinetics of CD137 upon AML-Stimulation

The following criteria should facilitate an optimal isolation of activated CD137 positive T cells:

1. Isolation at the temporal maximum of the percentage of CD137 positive cells. The separation procedure should start as soon as the maximum is reached to achieve an optimal cell yield. The isolation procedure and the subsequent quality control

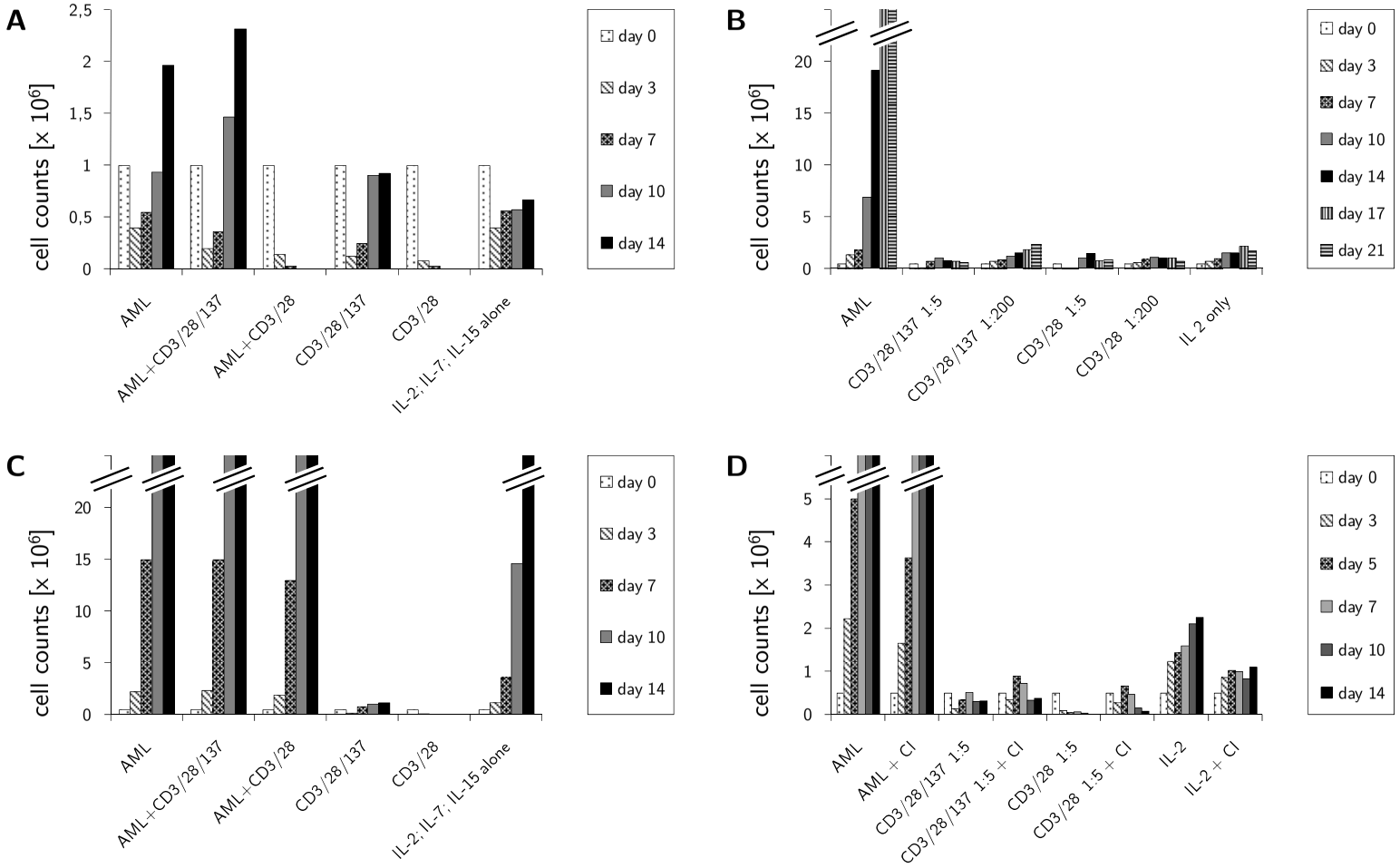


Figure 4.2: Bead-stimulation of AML-reactive CTL clones as described in the "Methods" section. Note the different y-axis scalings and the truncated y-axis in the panels B, C and D. A: clone 5H11, experiment V29, beads:cells 1:5. The slightly higher growth factor of the CTL stimulated with AML and CD3/CD28/CD137 beads could not be reproduced. B: clone 1C6, experiment V35. The bead:cell ratios 1:50 and 1:100 are not shown, as the results as the cell counts were almost equal to those obtained for 1:5. C: clone 8F11, experiment V32, beads:cells 1:5, note the considerable growth of the negative control with the triple cytokine combination. D: clone 8F11, experiment V40. All conditions were tested with and without pan-caspase inhibitor (CI).

takes several hours and should be completed before the decrease of the percentage of CD137 positive cells starts.

2. Also the CD137 expression level per cell – measured as the median fluorescence intensity (MFI) – should be high enough to provide a clear distintinction between a positive and a negative fraction.

3. A local minimum in the CD137 expression level should clearly separate a positive and a negative fraction. This does not only demand the previous requirement, but also a synchronized and uniform upregulation of CD137 in all activated T cells.

4.2.1.1 HLA-Matched and Single-Allele-Mismatched Systems

As different data on the expression kinetics of CD137 in human T cells have been published, the kinetics in the MLLC system used for the following work were determined first to meet the criteria stated above as good as possible. Five different leukaemia/donor systems were used (see also table 3.2): MZ653-AML/donor 069 (HLA matched), MZ529-AML/donor 430 (one HLA I C allele mismatched), MZ529-AML/donor 735, MZ574-AML/donor 274 (both one HLA I C sub-allele mismatch in genomic HLA typing) and MZ369-AML/sibling-donor 167 (matched sibling). Cytokines were used as described in the MLLC section (IL-21 was used in one HLA-matched setting). Data on CD137 expression were obtained before and after restimulation on days 7 and 14.

In all experiments the criteria stated above were met on day 14 of culture: Before restimulation no CD137 background expression was detected (less than 3% CD137 positive cells, sensitively gated, MFI of CD137 positive cells considerably lower compared to "real" CD137 positive cells after restimulation). The maximal percentage of CD137 positive cells together with maximal MFI of the CD137 positive cells was reached approximately 12 h after restimulation and stayed constant until approx. 18 h after restimulation. Afterwards the MFI of the CD137 positive cells decreased until the CD137 positive fraction was not clearly separable from the negative fraction at approx. 24 h after restimulation, but CD137 positive cells were still clearly detectable at 48 h. Figure 4.3 A. shows a representative example.

When whole CD8 T cells were used as starting population (MZ529-AML/Donor 430, MZ529-AML/Donor 735, MZ574-AML/Donor 274, MZ369-AML/Sibling-Donor 167), a marked CD137 background expression before restimulation was found on day 7, mostly not in terms of a distinct CD137 positive fraction but a shift into the positive gate of 10 to 20% of the T cells. This was never observed directly after thawing on day 0 or on day 14. Upon restimulation more T cells shifted into the positive gate, but no clearly separated positive fraction was seen. A representative experiment is shown in figure 4.3 B.

In two experiments with naive CD8 T cells as starting population (MZ653-AML/Donor 069, MZ369-AML/Sibling-Donor 167) on day 7 CD137 kinetics comparable to those on day 14 were found.

4.2.1.2 HLA-Mismatched Systems

As it was reported that the detection of naive T cells *ex vivo* from PBMC or isolated T cells by CD137 staining would be feasible [99], additional kinetics data were obtained for day 0 and day 7. In the culture medium no cytokines or 2 ng/mL IL-7 only were used to avoid Ag-independent reactivation of memory T cells. To obtain a clearly detectable response either PBMC or isolated CD8 T cells were stimulated with AML blasts mismatched in most of the HLA alleles. MZ911-AML and MZ964-AML were used as stimulator cells with HLA typed buffy coats from three healthy donors without cytokines. Within 72 h no CD137 expressing CD8 T cells could be detected (experiment A9, data not shown).

In a next step isolated CD8 T cells from two of the donors were stimulated with MZ911-AML either without cytokines or in medium with 2 ng/mL IL-7 and restimulated on day 7 under the same conditions. One condition without cytokines did not show any reaction. In the other three conditions similar reaction patterns were found which will be described in the following. No or little CD137 expression could be detected within the first 72 h. Afterwards a slow but constantly increasing percentage CD137 expressing CD8 T cells was found. The MFI of the CD137 positive cells was only slightly higher than the negative fraction, however the positive fraction reached a maximum of 4 to 8% at day 6 resulting in background expression of CD137 on day 7 before restimulation. Clear-cut CD137 expression (distinct population, high MFI) followed the restimulation on day 7. However the kinetics were more variable compared to matched systems with peaks of CD137 expression at 12 h to 48 h. Figure 4.4 shows a representative example.

4.2.2 CD137 Isolation Methods

One or several parallel CD137 enrichment steps were performed in 10 independent experiments including HLA-matched bulk cultures (MZ653-AML/donor 069, MZ369-AML/sibling donor 167) and CTL (5E10 from MZ653-AML/donor 069), single-/suballele-mismatched bulk cultures (MZ574-AML/donor 247, MZ529-AML/donor 430), complete HLA-mismatched bulk cultures(MZ911-AML/BC 0911834) and peptide-stimulated CD8 T cells. Several parameters were used to quantify the effectivity of the CD137 isolation procedure in terms of enriching CD137 positive cells, depleting the CD137 negative fraction and keeping the cell loss during the isolation procedure low. The IFN-γ secretion assay was used as an established standard procedure for isolating multi-antigen-specific T cells. The

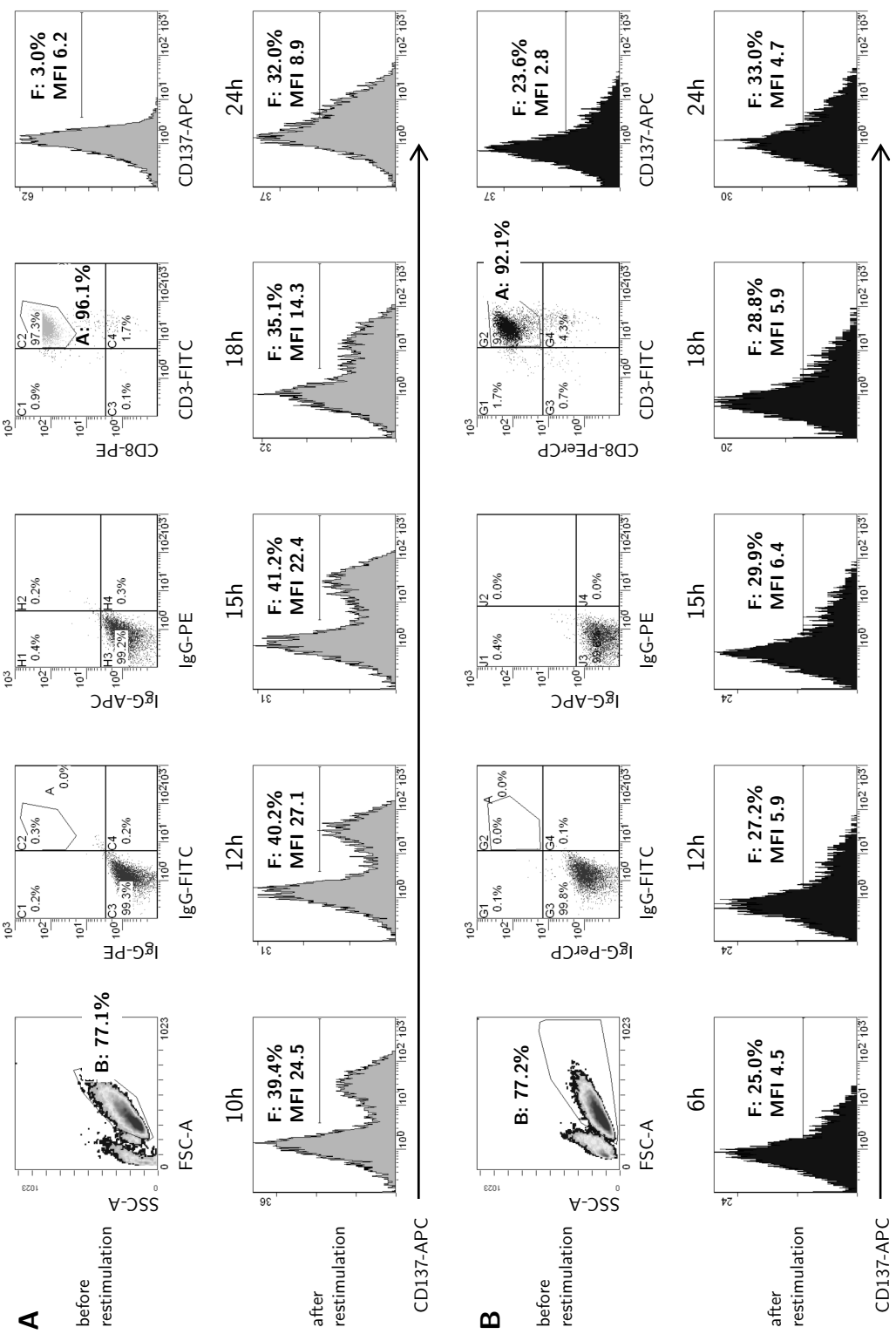


Figure 4.3: CD137-Kinetics in AML-reactive CD8 T cells. The top rows shows control stainings and CD137 staining of the T cells before restimulation. First events with sufficient forward scatter were selected in the FSC/SSC plot (gate B). The following two dot plots show isotype controls that were used for gating. In the next step CD3/CD8 positive cells were selected (gate A). Events from this gate are shown in the CD137 histogram. Events in Gate F were regarded as CD137 positive CD8 T cells. The MFI of the events in gate F ist stated in the respective dot-plot. A: CD137 kinetics of the MZ653-AML/Donor 069 systems on day 7/8 of culture. Similar kinetics were observed for day 14 in all other systems. B: In the MZ529-AML/Donor 735 sytem considerable background expression of CD137 was observed on day 7. Upon restimulation the percentage of CD137 and the MFI of CD137 positive events were increased. No clearly distinct CD137 positive T cell population could be detected.

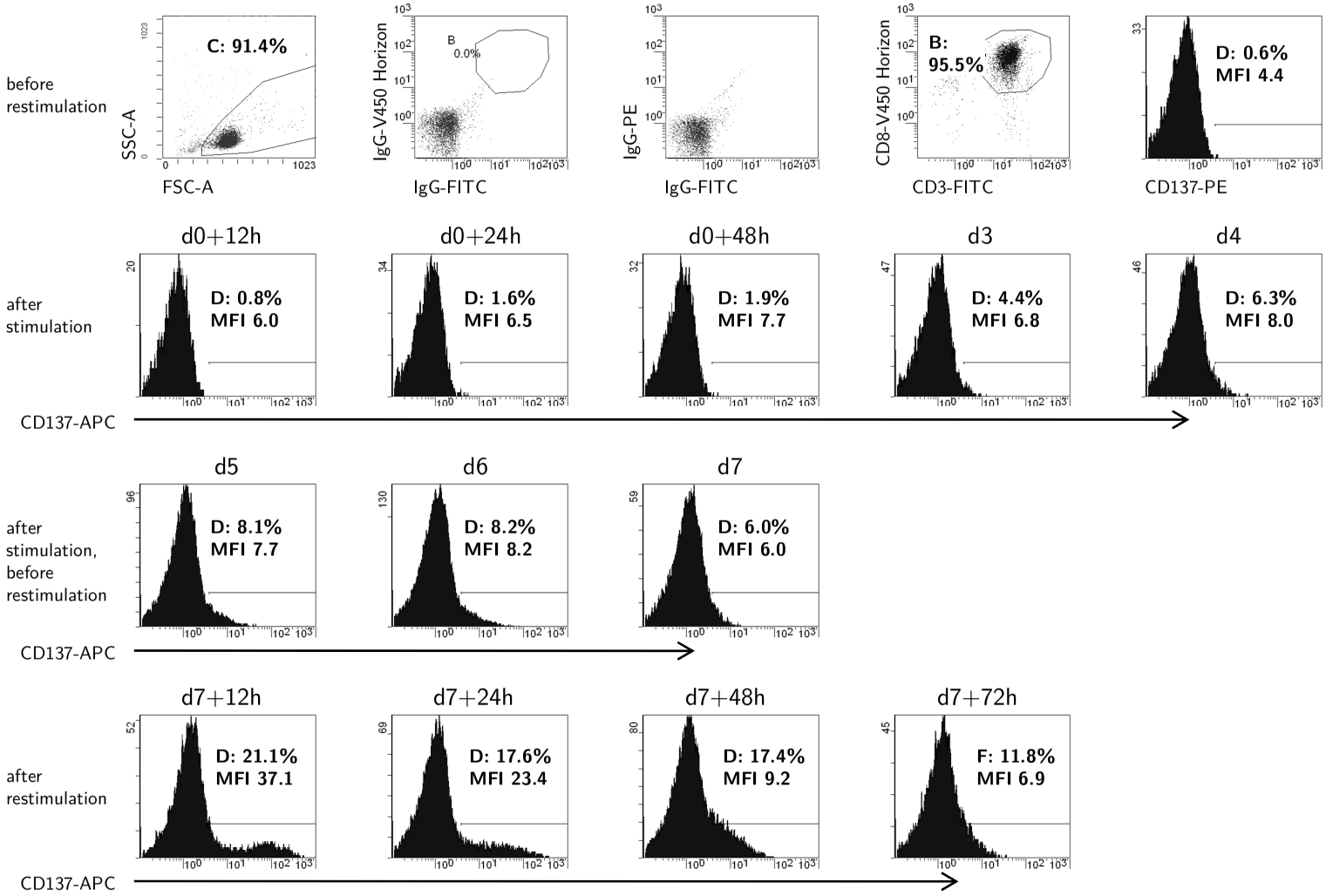


Figure 4.4: CD137-Kinetics in HLA-mismatch CD8 T cells, buffy coat 0911834 stimulated with MZ911-AML. The top rows shows the same control stainings and gating as figure 4.3. Rows two and three show the course of CD137 expression upon the inital stimulation with AML blasts. The increase of CD137 expression within the first 48h was only found in this experiment, but on day 3 clearly higher percentages of CD137 positive T cells were found in all three conditions. The bottom row shows the appearance of a new CD137high T cell population with restimulation on day 7.

values obtained with futher details are shown in table 4.1. All isolation methods yielded sufficiently pure CD137 positive fractions (Dynal direct not evaluable). To compare different methods simultanously all isolated fractions had to be large enough for further culture. Therefore high cell counts had to be restimulated (up to 3×10^8) due to the cell loss in each isolation condition and the incomplete depletion of the CD137 negative fractions. The priority of the different problems will be described in the discussion section.

Besides the efficacy of the procedure, the handling of the different methods was taken into account. The Dynal techniques required a flow cytometry run prior to the isolation to determine the percentage of CD137 positive cells and subsequent adjustment of Ab and beads. This extra effort did not lead to a better efficiency. Particularly for the direct method no apropriate quality control was possible after isolation. The tube-based approaches were not superior in terms of cell loss compared to column-based approaches at RT. Due to these circumstances in the majority of experiments column-based approaches were used. The biotin-approach was favoured because of better isolation efficacy and functional/proliferation data (see below) compared to PE as well as the possibility that this approach might be developed to clinical grade (GMP conditions).

Very high portions of dead cells in the trypan blue staining were found after the CD137 isolation procedures compared to other assays (e.g. CD8 isolation). The CD137 positive fraction always appeared to contain higher percentages of dead cells compared to the negative fraction, which was also in contrast to naive and whole CD8 isolations (positive fractions almost pure viable cells). This led to the hypothesis that the cooling of the freshly activated T cells might have adverse effects, as in standard cell culture only resting T cells are cooled. A comparison of the the Miltenyi Biotin CD137 isolation cooled and at RT simultanously with cells from the same culture showed a 7-fold higher CD137 yield and 44% less cell loss with better purity of the positive fraction in the RT condition (experiment A7). Also the performance of the PE-based approach was superior at RT.

In two experiments CD137/IFN-γ costaining was performed as part of parallel CD137 and IFN-γ secretion assay isolation. The costaining showed that 47 to 51% of the CD137 positive T cells did not secrete detectable amounts if IFN-γ while only 18 to 33% of the IFN-γ secreting cells did not express CD137 at a detectable level.

Different isolation methods were not only compared in terms of isolation efficiancy, but also because of the potential costimulatory signal via CD137 that could be advantagous during the expansion after isolation. Particularly the costimulatory effect mentioned by the manufacturer of the anti-CD137 beads remaining in the positive fraction of the Dynal direct approach could not be confirmed. Different other strategies to investigate and optimize influencing factors during the expansion period were used. AML blasts could remain in the CD137-negative fraction after isolation and have a stimulatory effect on the

Isolation Method	No. of Exp.	Pos. Frac. Yield	Pos. Frac. Purity	Neg. Frac. Depl. Lack	Total Cell Loss
Dynal direct	4	23,4 ± 14,7	n/a	47,3 ± 36,0	32,2 ± 13,9
Dynal indirect	3	28,3 ± 11,5	73,8 ± 35,1*	34,7 ± 9,6	34,0 ± 15,3
Miltenyi Biotin cooled	4	21.2 ± 18,3	80.2 ± 17,0*	51.1 ± 34,8	47.7 ± 20,3
Miltenyi Biotin RT	5	35,7 ± 22,3	81,8 ± 9,2	29,7 ± 13,9	13,8 ± 12,2
Miltenyi PE cooled	2	28,5 ± 3,0	95,5 ± 0,4	58,1 ± 13,8	50,2 ± 12,4
Miltenyi PE RT	3	36,5 ± 15,5	96,3 ± 3,0	43,9 ± 34,5	22,9 ± 19,9
Milt. IFN-γ secr. assay	2	24,7 ± 7,5	94,3 ± 1,0	64,5 ± 24,0	47,2 ± 7,3

Table 4.1: Comparison of the different isolation methods used. The number of experiments indicates in how many experiments the respective method was used. All further values are percentages with standard deviation. The CD137 positive yield is the ratio of cell number of the isolated CD137pos fraction compared to the number of CD137pos cells before isolation. The latter was calculated from the cell number used for isolation and the percentage of CD137 in the unseparated control. The purity of the positive fraction is shown as determined in flow cytometry. For the Dynal direct method no flow cytometry of the positive fraction could be performed (beads remain attached to cells). The overall cell loss is the percentage of the total cell number before isolation that was lost during the isolation procedure. The lack of depletion in the negative fraction was calculated as the ratio of CD137 positive cells left behind in the negative fraction (cell count of the neg fraction × percentage of CD137pos cells in neg fraction) compared to the number of isolatable CD137 positive cells. The cell loss during the isolation was assumed to affect CD137 positive and negative T cell equally. The number of isolatable CD137 positive cells is therefore total cell number × percentage of CD137pos cells × (100% – cell loss). Values marked with * may contain one possible outlier.

CD137-negative cells masking the advantage of the positive fraction. Isolated fractions were cultured with and without additional AML d2 (see methods section), but only a general increase of proliferation in positive and negative fractions due to the extra Ag presentation could be observed. Feeder cells after isolation were used in two experiments as this possibility has been described in the literature (details see below). As also described by others, restimulation with and without cytokines was evaluated in one experiment (A11). A moderate advantage for functional tests and a moderate disadvantage for proliferation was found for restimulation without cytokines. Furthermore the effect of restimulation 6 days after isolation was evaluated. The rationale for this was that differences between the different fractions might manifest at a later point of time in culture, which however could not be confirmed.

4.2.3 Proliferation of CD137 Isolated CTL

To estimate the T cell expansion all fractions after isolation were counted on day 7 after isolation. If possible all fractions were restimulated on this day and again counted on day 14 after isolation.

In matched and single-/sub-allele-mismatched systems of 20 separately cultivated fraction six CD137 positive fractions showed an expansion that was equal or above the unseparated control. In theses cases differences were moderate with approx. 1.3-fold (maximum 2.3-fold) higher cell counts in the positive fractions. Two of these cases showed the proliferation in fractions with additional AML blasts after isolation. In the vast majority of the cases CD137 negative fractions with additional AML had growth factors that were equal or above those of CD137 positive fractions without additional AML. If there were differences between the corresponding positive and negative fractions, the expansion of the positive fraction was higher (17 out of 20 fractions). However the majority of the experiments contained at least one inconsistent condition with unexpectedly high proliferation in the negative fraction (above unseparated/positive fraction). Almost all of these cases occured in fractions that were cultivated either with additional AML blasts or feeder cells after isolation (one exception in experiment A4, Dynal direct isolation). In 9 out of 13 separately cultured fraction without additional AML blasts or feeder cells irrespective of the isolation method all isolated fractions had a lower growth factor than the unseparated control at the end of the experiment. Experiments comparing all isolation methods showed a slight advantage of the Miltenyi Biotin isolation in terms of the growth factor compared to other methods. In two experiments an IFN-γ secretion assay was used for comparison. No clear differences between the different fractions in terms of cell expansion were found.

In contrast one experiment (A11) in a HLA-mismatched system showed a marked

advantage for the CD137 positive fraction if feeder cells were used. There was no major influence of the feeder cells on the negative fractions and the unseparated control. These results were similar for two differernt restimulation methods (restimulation in medium with/without cytokines). Unfortunately these results for the feeder cells could not be reproduced in a HLA-matched setting. A similar setup led to highest expansion in the CD137 negative and lowest in the CD137 positive group irrespective whether naive or whole CD8 T cells were used for the MLLC. Data of these experiments are shown in figure 4.5.

One cell sorting experiment (experiment A19, see below) included a direct comparison between column-based isolation and cell sorting. All separated fractions had a lower growth factor compared to the unseparated control, and the expansion of column-isolated T cells was approx. 30% lower than the corresponding sorted fraction. However all differences were moderate with the maximal difference between the unseparated control and the column-isolated negative fraction being 2.4-fold.

4.2.4 Functional Testing of CD137 Isolated CTL

In seven independent experiments each comparing several isolation methods and subsequent culture conditions, ELISPOT assays were used to evaluate functional differences between the different conditions. Only one experiment (A3) showed a stronger response in the CD137 positive fraction compared to the unseparated control for three out of four isolation methods. The positive fractions had 2.5 to 3-fold higher spot count compared to the unseparated fraction and 1.6 to 6 fold higher spot counts than the negative fractions with and without AML d2. In five experiments no difference between the CD137 positive fraction and the unseparated control were found.

One experiment (A4) showed higher spot counts in the negative fraction on day 4 after isolation and no differernces on day 7+4 after isolation for all almost all isolation methods. The only exception were the Miltenyi Biotin and PE isolated positive fractions that had up to 2-fold higher spot counts compared to the negative fractions on day 7+4 after isolation. Nevertheless these spot counts were equal to the unseparated control. Higher spot counts in fractions cultivated with AML d2 compared to the counterparts without were observed in two experiments. A chromium release assay in one experiment (A11) with HLA-mismatched T cells showed higher a SL in all CD137 positive fractions compared to the negative fractions. However the SL of the positive fractions was not superior compared to the unseparated control and the result could not be reproduced in a HLA-matched system on day 4 and day 7+4 after isolation (experiment A12).

In all ELISPOT assays that included B-LCL, reactivity against patient B-LCL but

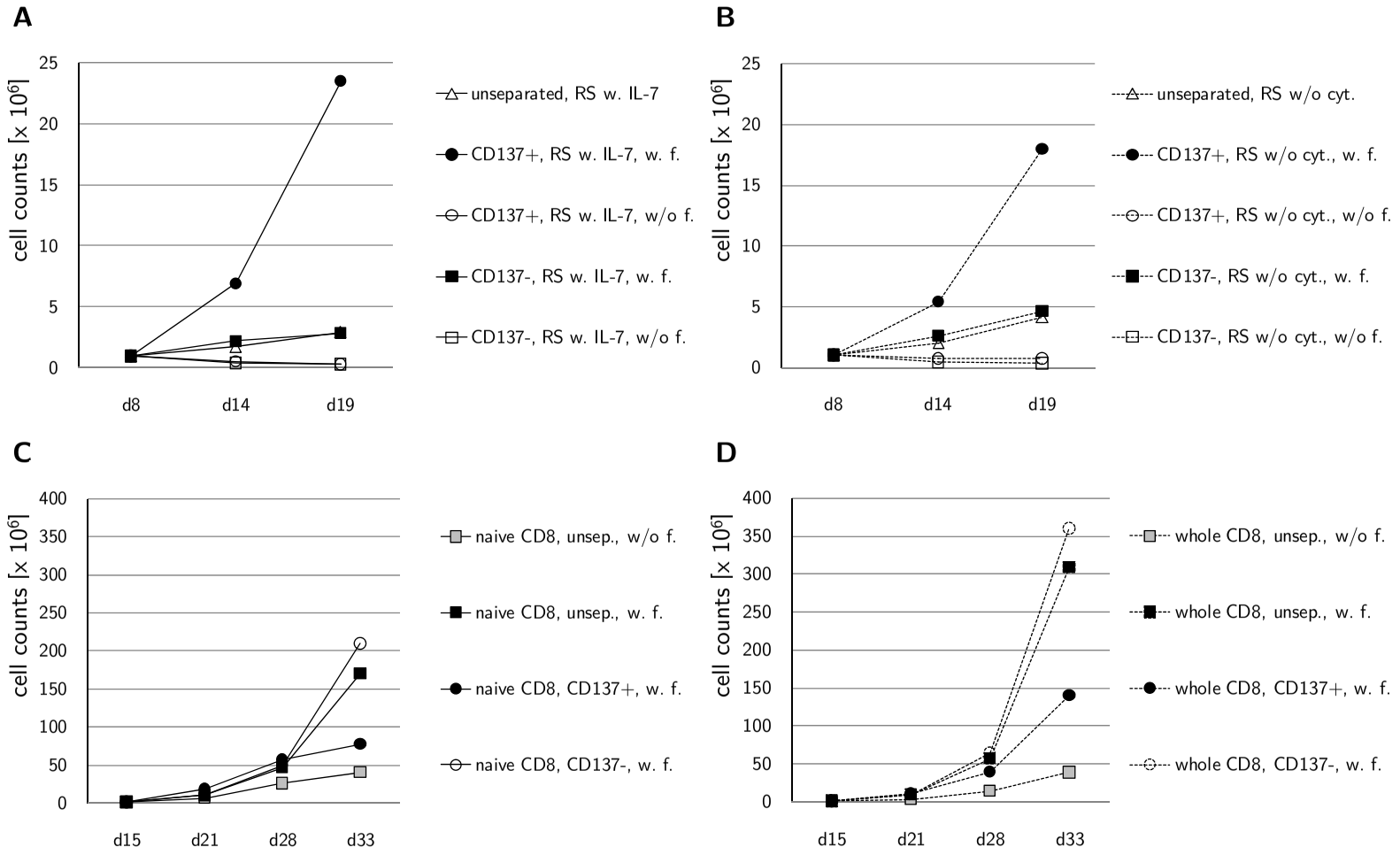


Figure 4.5: Proliferation of CD137-isolated fractions and corresponding unseparated controls in HLA-mismatched and -matched systems. Note the different axis scalings; cell counts are extrapolated. The top row shows the MZ911-AML/BC 0911834 (HLA-mismatched) system (experiment A11, Miltenyi Biotin isolation at RT). CD137 isolation was performed on day 7+1, 12 h after restimulation in either medium with 2 ng/ml IL-7 (w. IL-7, panel A) or without cyokines (w/o cyt., panel B). Isolated fraction were cultivated with (w. f.) or without feeder cells (w/o f.). Unfortunately in some fractions all cell were needed for functional testing on day 19, so that no further cell counts are available. The bottom row shows experiment A12 in the HLA-matched MZ369-AML/sibling donor 167 system under similar conditions as experiment A11 (same isolation method, feeder cells after isolation). Cytokines were used according to the established MLLC standard. The CD137 enrichment was performed on day 14+1 as a very high CD137 background expression on day 7 before restimulation made the isolation impossible at that point of time. Either naive (panel C) or whole CD8 T cells (panel D) were used as starting population. A transiently higher growth factor was observed from day 15 to day 21 for the CD137 positive fraction isolated from naive CD8 T cells (1.6-fold of CD137 negative population).

not or at low levels against donor B-LCL and K562 were found. In one experiment (A12) reactivity below the AML reactivity level against K562 was found in two subsequent chromium release assays for T cells from a naive CD8 starting population. A flow cytometry analysis showed phenotypically no NK cells in the culture.

4.2.5 Phenotype of CD137 Isolated CTL

In two experiments the different conditions were phenotyped in flow cytometry for differentiation markers after the functional tests. T cells from a naive starting population (experiment A1) on day 21 of culture showed 50 to 60% CD45RA and over 94% CD45RO expression. Over 90% of the T cells stained negative for CCR7 and over 84% positive for CD62L. More than 90% expressed CD27 and CD28. Over 73% were positive for CXCR4 and over 65% positive for CD127. No striking differences were found between unsparated and isolated fractions. The culture did not contain any NK cells.

In contrast an experiment (A3) with whole CD8 T cells as starting population was also phenotyped on day 21 of culture and the conditions isolated with Miltenyi Biotin, PE and Dynal indirect methods showed a consistent pattern. Of all T cells over 96% expressed CD45RO, while CD45RA was expressed by approx. 5% of the CD137 positive fractions, 20% of the negative fractions and 8% of the unseparated control. Approx. 30% of all T cell conditions expressed CD62L, over 60% expressed CXCR4 and below 3% expressed CCR7. CD27 staining was higher in CD137 positive fractions (over 70%) compared to the other conditions (approx. 50%). A similar pattern was found for CD28 with over 80% in positive fractions, 80% in the unseparated control and approx. 63% in negative fractions without AML d2. Furthermore a higher percentage of T cells expressed CD127 in the positive fractions (over 45%) compared to the unseparated control (30%) and negative fractions (11 to 25%). Eventually up to 5% NK cells were found some conditions, but the CD137 fractions were free of NK cells.

4.2.6 Vital Staining

The considerable cell loss during the isolation procedure with prominent cell debris especially in the isolated CD137 positive fractions led to the question whether evidence for apoptosis (for example in terms of AICD) or necrosis could be found either upon restimulation or subsequent to the isolation procedure. Furthermore this would provide an explanation for the variable, often dissatisfying proliferation and effector function.

Vital staining was performed before and 12 to 18 h after restimulation as described above. As no standard positive control for the annexin V staining is established, the strongly annexin V binding cell detritus was used as a positive control. An ungated histogram of

annexin V binding showed viable T cells and cell detritus as two distinct populations. The local minimum inbetween was used as threshold for the positive gate.

4.2.6.1 Bulk Cultures

In the patient/donor systems MZ574-AML/donor 247, MZ911-AML/unmatched donor and MZ369-AML/sibling donor 167, vital staining was used as a part of CD137 enrichment experiments. In addition to vital staining before and after restimulation, also all isolated cell fractions were stained with annexin V and 7-AAD after isolation. In all experiments a higher annexin V binding (1.7 to 6.3-fold) in CD8 T cells was observed after restimulation compared to the level of resting T cells before restimulation. However these values obtained for unseparated control groups still ranged at the lower end of the annexin V negative gate. 7-AAD staining did not yield any helpful information as the "lymphocyte-population" in the FSC/SSC plot contained only a very low percentage of 7-AAD positive events (always below 4%, all events also annexin V positive).

A positive correlation for CD137 expression and annexin V binding in the respective dotplot was observed in all systems. CD137 positive T cells had a higher annexin binding compared to CD137 negative T cells and 18 to 35% of the CD137 positive T cell were above the threshold for positive annexin V-staining. Also a population (up to 15% of CD8pos events) of CD8pos/CD137neg/annexin V positive cells was found in two experiments after restimulation. This population appeared clearly distinct in a CD137/annexin V densitiy plot, while the majority of the events showed a continuous distribution from CD137neg/annexin V neg to CD137pos/annexin V pos. Furthermore the annexin V-MFI of isolated CD137 positive fractions was 2.3 to 6.6-fold higher compared to the negative fractions directly after the isolation procedure. A representative plot series is shown in the top row of figure 4.6. In two experiments the different fractions were also stained on day 2 after restimulation (24 h after isolation) and the difference in annexin V binding between the CD137 positive fraction and the negative/unseparated fractions could still be observed. Unmanipulated control fractions showed an annexin V-MFI that was below or equal to the CD137 negative fraction in all experiments.

In the HLA-matched MZ369-AML/sibling donor 167 system cultures from a naive CD8 and whole CD8 starting population were compared. annexin V binding was always lower at all points in time (before and after restimulation) for T cells from the naive starting population. In the MZ574-AML/donor 247 system an additional IFN-γ secretion assay enrichment was performed simultanously to the CD137-based enrichment. All findings in terms of annexin V-binding stated for the CD137 enrichment were also found for the IFN-γ-based enrichment. The correlation between IFN-γ secretion and annexin V-binding

was even more pronounced than for CD137 with 56% annexin V binding cells in the IFN-γ positive fraction (figure 4.6, bottom row). Another flow cytometry measurement on 2 after restimulation showed similar results with again a very strict correlation of secreted IFN-γ and annexin V binding in the positive fraction.

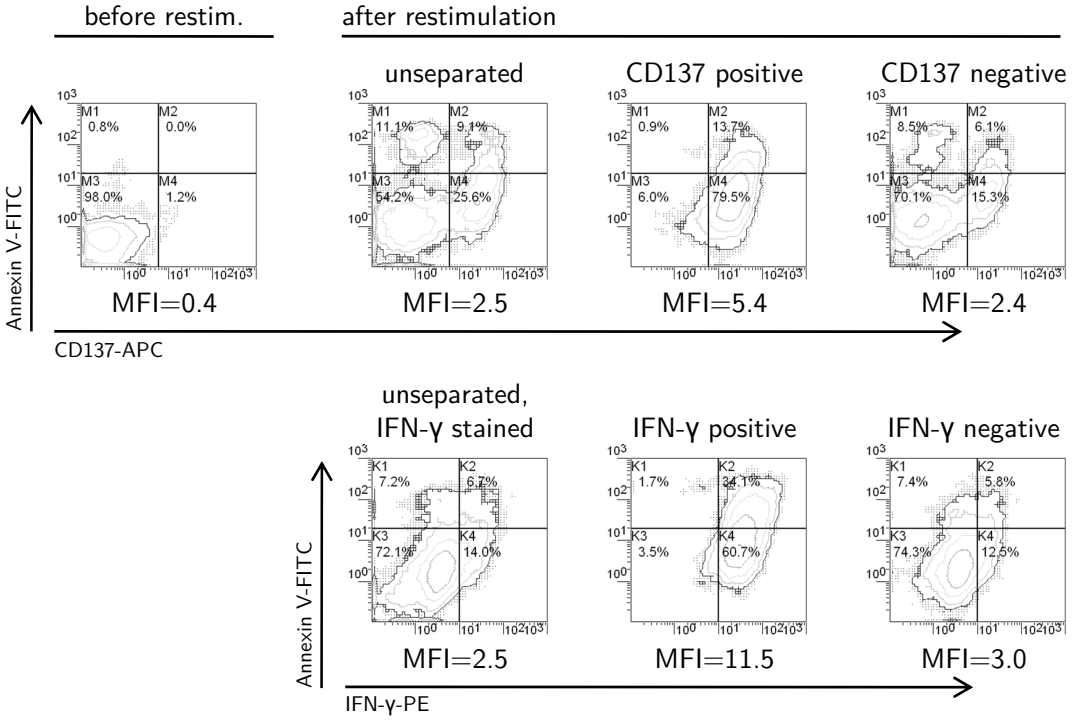


Figure 4.6: Vital staining after cell separation. All data are from simultanous CD137 enrichment (top row) and IFN-γ secretion assay enrichment (bottom row) in the experiment A7 (MZ574-AML/donor 247). All events shown are gated in the FSC/SSC plots like shown above and CD8 posititve. Density plots were used to visualize the positive correlation between annexin V binding and CD137/IFN-γ secretion. The MFI values indicate the annexin V-FITC median of the whole cell population.

4.2.6.2 CTL Clones

In six independent experiments costaining of CD137, annexin V, CD8 and 7-AAD was performed with CTL clones from three different systems of AML-reactive CTL clones. Additionally IFN-γ was costained in some experiments in a IFN-γ secretion assay. Restimulation of CD137 negative CTL caused an upregulation of CD137 in the whole CTL population which shifted 58 to 89% of the CTL in the positive gate. In all proliferating cell cultures no or a minor increase in annexin V staining (MFI) upon restimulation was observed. If the annexin V staining was at a higher level after the restimulation, at least 90% of the CTL were still below the threshold of the "positive"

gate. When an IFN-γ secretion assay was performed 21 to 99% of the CTL were found to secrete IFN-γ. No positive or negative correlation between annexin V and CD137 or IFN-γ was seen in the respective dotplot. Both annexin V-PE and annexin V-FITC were used and led to similar results. As in previously performed experiments with bulk cultures 7-AAD stainings had been always negative, only in one experiment 7-AAD was used. At all points in time the percentage of 7-AAD positive cells was below 5%.

One long-term cultured CTL (MZ987-AML/donor 940 clone 2B8) stopped to proliferate reproducably upon the restimulation on a particular day of culture (day 84). Upon this restimulation over 70% of the CTL stained positive for annexin V after 12 h, while one week before over 70% remained negative. During the following week from day 84 on the cell number declined with a growth factor of 0.3 per week.

4.2.7 Cell Sorting Experiments

A positive correlation between CD137 expression, IFN-γ secretion and annexin V staining had been found in bulk cultures but not in CTL clones. Additionally only small or no advantages of the CD137 enriched fractions were found in terms of proliferation and effector function. To address the question whether in the specific setting of AML-reactive CTL CD137 expression could be associated with impaired proliferation and function, established CTL clones were sorted for CD137 expression. In bulk cultures differences in proliferation, phenotype and function can be attributed to the various TCR specificities, so clones were chosen for these experiments. Furthermore these experiments were carried out to evaluate whether the bead-based isolation could be a cause of the dissatisfying proliferation and function.

CTL clones with known intact proliferation and effector function were thawed, restimulated and tested after one week for CD137 upregulation, annexin V binding and IFN-γ secretion upon restimulation. After restimulation on day 14 (day 7 in one experiment) 12 h later the CTL were divided into a CD137low, a CD137intermediate (CD137int) and a CD137high fraction by sorting. The gates for sorting were set in a manner that the T cell population was divided into three fractions of equal cell number (each 33% of the total cell number). This strategy was chosen to ensure optimal reproducibility inbetween different CTL, that exhibit a varying CD137 distribution. Furthermore most CTL clones did not show distinct CD137 positive/negative populations but a distribution around the positive gate-threshold in the histogram. Simultanously to the sorting, the CTL were stained for CD137, annexin V and 7-AAD. Five days after restimulation the sorted CTL and an unseparated control were stained for TCR expression (Vβ chain) and tested in IFN-γ-ELISPOT and chromium release assays.

Four independent experiments with three CTL from two patient/donor systems (MZ653-AML/donor 069, clones 7C12, 5E10; MZ201-AML/donor 332, restimulated with MZ418-AML, clone 2A7) were performed. While sorting 41 to 77% of the CTL were CD137 positive and over 90% of the CTL were negative for annexin V in all experiments. The reanalysis after cell sorting always showed clearly separated CD137high/int/low fractions with on average 87% of the sorted cells in the respective gate and a 7.5 to 42-fold higher CD137-MFI of the CD137high fraction compared to the CD137low fraction. (figure 4.7 A)

In the sorted cell fractions the proliferation (cell counts) was the highest for the CD137-high (however only approx. 1.5-fold higher growth factor) and the lowest for CD137low fraction in all experiments. Nevertheless in three of four experiments the proliferation of the unseparated fraction was still higher than in the CD137high fraction. (In the remaining experiment the proliferation was generally impaired by CFSE staining.) A represenative graph is shown in figure 4.7 B. In two experiments CFSE stainings were performed to assess the proliferation more accurately. The CFSE-MFI was always consistent with the cell counts, but an unseparated, non-CFSE stained control showed that the CFSE staining decreased the proliferation considerably in general. Lower concentrations of CFSE than 10 µmol (see also methods section) were found to be insufficient to label the cells for one week.

In five of six ELISPOT assay on the days 5 and 7+5 after restimulation the CD137low fraction showed the highest IFN-γ release among the sorted fraction with up to 2-fold higher spot counts comapred to the CD137high fraction. However the unseparated controls did not show a consistent pattern with spot count above and below the sorted fractions. An example is shown in figure 4.7 C. In all experiments the chromium release assays showed equal cytotoxicity for all fractions (figure 4.7 D) and no differences in TCR expression by Vβ chain staining were found.

For one clone cell-sorting and bead-based separation (Miltenyi PE-based method) were compared. In the bead-isolated fractions an approx. 35% lower growth factor, equal cytotoxicity and a comparable ELISPOT response (negative fraction stronger than positive) was found compared to sorted CTL.

4.2.8 CD137 Isolation of Peptide-Stimulated T cells

The generation of AML-reactive CTL with leukaemia blasts was considered as a possible cause of the ineffective CD137 enrichment, as the vast majority of the data on CD137 enrichment was obtained after stimulation with peptide loaded APC (DC, monocytes, PBMC) as shown in the literature discussion. DC generated from AML blasts have

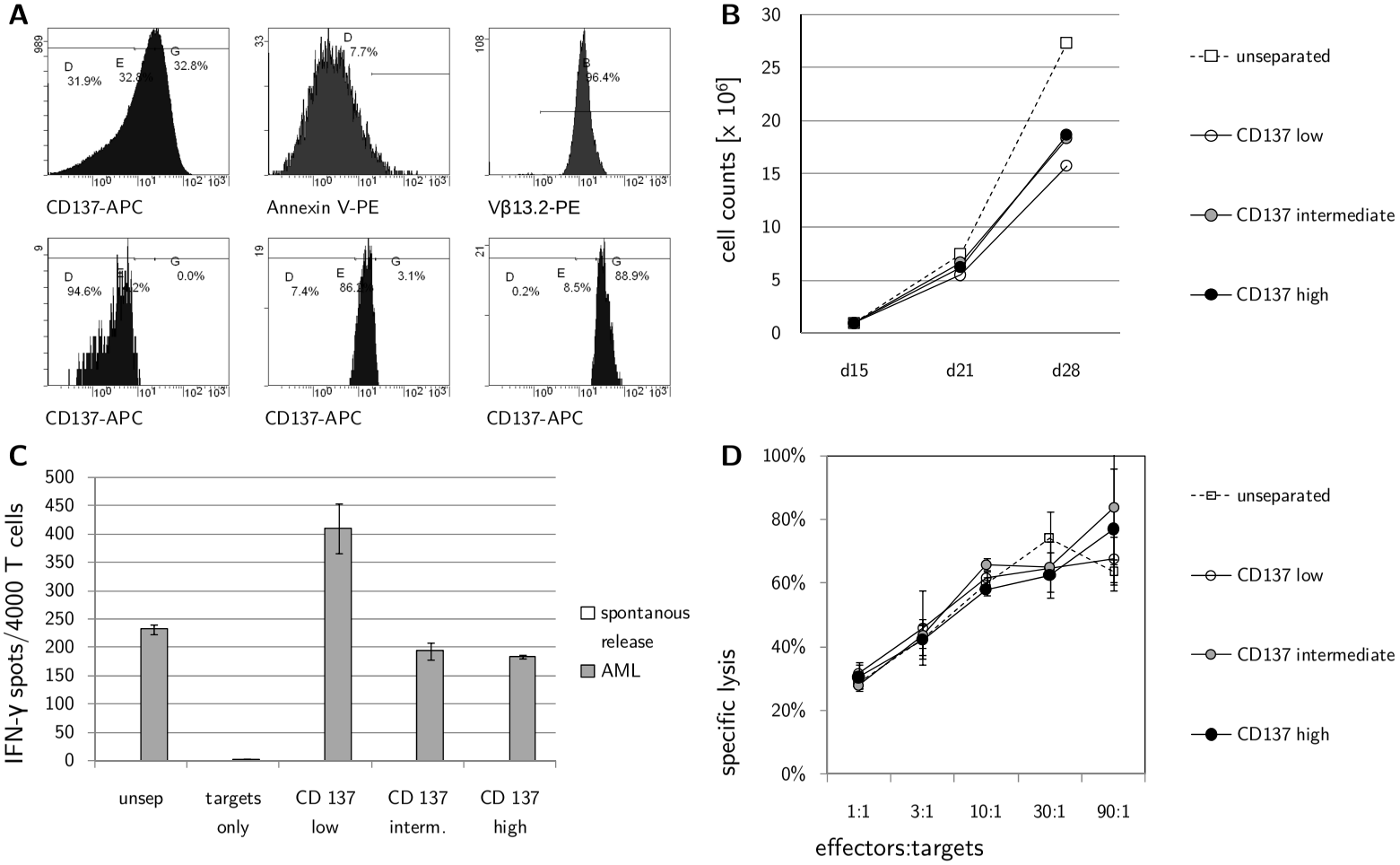


Figure 4.7: Experiment A17, sorting of AML-reactive CTL clones, MZ201-AML/donor 332, restimulated with MZ418-AML, clone 2A7. A: All histograms show events gated in the FSC/SSC plot like previously shown and subsequently gated on CD8. The top row shows data of the CTL before sorting. Each of the gates D, E and G contains approximately 33% of the events in the CD137 histogram. The majority (92.3%) of the CTL does not bind annexin V and 96.4% express Vβ 13.2. The bottom row shows the reananlysis of the sorted CTL, with over 86% the CTL in the respective target-gate. B: Growth of the different CTL fractions, extrapolated by the growth factor from a hypothetical starting number of 1×10^6 cells on day 15. C: ELISPOT on day 21+5 of culture (11 days after sort), target MZ418-AML, 10,000 CTL/well, 50,000 targets/well. D: Chromium release assay on day 21+5 of culture, target MZ418-AML

disadvantageous properties for induction of CTL responses [19] and impaired formation of immune synapses with AML blasts has been found in T cells from AML patients *ex vivo* [149]. To evaluate a comparable setup to the established MLLC protocol, the tumour-associated, HLA-A*0201 binding peptide Melan-A (ELAGIGILTV) was chosen to stimulate a naive starting population. Naive CD8 T cells from two different HLA-A*0201 positive healthy donors were isolated and stimulated as described in the methods section.

Both donors showed a vigorous response to the peptide with uncommonly rapid proliferation of naive T cells (growth factor 2.7 to 3.2 within the first week) and 55 to 61% tetramer-binding cells after one restimulation (day 7+5). A staining for CD3, CD8 and CD16 on day 7+5 in one experiment did not show any NK cells. CD137 enrichment assays were performed 24 h after restimulation on day 8 and 15, respectively. Both points of time yielded a pure (98%) CD137 positive fraction and a slightly depleted negative fraction (48 to 33% and 12 to 9% remaining CD137 positive T cells, respectively).

Five days after restimulation the tetramer binding of the unseparated fractions was 61 to 79%. Tetramer binding of the negative fractions was comparable to or approx. 10% lower than the unseparated control, while in the positive fraction it was 13 to 28% higher compared to the unspearated frations (figure 4.8 B). Chromium-release assays on the same days showed strong respones to peptide loaded T2 cells without differences betweeen the fractions and no response to T2 cells without peptides (figure 4.8 C,D). Also ELISPOT tests showed a vigorous recognition of peptide loaded T2 cells and no response to T2 cell without or with an irrelevant peptide. The spots were not countable due to the very strong response (even 2,000 CTL/well confluent), but the wells of the CD137 positive fraction showed a more intense red staining. The growth factors of the positive fraction were always higher compared to the negative fractions, however the differences were not pronounced (less than 2-fold). Additional feeder cells after the isolation increased the growth factor over 4-fold without any influence on functional tests.

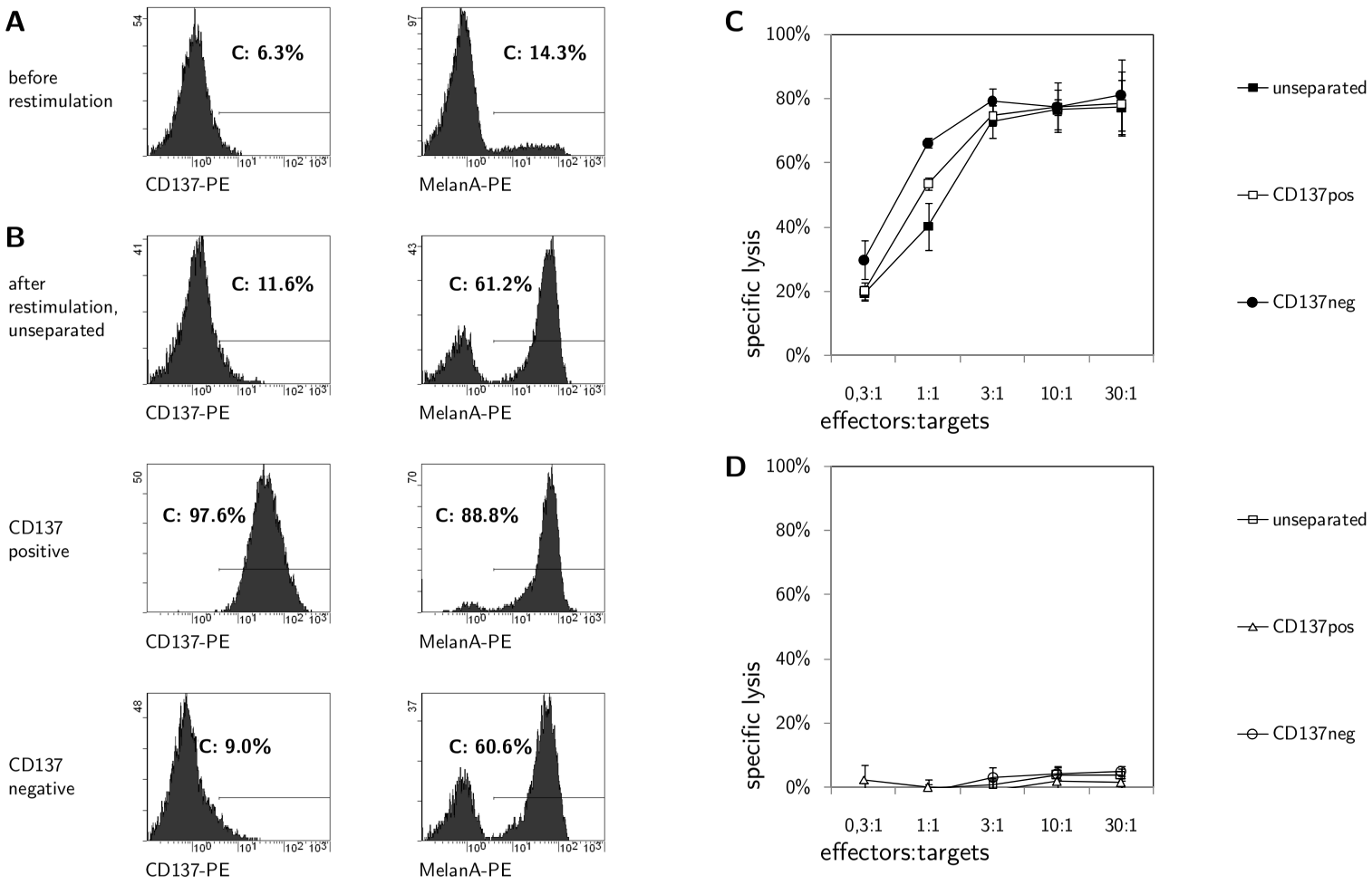


Figure 4.8: Isolation of Melan-A-specific CTL, experiment A21. A: CTL on day 7 before first restimulation. B: CD137-fractions on day 7+1 after isolation (left column), tetramer-staining on day 7+5 (right column). Note the increase in tetramer binding without enrichment procedure. C: Chromium-release assay on day 7+5, target: T2 cells loaded with Melan-A peptide. Note the comparably low effector:target ratios. D: similar chromium-release assay as above, but different target: T2 cells without peptide.

5 Discussion

5.1 Stimulatory and Adverse Effects of Bead-Stimulation in Tumour-Reactive CTL

5.1.1 RCC-Reactive CTL, Bead:Cell Ratios

In accordance with the previous results (published in [116]) for RCC-reactive CTL the data shown in the results section confirm that bead-stimulation in the first four weeks of cell culture is an appropriate way to expand tumour-specific CTL without using malignant stimulator cells. The novel CD3/CD28/CD137 beads were more effective in terms of cell expansion than the standard CD3/CD28 beads and equal to RCC-stimulation. All cultures were performed in medium supplemented with 100 IU/mL IL-2 which alone had a negligible effect on CTL expansion. Simultaneous stimulation with beads and tumour cells did not show a synergistic effect. However at least in this setting there seems to be a time frame concerning the age of a CTL line in which bead-stimulation is feasible. The favourable results for bead-stimulation were found for CTL in approx. week 3 of culture, while T cells in week 8 of culture showed no response to the bead-stimulation. Unsuitable bead-concentrations are unlikely to be a cause of the inefficient stimulation as higher bead-concentrations were tested previously (D. Teschner, unpublished data) and lower bead-concentrations were tested as shown. Similar to the consistent differences found for the RCC-specific CTL with better growth factors for higher bead-concentrations, others also described the same correlation in tumour-reactive T cells [150]. In contrast data for PBMC obtained by another group showed equal efficiancy for bead:cell ratios from 1:5 to 1:80 [151]. An advantage for an additional CD137 signal with artificial APC has also been found by others [105, 122]

The functional data from chromium-release assays however revealed a lack of cytotxic function for bead-stimulated RCC-reactive CTL that had no correlate in previous ELISPOT assays [116]. This can be either interpreted as a loss of Ag-specificity by unspecific stimulation or as an effect of differentiation modification by bead-stimulation. Similar results were found for bead-expanded umbilical cord blood T cells that showed a clear type 1 differentiation with IFN-γ secretion, but a lack of cytotoxicity and low granzyme B and perforin expression [152]. Also a loss of tumour-specificity in oligoclonal cultures during

bead-expansion [153] has been described, which is often attributed to skewing of the TCR repertoire by unspecific stimulation [154].

In contrast others found that bead-stimulation (CD3/CD28) can promote rapid expansion (84-fold in 14 days) of human PBMC and the development of effector functions in chromium-realease assays [150,155] and secretion of type 1 cytokines in the supernatant [155]. The T cells generated lysed various tumour cell lines, however in a HLA-mismatched setting. Early removal of the stimulation beads (day 4, day 7) improved the viability of the culture, the expansion and the IFN-γ secretion considerably compared to bead-stimulation during the full 14 days of cell culture [155]. The relatively strong effector functions are in keeping with results that show more T cell differentiation to effector phenotypes by CD3/CD28-stimulation compared to Ag-independent expansion with mitogens (PHA) [156]. Also for naive umbilical cord blood T cells a differentiation towards a more mature phenotype during bead-stimulation was found in CD8 but not in CD4 T cells [157]. Nevertheless these results are not fully comparable to HLA-matched tumour-specific T cells as no Ag-dependent control was included in the experiments.

Recently others compared CD3/CD28 beads and CD3/CD28/CD137 beads for the expansion of tumour-specific CD8 T cells of patients that had been vaccinated with tumour-specific DC-vaccines (melanoma and RCC) previously. Direct *ex vivo* bead-stimulation of T cells from vaccinated patients did neither expand tumour-specific nor virus-specific (CMV) T cells. With an *in vitro* restimulation-step using peptide-loaded PBMC or DC prior to bead-stimulation an efficient expansion of Ag-specific T cells was achieved. The response to the peptide-stimulation was considerably stronger for CMV-peptides than for a tumour-peptide (94% vs. 14% specific T cells, respectively). The subsequent bead expansion was efficient in terms of achieving high cell counts, but the percentages of specific T cells in the cultures decreased (44% and 11% specific T cells, respectively) [150]. These results are in keeping with a previous publication showing that CD3/CD28 beads can be used to expand melanoma-specific T cells from PBMC of tumour-vaccinated melanoma-patients and that the bead-expansion is dramatically improved with a prior Ag-specific restimulation [158].

Concerning the melanoma and RCC patients higher bead-concentrations were found to be more effective for T cell expansion, but caused a stronger decrease in the percentage of specific T cells. CD3/CD28 and CD3/CD28/CD137 beads yielded similar results for the exansion and functionality of tumour-specific CD8 T cells. Furthermore the possibility to restimulate multiple T cell specificities simultanously with tumour-lysate-loaded DC was demonstrated. However the lysate-stimulation did not expand all multimer-detectable tumour-reactive T cell specificities and no data for subsequent bead-expansion were published [150]. These results can be seen as a more specialized case of earlier data showing

a loss of Ag-specific T cells with unspecific T cell expansion [105]. The overall data of these publications are in accordance with the data on RCC-reactive T cells from the results section. Nevertheless the lack of data on the bead-expansion of polyspecific T cells in [150] might indicate together with decreases in the percentages of specific T cells during bead-stimulation that the simultanous expansion of multiple specificities in one culture by bead-stimulation may be problematic. Also an experiment series investigating bead-expansion of T cells from CLL patients showed some evidence, that bead-expansion might reduce the percentage of specific T cells in a culture promoting a broader repertoire of TCR specificities. In this specific context the shift from a skewed TCR repertoire as a symptom of the CLL (detected by TCR spectratyping [159]) to a distribution similar to healthy individuals was seen as a favourably possibility to improve patients' T cell immunity by adoptive T cell transfer [160]. However the results are in keeping with those showing the possibility to loose specificity during bead-expansion. The loss of some specificities might explain the loss of cytotoxicity during bead-stimulation as shown in the results section.

5.1.2 Phenotype of Bead-Expanded T Cells

Phenotypical analysis of the differently stimulated CTL cultures revealed higher levels of CCR7 and CD62L for bead-stimulated CTL while no significant influence was found for CD27, CD28, CD45RA, CD45RO and CD57. However all stimulation methods caused a downregulation of CCR7 and CD62L (CD62L) compared to the initial level [116].

The higher levels of CD62L and CCR7 expression [116] could be consistent with a more early effector phenotype [51] compared to CTL stimulated with the specific Ag. As repetitive Ag-exposure is usually regarded as the key trigger of differentiation to an effector phenotype, one could speculate whether Ag-independent activation might preserve an earlier differentiation stage, which is usually associated with less pronounced *in vitro* cytotoxicity. However the phenotypical differences are moderate and not consistent for all markers (e.g. CD27). Furthermore a TCR activation by anti-CD3 should cause similar downstream signaling as Ag-specific activation, which makes this explanation less plausible. Furthermore CD62L is shed from the cell surface after Ag-specific TCR activation. This phenomenon has also recently been demonstrated to be associated with cytotoxicity in human tumour-reactive T cells [161]. As the bead-stimulation mimicks a TCR-signal a decrease of CD62L in a flow cytometry analysis after bead-expansion would be as expected. In the literature high expression levels of CD62L have been reported for bead-stimulated umbilical cord blood T cells [152] and human melanoma-reactive T cells [158]. In the latter experiment series the high CD62L was found in the context of a relatively early T cell phenotype (CD28 high, CD27 intermediate, CCR7 low, CD57 low). As the starting

population contained mainly terminally differentiated T cells, the data could indicate that Ag-specific restimulation followed by bead-expansion preferentially expands a less differentiated T cell population [158]. This interpretation would be in keeping with the own data for bead-expansion of RCC-reactive CTL at different points in culture time. Contrastingly other data for human tumour-specific CD8 T cells showed lower CD62L expression after bead-expansion together with an effector phenotype [150].

Recently data on the effects of bead-stimulation on T cell differentiation were published for human CD4 and CD8 T cells stimulated with CD3/CD28 beads or soluble anti-CD3 with F_C-receptor bearing feeder cells. For CD8 T cells the latter approach was superior in terms of expansion and T cells generated form a naive starting population retained a less differentiated phenotype compared to bead-stimulated T cells [151].

5.1.3 AML-Reactive CTL

Compared to RCC-reactive T cells contrasting results for AML-reactive CTL showed consistently a dose-dependent deleterious effect of the bead-stimulation irrespective of the bead-format used. This was more pronounced for CD3/CD28 beads as the decline in cell number was the fastest for this kind of beads. In a very robust system the adverse effects of the beads were overridden by additional AML blasts as stimulators, while in a less robust system this was only possible for CD3/CD28/CD137 beads but not for CD3/CD28 beads. Finally minimal stimulatory effects were observed for low concentrations of CD3/CD28/CD137 more than for CD3/CD28 beads. This again emphasizes the stronger growth-inhibition by CD3/CD28 beads. Notably the results show an adverse effect of the beads that goes beyond the neglect due to absent stimulation as cell counts of the negative control (maintenance in medium with cytokines only) were mostly higher than those of bead-exposed T cells.

Despite the difficulties described for RCC-reactive CTL, AML-specific CTL generated as described in the methods section seem to represent an exceptions as they respond inversely to bead-exposure. Bead-expansion of T cells not only for viruses and solid tumours but also for haematological malignancies has been shown to be feasible and clinically relevant in various publications.

As CLL patients often lack a sufficient T cell immunity, one group tested the possibilities of *ex vivo* T cell-expansion for adoptive transfer by CD3/CD28 beads. Very high growth-factors (1400-fold in two weeks) were achieved by a combination of bead-stimulation and magentic isolation of bead-bound cells with a considerable decrease of CLL in the culture. The expanded T cells showed an activated phenotype with expression of CD25, CD134 and CD137. Leukaemia reactivity (ELISPOT and cell killing in flow cytometry) was

found in bead-stimulated cultures, but not in native T cells. However the estimated frequencies of leukaemia-reactive T cells ranged from 1 in 200 to 1 in 2500, which was too low to explain the extensive cell killing observed in flow cytometry. Restauration of susceptibility to Fas-mediated apoptosis of CLL cells by cell-cell interaction with expanded T cells was discussed as a possible explanation because there was no direct evidence for leukaemia specific, TCR-dependent cytotoxicity [160].

A preclinical experiment series compared CD3/CD28 bead-stimulation of marrow infiltrating lymphocytes and peripherial blood lymphocytes from multiple myeloma patients. The bead-stimulation was considerably more successful for marrow infiltrating lymphocytes in terms of cell expansion, proliferative response to malignant cells, growth inhibition of malignant cells and tumour-specific cyotoxicity. Both peripherial blood and marrow infiltrating lymphocytes showed an oligoclonal TCR Vβ chain repertoire, which can be regarded as sign of a polyclonal tumour-specificity. During bead-stimulation the Vβ chain pattern of marrow infiltrating lymphocytes was preserved, while in peripherial blod lymphocytes the repertoire tended to normalize [153]. Overall this experiment series could indicate that the T cell microenvironment can subtly influence the response to bead-stimulation. As for T cells from different compartments in the same patient bead-stimulation was more or less effective, one could speculate that *in vitro* generated T cells might exhibit similar differences depending on various factors in the cell culture.

Also for multiple myeloma patients a phase 1/2 study with CD3/CD28 bead-stimulated T cells was conducted after preliminary data [162, 163] had been obtained. The objective of the trial was the restauration of immunity in patients with active disease by a combination of vaccination (tumour-peptides, CMV and pneumococcal conjugate vaccine) and a CD3/CD28 bead-stimulated T cell infusion after autologous HSCT. The control group recieved only the pneumococcal conjugate vaccine. Despite better immunological laboratory parameters for the experimental branch, no difference in overall or event-free survival was found [164].

In a phase 1 study patients with different relapsed haematological diseases after HSCT received *ex vivo* CD3/CD28 bead-expanded DLI products in a dose-escalating regimen. During 12 days of culture the expansion of CD3 positive cells was 113-fold on average. Eight of 18 patients were in CR after the administration, including two of four patients with AML. One of those patients (diagnosis: acute promyelocytic leukemia) had a durable CR of over one year at the time of the publication [165].

Recently tumor-derived donor lymphocyte infusions have been generated by CD3/CD28 bead-stimulation for patients with refractory B cell neoplasms after allo-HSCT. Over 12 days median culture time a median T cell expanion of 57-fold was achieved. Unabated disease progression but also positive and mixed responses without GVHD were found in

positron-emission tomography scans after infusion [166].

The heterogenous data on bead-stimulation of tumour-specific T cells might indicate that in some experimental setups certain unknown stimulatory factors are provided by the APC, that can not be fully and sufficiently imitated by beads. This is further supported by a comparably simple experiment series in which CD3 and CD137 signals were able to induce IL-2 production, but no proliferation. Purified human adult CD8 T cells did not produce IL-2 upon unspecific TCR-stimulation with OKT3, but did so when a CD137 signal was added. Remarkably this did not lead to proliferation unless accessory cells (providing further unknown signals) were added, too. In proliferating cultures stimulated with TCR and CD137 signals and accessory cells, the level of IL-2 in the culture medium was lower than in non-proliferating cultures stimulated with just TCR and CD137 signals, which could indicate IL-2 consumption of proliferating cells. Furthermore the IL-2 receptor α-chain (CD25) could not be induced by CD137 [98]. Furthermore inter-donor variability with unexpected drops in Ag-specific T cell counts are even known in more robust virus-immunity settings [95].

The data in the results section show considerable proliferation in the negative control (no Ag-specific restimulation, only medium supplemented with cytokines) for some AML-reactive CTL. A very consistent pattern was found in experiments with the clone 8F11 (MZ653-AML/donor 069) with rapid growth for a triple cytokine combination (high dose IL-2, IL-7, IL-15), intermediate growth for high dose IL-2 only, and slower expansion for low dose IL-2.

The proliferation of memory T cells upon cytokine-exposure without Ag-specific activation is a well-known phenomenon particularly for IL-2, but also for IL-7 and IL-15. The cytokine-driven proliferation corresponds to the *in vivo* T cell proliferation triggered by lymphopenia. This process called homeostatic proliferation has been reported to be dependent on IL-7 and IL-15 for CD8 T cells. Especially memory, but even naive CD8 T cells show homeostatic proliferation. Over a longer period exposure to IL-2 and IL-7 without Ag-presentation leads to the programmed shutdown of the immune response [167].

5.1.4 Apoptosis and AICD

To address the question whether the adverse effects of bead-stimulation in AML-reactive T cells could be mediated by apoptosis, a pan-caspase inhibitor was used to block the caspase-cascade. No change in the adverese effect of the bead-stimulation on the T cell expansion (cell counts) could be seen with the caspase-blocking. IL-2 was the only cytokine used in this experiment as IL-2 without IL-15 has been reported to promote AICD [167]. Thus apoptosis-dependent effects should be more pronounced in medium

with IL-2 only. Z-VAD-FMK has been shown to reduce apoptosis, particularly AICD in tumour-reactive T cells effectively [147] and apoptosis has been reported in the context of CD3/CD28 bead-stimulation. In human T cells apoptosis upon bead-stimulation occurred predominantly in CD8 T cells and despite high Bcl-X_L levels. As a consequence of this one publication showed lower expansion of CD8 T cells compared to CD4 cells [168] which contradicts other data showing an outgrowth of CD8 T cells during bead-stimulation of mixed cultures [155] or umbilical cord blood [152]. Another experiment series suggested that AICD can not only occur during primary bead-stimulation but also even more pronounced in the context of restimulation with CD3/CD28 beads. This phenomenon was especially seen in CD8 T cells and for relatively early restimulation (less than 20 days after primary bead-stimulation) [151]. Again for umbilical cord blood T cells contrasting results were found with higher rates of apoptosis during bead-expansion among CD4 T cells [152].

Melanoma-reactive CTL can undergo apoptosis upon MHC class I dependent recognition of their targets and upon stimulation with immmobilized anti-CD3. Both kinds of AICD seem to be Fas-dependent as they can be reduced by anti-Fas Ab. Notably the tumour cells did not express Fas ligand. This is a major difference to AML-reactive CTL in which Fas-mediated cell killing seems to be of particular importance. Caspase-blocking with Z-VAD-FMK was also capable of decreasing both variants of AICD and did not impair tumour-cell lysis or IFN-γ scretion of the CTL [147]. Also for the Jurkat cell line Z-VAD-FMK was shown to inhibit caspases effectively. The inhibition activity was measured by the cleavage of the CD3 ζ chain by caspases upon Fas-signals [148].

The slightly decreased cell growth in the control groups with caspase inhibitor is in accordance with results from the literature. Different caspase-inhibitors have been reported to decrease the proliferation of T cells stimulated by CD3/CD28 Ab. Notably the concentration needed for proliferation inhibition in this setting was 10-fold higher than the one used in the experiment with AML-reactive T cells. The IL-2 and IFN-γ production during T cell activation was nearly unaffected by caspase-blocking. Interestingly in contrast to the apoptosis-inhibition, the proliferation-inhibition was not dependent on the caspase-blocking function of the respective agents [169].

The results from the literature suggest that using the caspase-inhibitor in this context was suitable to address the problem, as the same substance has been successfully tested for human tumour-reactive T cells. The dose of caspase-inhibitor used was lower to the one published, however the efficacy of the inhibitor could be seen by the effect of proliferation inhibition in the control groups. As higher doses have been reported to decrease the sensitivity to CD3/CD28-stimulation, a dose escalation would have most likely not yielded further clarification. The results alltogether suggest, that the dose-dependent deleterious effects of both kinds of beads do not include apoptosis as a mechanism of cell deletion. The

varying disadvantageous effects of bead-stimulation reported by others together with own results could indicate, that relatively specific problems with bead-stimulation exist for the respective experimental setup. More problems have been reported for the restimulation of CD8 T cells and the majority of experiments was performed in a non-Ag-specific setting. Notably many reports of successful bead-expansion do not show a direct comparison of bead-expansion to Ag-specific restimulation and often no functional data. Several experiments have been performed with mixed CD4 and CD8 T cell cultures or PBMC. In these mixed and polyspecific or non-Ag-specific cultures cytokines and other bystander cell factors support CD8 T cell growth [150, 160]. As these supporting factors are absent in a setup with bead-stimulated tumour-specific CD8 T cells, this experimental setup is expected to be more challenging. Furthermore most data were obtained for autologues T cells in a setting without HSCT or for allogenic T cells *ex vivo* after HSCT, but not for allogenic HLA-matched T cells from healthy donors. No data on successful *in vitro* generation and bead-expansion of allogenic HLA-matched T cells stimulated with AML blasts are currently available. Eventually Ag-independent bead-expansion does not seem to be a favourable option for the expansion of prevoiusly stimulated Ag-specific CD8 T cells for clinical use.

5.1.5 CD137L Expression of Leukaemia Blasts

The published data on CD137L expression on AML blasts could be confirmed. This can theoretically support the assumption that additional CD137-costimulation on beads should mimic the AML-stimulation of leukaemia-reactive T cells more effectively. However it needs to be considered that the secretion of soluble CD137L is pronounced in AML compared to other haematological malignancies, especially in high-risk subtypes and cases with high malignant cell load. The serum levels of soluble CD137L do not correlate with the cellular expression, but it has a prognostic impact [77]. It can be regarded as a marker for immune escape as loss of CD137L from the surface of AML blasts could prevent the reverse CD137L signaling, which has been shown to induce apoptosis in lymphocytes and monocytes. Therefore AML blasts may gain a survival benefit by shedding CD137L to reduce apoptotic signals [170].

Soluble CD137L does not stimulate T cells or even inhibit the growth of anti-CD3 stimulated PBMC, whereas the trimeric bead-bound or immobilzed molecule provides a costimulatory effect [67, 171]. The soluble, non-aggregated CD137L is thought to interfere with the CD137/CD137L interaction and subsequent T cell activation [64]. One could speculate, that AML-reactive CTL stimulated with AML blasts were exposed to high levels of soluble CD137L. In contrast *ex vivo* expanded or peptide-stimulated CTL did

not encounter soluble CD137L but cellular expressed CD137L on APC. Therefore AML blast-stimulated CTL should be rather insensitive to CD137 signals as otherwise they would be exposed to the deleterious effects of the soluble CD137. According to this hypothesis only T cell clones with abnormal responses to CD137-signals will grow to high cell counts in a limiting dilution assay under stimulation with AML blasts. This could possibly explain atypical responses to CD137 Ab in bead-stimulation or CD137-isolation procedures.

5.2 CD137 Kinetics

Clear expression kinetics without background expression were found for MLLC upon restimulation on day 14 of cell culture. Peak expression was reached approx. 12 h after restimulation and sustained until 18 h. CD137 positive T cells formed a clearly distinct population in a CD137 histogram with maximal MFI of this population around 12 h. This is compatible with results from the literature [98, 111], while most publications show a CD137 peak around 24 h after restimulation [33, 99, 110]. However varying data from the literature (see literature discussion) might indicate that the kinetics are dependent on the particular T cell subtype and the cell culture conditions.

The T cell subset seems to be of particular importance if Treg are contained in the cell culture. One experiment series with human virus and tumour specific T cells showed that CD4 Treg can be activated by autologous APC and subsequently express CD137. The CD137 surface expression on Treg occurred more rapidly compared to naive and memory T cells [121]. Assuming that also CD8 Treg might show similar CD137 kinetics, CD137 enrichment early after restimulation might preferentially enrich Treg in addition to conventional Ag-specific T cells. CD8 Treg may have a similar phenotype as acitvated T cells and it may be difficult to distinguish between both in flow cytometry [172]. In contrast to CD4 Treg that interfere with activation of resting and the priming of naive T cells, some of the CD8 counterparts target especially activated T cells [172]. Furthermore for human AML Treg-mediated immune escape has been shown (induction of CD4 Treg by AML blasts and elevated frequencies of Tregs in the blood of AML patients) [173]. Several results for autoimmunity models suggest that CD137 signals can promote the development of CD8 Tregs [103]. Altogether these data show the possibility that CD8 Treg might be present in the MLLC and subsequently expanded under the influence of CD137 and AML blasts. After the CD137 enrichment these Treg might be contained in the CD137 positive fraction and dampen the response of activated leukaemia-reactive T cells in the same fraction.

In accordance with data from the literature only negligible baseline CD137 expression

was found before restimulations with exception for day 7 of culture in some experiments. CD137 background expression on day 7 was mostly observed in single allele mismatched systems, but can most likely not be attributed to this. In the matched sibling-donor sytem parallel branches with naive and whole CD8 T cells were set up. The naive branch showed no background on day 7 while in the other arm a very high background of 40% was found. The correlation of a CD137 background with mismatched systems is most likely coincidental as in these systems whole CD8 T cells were always used. Finally the phenomenon was present and absent under conditions of the regular MLLC cytokine conditions and with additional IL-21. This suggests that the background is a phenomenon that derives from the type of starting population and is independent from HLA matching, the patient-donor system and cytokines.

The slow increase in CD137 positive cells during the end of the first week of stimulation observed in completely mismatched T cell stimulations is consistent with the background expression of CD137 observed before restimulation on day 7 in matched/single-allele mismatch systems. Most likely the day 7 background in other experiments was the end point of the process observed during the first week. As repeated CD137 stainings during the first weeek were not performed in other experiments, the increasing CD137 had remained undetected until the first restimulation. The lower percentages of CD137 positive T cells on day 7 in the mismatch experiments could be due to the use of no or low dose of cytokines compared to the matched setting. Published results confirm this phenomenon of gradually incrasing CD137 expression in primarily stimulated human T cells with similar percentages of CD137 positive cells at day 5 of culture [174].

Alltogether these data could indicate that the T cells of the bulk MLLC need to be "synchronized" in their activation process by restimulation before uniform CD137 kinetics with distinct CD137 population can be detected. This could also explain the lack of clearly CD137 positive T cells in *ex vivo* stimulations. This hypothesis is in accordance with data which show that for IFN-γ secretion-based isolation the activation of T cells needs to be synchronized. The synchonisation seems to be especially important for primary responses to a new Ag and less for memory or other *in vivo* preactivated T cells [175]. This could explain other *ex vivo* CD137 kinetics found for example in virus-reactive T cells.

5.3 CD137 Isolation

Various isolation methods were compared for enriching leukaemia-reactive T cells via CD137 12 h after restimulation. If evaluable all isolation methods yielded sufficiently pure CD137 positive fractions. Major problems of the isolation procedure were the insufficient depletion of the CD137 negative fraction and the high cell loss during harvesting the

cells 12 h after restimulation and the CD137 enrichment procedures. As described above the column-based methods were favoured compared to the tube-based procedures due to better performance of the isolation, functional data and practical handling aspects. In the two column-based approaches the cell loss during the isolation could be dramatically reduced (table 4.1) with performing the enrichment at room temperature compared to 4 °C. Despite the manufacturers recommendation to perform the isolation procedure under cooled conditions, in our lab by experience activated T cells are handeled at room temperature. Also in this context the advantage for RT was confirmed with considerably lower cell loss without compromised specificity. The slightly lower purity of biotin-isolated positive fractions might be an artifact, as the biotin-labeled cells were stained for flow cytometry with anti-biotin-APC or GAM, while PE-isolated fractions were already stained. Results from some experiments suggested, that the secondary-staining of biotin might be suboptimal.

The major problem however was the lack of advantages in terms of functional data and prolifertion for the isolated CD137 positive fractions which often rangend below the unseparated controls. As this question was addressed first, less effort was put on increasing the yield in the isolation procedure. Although a complete depletion of the negative fraction might be possible by increasing Ab- and/or bead-concentrations during the isolation, lower Ab concentrations should favour the isolation of T cells with high CD137 expression. As it was expected to find the most marked differences between the isolated fractions when only CD137 high-expressers were contained in the positive fraction, the problem concerning the yield was postponed. The generation of sufficient cell numbers for adoptive transfer was a major objective of the project. Even with column-based isolation at RT the overall cell loss during harvesting freshly restimulated cells and the isolation procedure was too high to make the CD137 isolation procedure a promising candidate to achieve this aim. The cell count of the CD137 positive fraction ranged from 8.7% (robust HLA-mismatched system) to 0.68% (naive CD8 HLA-matched system) of the cell number used for restimulation. Even with optimized yield during isolation and superior T cell expansion in the CD137 positive fraction afterwards it seems rather unlikely that the isolation procedure is a helpful means to increase the cell counts that can be achieved within a short culture period.

The majority of the functional data showed similar results for the isolated and unseparated fractions. Even with remaining leukaemia-reactive T cells in the CD137 negative fractions one would expect marked differences between enriched and depleted fractions. Several explanations may account for this unexpected finding. As described above the isolated CD137 positive fraction might also contain enriched and activated CD8 Treg that might reduce proliferation ans functionality of the isolated T cells. However similar results were found in a setting with a naive CD8 starting population. Multiple surface markes are used

to deplete non-naive T cells from PBMC and a uniform, pure population of naive CD8 T cells was isolated, which makes this explanation less likely.

Shortening the culture period required for the generation of sufficient numbers of T cells for adoptive transfer was one of the major aims of the work. Therefore the funcional tests were performed after a relatively short culture period of 14+5 days. At relatively early differentiation stages (central memory cells) T cells usually do not secrete large amounts of IFN-γ or exhibit strong effector functions. This could be regarded as a possible explanation for the generally small differences found betweeen the different isolated and unseparated fractions. Nonetheless the functional data of these T cell were comparable to those of leukaemia-reactive effector CTL clones created in MLLC [12]. Furthermore in experiments more differentiated T cells of effector-memory phenotype were generated within the same time period under less stimulatory and differentiation-promoting cytokine conditions [121]. Another common problem in T cell bulk cultures is the off-target proliferation of bystander cells [121]. As no Ag/TCR-independent CD137 has been reported in the literature and sufficient responses were found in functional tests, outgrowth of nonspecific T cells has most likely no relevance in the given context. AICD of activated leukaemia-specific T cells could particularly affect the CD137 positive fraction and reduce especially the expansion of the positive fraction. This possible cause of the dissatisfying isolation will be disussed below in together with the results of the vital staining with timing effects in particular.

Prior to CD137 enrichment the MLLC mostly contained more than 10% leukaemia-reactive T cells according to the CD137 staining of the unseparated fraction after restimulation. This contrasts data from the literature that show undetectably low frequencies of minor histocompatibility Ag-specific T cells after two weeks under similar culture conditions [121]. Although one would expect higher percentages of responding T cells in polyclonal cultures compared to a single peptide-Ag, the difference is striking. Alloreactivity due to the single/sub-allele HLA mismatches is unlikely to account for the high percentages, as similar frequencies of leukaemia-reactive T cells were found in HLA matched setups.

To compare the CD137 enrichment to a more established standard method, an IFN-γ secretion assay was performed in parallel. Compared to the column-based CD137 isolation methods at RT the secretion assay was not only more laborious concerning the handling but also less effective in terms of cell isolation. The positive fraction yield (ratio of cell count in the isolated positive fraction and IFN-γ positive cells before isolation) was comparable to tube-based and cooled, coulumn-based isolation methods. Consistent with this the IFN-γ negative fraction was less efficiently depleted than in all CD137 isolations. Also the cell loss was similar to cooled, tube-based isolations, which can be attributed to the compulsory cooling step and the numerous centrifugation steps in the IFN-γ secretion assay.

The IFN-γ secretion assay has been reported to be suitable for the clinical grade production of leukaemia-reactive T cells within three weeks [175]. Clinical trials with adoptive transfer of IFN-γ secretion assay-selected T cells for CMV treatment [176, 177] but not malignancies have been published. Some reports showed that the CD137 approach has advantages regarding handling, cell yield and proliferative capacity compared to the IFN-γ method [99, 111, 119]. In addition CD137 has been reported to select a wider range of T cells compared to cytokine-secretion assays [99, 119], for which reason some own experiments were designed to allow a CD137/IFN-γ costaining. For simultanous detection of CD137 and IFN-γ the secretion assay was performed during the period of maximal CD137 expression, i.e. 12 to 18 h after restimulation. This period is later than the usual period for the IFN-γ secretion assay recommended by the manufacturer, but depending on the type of stimulation (peptide only, professional APC) up to 16 h post restimulation are recommended. Nevertheless the results above and data from the literature [174] show that sufficient IFN-γ detection is sill possible after 12 to 18 h. CD137/IFN-γ costaining in two experiments confirmed data published in the literature [99, 119] that CD137 isolation does select a wider population than an IFN-γ secretion assay. This was indicated by the considerable proportion of CD137 positive cells, that did not secrete detectable amounts of IFN-γ. However it has to be considered that the staining was performed during the CD137 peak expression and not during the earlier period of peak IFN-γ secretion.

The purity of positive fractions in enrichment assays can differ considerably with the Ag. Even for tetramer-based enrichment, which usually leads to comparably high purity [121], the enriched positive fraction can contain 8% (Melan-A) to 96% (CMV pp65) tetramer positive T cells [178]. Furthermore a publication shows that in some contexts a CD137 enrichment assay can enrich T cells with low precursor frequencies to moderate percentages (approx. 10%). However the percentage of tetramer-positive T cells in the CD137 positive fraction could only be enhanced further by restimulations or tetramer-based isolation, but not by CD137 isolation [121]. One could speculate that a similar effect might account for the data shown in the results section, in which most percentages of leukaemia-reactive T cells before CD137 isolation were already above 10%.

5.4 T Cell Survival and Vital Staining

Vital stainings with annexin V and 7-AAD were used to assess bulk cultures and T cell clones for viability during the restimulation and isolation procedures (CD137 and IFN-γ). Restimulation caused an increase in annexin V binding that was more pronounced in bulk cultures than in T cell clones. Furthermore bulk cultures showed a positive correlation between CD137 expression levels and annexin V binding with T cells that shifted into the

annexin V positive gate. The positive correlation was seen as a continous distribution in a CD137/annexin V density plot and as a higher annexin V-MFI of isolated CD137 positive cells. Both phenomenons were not found in proliferating T cell clones. An even more strict correlation with annexin V binding was found for secreted IFN-γ than for CD137. A direct comparison of cultures with naive and whole CD8 T cells as starting population showed lower annexin V binding in the naive culture at all points in time (before/after restimulation/isolation).

Exposure of phosphatidyl serine on the outer leaflet of the plasma membrane and subsequent strong annexin V binding has been reported to occur in murine CD4 T cells upon activation without involving apoptosis [179]. Although the publication describes the phosphatidyl serine exposure as a all-or-none effect, it could be possible that in this case of *in vitro* generated human CD8 T cells the effect described is responsible for the higher, but not above-threshold annexin V binding after restimulation. That a similar effect might account for the positive correlation between CD137 expression and annexin V binding seems rather unlikely as CTL clones stayed reproducably annexin V negative after restimulation. One should take into account that most likely not all apoptotic T cells are captured during the flow cytometry measurement. Induction of apoptosis is particularly fast in cells of hematopoietic lineage and the actual apoptosis takes less than 1 h, although apoptotic cells mostly stain positive for annexin V for a longer period. Furthermore there are distinct apoptosis pathways that do not include phosphatidyl serine exposure on the outer leaflet of the plasma membrane. During and after apoptosis the light scatter properties of the respective cells usually show characteristic changes [180], which explains that no late-stage apoptotic, 7-AAD positive T cells were found within the "lymphocyte" gate in the FSC/SSC plot.

An increased annexin V-MFI in isolated CD137 positive fractions on day 2 after restimulation was found, which could indicate that the majority of apoptotic cells is not detected as annnexin-positive directly after isolation. An increasing percentage of apototic cells around day 2 after restimulation could explain the varying results for functional tests and proliferation after isolation. Espectially the lack of striking differences between the isolated fractions would be well explained by an assimilation of positive and negative fractions due to apoptosis in the positive fraction. Lower annexin V binding in the unseparated fractions could indicate, that the extensive handling of the T cells directly after restimulation could contribute to apoptosis of activated T cells that might be specially susceptible to adverse stimuli. This is in keeping with results from cell sorting experiments (see below).

Compared to other CTL and the previous data for the same CTL, the long-term cultured CTL 2B8 displayed a significantly higher annexin V binding upon restimulation directly

prior to a rapid decline in cell number. This indicates that the annexin V/7-AAD staining method and the gating strategy used were apropriate to detect apoptosis in T cell cultures. The number of apoptotic cells detected (approx. 70%) corresponded well with the cell counts (decline to approx. 30% of previous cell count). AICD has been described to be mediated by intrinsic or extrinsic apoptosis pathways in previously activated restimulated T cells. IL-2 renders T cells more susceptible to AICD, which may be reversed by interleukin-15 (IL-15). Also CD8 T cells primed without CD4 T cell help are prone to apoptosis upon restimulation. AICD is thought to be especially important for the elemination of chronically activated and therefore potentially autoreactive T cells but also for the shutdown of an immune response [181, 182]. DC-stimulated [115] and long-term-cultured memory-phenotype-like human T cells have been shown to be susceptible to TCR-mediated AICD [183]. Therefore AICD seems to be the most likely corresponding phenomenon to annexin V binding in this context. This is further supported by the observation that lower percentages of annexin V binding cells were found in cultures with naive CD8 T cells as starting population compared to whole CD8 T cells as the latter contain a larger portion of Ag-experienced T cells that are more prone to AICD.

Also the pronounced correlation of annexin V binding with secreted IFN-γ may explain some results in functional tests. Assuming that T cells that secrete the highest amounts of IFN-γ are most prone to apoptosis, it seems plausible that differences between isolated fractions are not well detected in an IFN-γ-ELISPOT. As often more differentiated effector-cells secrete high amounts of IFN-γ and exhibit the strongest cytotoxicity, a chromium release assay might also be a suboptimal readout in this very specific context.

When designing the experiment series CD137 was not only chosen due to the favourable characteristics as an activation marker, but also due to the costimulatory activiy. In different experimental setups the CD137 costimulation has been shown to provide antiapoptotic effects [68, 69, 90, 95, 98, 105] and CD137-isolated T cells have been reported to show better growth than tetramer or CD107a isolated T cells [111]. Cross-linking of anti-CD137 Ab is necessary for the stimulatory effect, which is provided by the bead-cound Ab. But as described in the literature discussion there are data that suggest that an anti-CD137 Ab signal may have differential effects depending on the time of Ab administration after stimulation. In influenza mouse models a CD137 signal within the first 24 h after Ag challenge in the presence of APC led to a collapse of CD8 T cell immunity due to AICD. Later onset of the CD137 signal had the expected, well-decribed stimulatory effects on T cell immunity [107–109]. In the literature on CD137 enrichment the isolation procedure was always performed from 24 h post stimulation onwards [33, 99, 115, 119–121]. One could speculate that the possible AICD observed in the vital staining experiments was an effect of the early onset (12 h post stimulation) of the CD137 signal in presence of AML blasts

serving as APC. This may contribute to explain that the results on functional data and proliferation after the CD137 isolation contradict data published by others.

As various factors regarding the cell culture conditions have been reported to favour or prevent AICD the conditions used were compared to those published by others. All other groups used a single or mixed common gamma chain cytokines for cell culture, as IL-2, IL-7, IL-15 and IL-21 have well described functions [184] and are widely used for *in vitro* T cell generation. However for the period before CD137 isolation the protocols by others tend to use less cytokines both in concentration and number of cytokines used at any one time. The protocols comprise of 5 ng/mL IL-7 [121], 10 IU/mL IL-2 [120], 50 IU/mL IL-2 [111], no cytokines (24 h), 150 IU/mL IL-2 [33]. One publication described the same cytokine combination as used from day 7 on (see methods section), but the restimulation was performed in medium without cytokines and IL-2, IL-7 and IL-15 were added 24 h later [99]. The delayed administration of cytokines has been reported to support the generation of Ag-specific T cells [185], however own data on a direct comparison of delayed or continuous cytokine administration only showed a tendency towards better results for delayed administration. For culturing isolated CD137 positive T cells conditions with higher cytokine concentrations were used: 100 IU/mL IL-2 + feeder cells + PHA [121], 50 IU/mL IL-2 + 10 ng/mL IL-15 + feeder cells [120], 1000 IU/mL IL-2 [111], 50 IU/mL IL-2 + 5 ng/mL IL-7 + feeder cells [119]. That stronger stimulatory conditions were applied after CD137 isolation might indicate that others encountered similar problems concerning high cell loss and expansion of the isolated T cells. The cytokine combination described in the methods section has been successful in the generation of various leukaemia-reactive CTL [11, 12, 32]. IL-7 is regarded as the most important cytokine for survival of naive and memory T cells and IL-15 promotes formation of CD8 memory. As naive CD8 T cells produce only low levels of IL-2 and no IL-21 [184], pure naive CD8 T cell cultures could need more cytokine supplementation than whole CD8 T cells, mixed T cell cultures or whole PBMC.

Feeder cells are used by several groups (see above), as these should not only promote cell survival and expansion but also improved memory formation which has been described as an effect of bystander cells [167]. Increased proliferation was found with the use of feeder cells mostly without a clear pattern regarding the different fractions. Similar effects were observed for AML blasts that were actually added to correct possible stimulatory effects of remaining leukaemia cells in the CD137 negative fraction (see results section).

Only in a publication from our lab AML blasts are used as allogenic stimulator cells together with CD137 isolation [33]. Other methods used were adding peptide to PBMC [111, 119, 120] and autologues peptide-loaded DC/PBMC/monocytes [99, 121]. Primary stimulation with mature DC and restimulation with PBMC has been reported to be

efficient for generating peptide-specific T cells as PBMC tend to cause less AICD during restimulation [185,186]. Restimulation were either weekly [33,99] or every 10 days [121]. On the one hand earlier restimulation could favour AICD [151] but on the other hand a shorter restimulation cycle could be useful to shorten the *in vitro* culture period and preserve an earlier phenotype of the T cells. Weekly restimulations have been successfully used perviously, which suggests that this is most likely not a major cause of the discrepancies between the own results and the literature.

In all publications column-based isolation methods (Miltenyi) were used for CD137 isolation. The primary Ab was either labeled with biotin [33], PE [119] or APC [99,120,121] or anti-murine Ab-coated beads were used [111]. Cooling during the enrichment procedure was reported for PBMC [120] and prestimulated T cells [99, 111, 119, 121]. In contrast for the CD137 depletion of alloreactive T cells 6 to 12 °C were used [33]. No published data comparing activated T cell isolation at different temperatures are currently available.

Furthermore when comparing both the results on CD137 isolation and bead-stimulation to published data, it needs to be considered, that several experiments were performed in mixed CD4 and CD8 cultures [119–121] which are generally more robust than pure CD8 cultures. But also in particular for CD137, mixed CD4 and CD8 cultures have shown considerably better responses to CD137-stimulation than CD8 T cells alone [14]. The costimulatory potential of anti-CD137 Ab can vary depending on the type of APC and even reverse to adverse effects in some experimental setups [75], especially with DC [98]. As AML blasts can differentiate spontanously into DC [24], it seems possible that CD137 signals together with AML as APC may show different effects than with other APC.

5.5 Cell Sorting Experiments

Leukaemia-reactive CTL clones with known intact proliferation and effector function were sorted upon restimulation into CD137low/intermediate/high fractions to evaluate whether possible disadvantages of CD137 high-expresser T cells could be found independently from the TCR specificity. The purity of the sorted fractions was comparable to bead-isolatd CD137 positive fractions. As no clear-cut populations were sorted and CD137 expression can be transient over the sorting time period, the purity was regarded as sufficient. annexin V stainings in parallel to the sorting procedure showed low percentages of apoptotic cell compared to bulk cultures. CFSE stainings in order to obtain data on cell divisions in the sorted fractions correlated well with the cell counts, but impaired the growth of the stained T cells considerably compared to unstained controls. For the CD137high fraction moderate advantages in growth compared to the CD137low and inversely higher ELISPOT responses in the CD137low fraction were found. No differences in cytotoxicity

and TCR expression were seen. Consistently with the data obtained in the prevoius vital staining experiments with T cell clones, no correlation of higher apoptosis rates with CD137 expression levels was seen. Therefore one needs to consider that in a relatively uniform culture of CTL clones only moderate differences upon sorting of the population can be expected.

The higher proliferation (cell counts and CFSE) in unseparated compared to all sorted and bead-isolated fractions may indicate that the extensive cell manipulation during the time period directly after restimulation could have an adverse influence on the T cells, resulting in impaired cell growth. Also others describe efforts to reduce the manipulation of T cell cultures during restimulation [111]. Furthermore the bead-based isolation procedure seems to impair the T cell growth more severely than the sorting procedure. This could be either due to the manual handling or due to apoptosis triggered by cross-linked CD137 Ab on isolation-beads. As shown in the literature [107–109, 171] CD137 signaling (including possible pro-apototic effects) is only caused by aggregation of CD137 which should not be caused by adding staining Ab for cell sorting.

5.6 Melan-A

To generate responses against the Melan-A peptide, naive CD8 T cells were stimulated with peptide loaded mature DC for one or two weeks respectively. Vigorous proliferative responses of CD8 T cells with expansion of tetramer-binding T cells to high percentages were observed. CD137 isolation was performed after one or two weeks resulting in enrichment of tetramer-binding T cells in the positive fraction and moderate depletion in the negative fraction. Nevertheless the generally very strong responses in all fractions caused an assimilation of frequencies of tetramer-binding T cells, so that no differences between the different fractions in functional tests and small growth advantages for the CD137 enriched fractions were found.

Remarkably CD137 enrichment was possible on day 7+1 of culture despite no clearly distinct CD137 positive fraction in the unseparated control (figure 4.8). A lower percentage of tetramer-binding than CD137 expression was found in the isolated CD137 positive fraction. This might at first suggest an Ag-unspecific expression of CD137 but can also be explained by the well-described phenomenon of TCR downregulation upon activation of T cells [187]. However the tetramer-stainings before and after restimulation in this experiment are not compatible with the hypothesis of TCR downregulation. Furthermore the tetramer-staining of the useparated control and the CD137 negative fraction show, that the majority of tetramer-binding T cells did not express CD137 at that point of time, which explains the incomplete depletion of the CD137 negative fraction.

A 24 h incubation period between restimulation and CD137 isolation was used, as this had been extensively published for human peptide-stimulated T cells [99, 111, 115, 119, 121]. On day 14+1 the CD137 staining of the unseparated control confirmed that clear-cut positive and negative populations were detectable at that time. In a second experiment with CD137 enrichment on day 7+1 no distinct CD137 positive population was found in accordance with the data for bulk MLLC. Compared to AML-stimulated T cells the CD137 expression of peptide-stimulated T cells seems to be sustained longer. Also data by others comparing CD137 enrichment at different points in time after stimulation confirm that the isolation of human peptide-stimulated T cells is possible up to 96 h after stimulation [120].

The data on isolation, proliferation and functional tests of Melan-A-specific T cells are compatible with the results from the literature that show the feasibility of enrichment of peptide-specific T cells by CD137 assays. The minimal differences found in this constellation can be attributed to the vigorous response of both donors to the Melan-A peptide. When frequencies over 60% Ag-specific T cells after only one restimulation can be obtained without any enrichment assay, the additional effect of a non-tetramer enrichment should only have a negligible effect. As previously published [178] the precursor frequencies for the Melan-A peptide-specific T cells among naive CD8 T cells are exceptionally high compared to other tumour-associated and even viral Ag. However the responses to the Melan-A shown in the literature [99, 188] resulted in much lower frequencies of multimer-positive CD8 T cells than those shown in the results section, which could indicate that the cell culture conditions used are optimal for the generation of tumour-reactive CTL.

Notably Melan-A-specific T cells with memory phenotype can be found in the blood of healthy adults [188]. As a pure naive starting population was used for the experiments, a rapid expansion due to reactivation of contaminating memory T cells is very unlikely to be responsible for the unanticipatedly strong response. High IFN-γ release was observed in ELISPOT tests in both experiments, which is in keeping with data from the literature showing that over 85% of expanded Melan-A-specific T cells from healthy adults produce IFN-γ [188]. The magnitude of immune responses is known to correlate with the precursor frequency of naive T cells [189, 190]. Enrichment experiments with other tumour-associated peptides (e.g. WT1, gp100) with lower corresponding precursor frequencies of naive T cells could clarify further if the specific CD137 enrichment assay used would reproduce the data from the literature. Unfortunately these experiments could not be performed due to a lack of time.

6 Summary

In relapsed leukaemia patients having undergone allogeneic haematopoietic stem cell transplantation, donor lymphocyte infusions (DLI) can induce durable complete remissions. T cells in the DLI mediate the potentially curative graft-versus-leukaemia effect as well as the potentially life-threatening graft-versus-host-disease (GVHD). Administration of leukaemia-reactive T cells could provide a selective graft-versus-leukaemia effect and long-term protection from relapse due to immunological memory directed against the malignancy. Our group developed protocols for the *in vitro* generation of leukaemia-reactive T cells that show high cytolytic activity against acute myeloid leukaemia (AML) with minimal recognition of possible GVHD targets.

For translation to clinical application a protocol for the fast generation of sufficiently high T cell counts for adoptive transfer within approximately three weeks are needed. The shortening of the culture period could yield T cells of a central memory or early effector phenotype that have been shown to exhibit better *in vivo* effector function and persistence compared to T cells from prolonged culture. The activation marker and costimulatory receptor CD137 can be used to detect and isolate antigen-specific T cells without knowledge of the exact antigenic peptide. A CD137 enrichment step with clinical grade reagents could be an efficient *in vitro* means to improve the production of leukaemia-reactive T cells resulting in increased numbers of antigen-specific T cells not only due to positive isolation but also costimulation during early culture periods.

In this MD thesis mixed lymphocyte leukaemia cultures with CD8 T cells of healthy donors and HLA-matched or single HLA-allele-mismatched AML blasts were generated and restimulated weekly. After two weeks leukaemia-reactive T cells were positively selected by CD137 12 h after restimulation and cultured subsequently. The isolated fractions and unseparated controls were tested in ELISPOT and chromium release assays on day 5 after restimulation. No consistent major advantages were found concerning growth and functionality of the isolated CD137 positive fractions compared to the unseparated controls. Different isolation-methods, patient-donor-systems, modes of restimulation, temperature conditions, cytokine combinations and modes of cytokine administration as well as additional feeder cells or AML blasts after isolation did not substantially influence growth, functional data and the considerable cell loss during the isolation procedure. Live/death cell staining assays showed that activation induced cell death of CD137 positive

T cells could contribute to the results. As a major difference to AML blast-stimulation, successful CD137 enrichment has been reported for peptide-stimulated T cells. Different CD137 kinetics, activation induced cell death and regulatory T cells are potential factors that could make CD137 enrichment an unsuitable method in the context of AML blast stimulation.

The stimulatory effect of CD137 signals in tumour-reactive CD8 T cells was compared using CD3/CD28 and CD3/CD28/CD137 antibody coated magnetic beads. In renal cell carcinoma-reactive T cells CD3/CD28/CD137 beads were equally efficient as tumour stimulator cells and more efficient than CD3/CD28 beads. Both kinds of beads were suitable for stimulation during the first weeks of culture, so the additional CD137 signal could be useful for clinical grade expansion of tumour-reactive T cells. The bead expansion did not alter antigen-induced IFN-γ secretion in the ELISPOT assay, but caused moderate impairment of cytotoxicity as measured by chromium release assay. In contrast in AML-reactive CD8 T cells both kinds of beads showed a non-apoptosis-mediated, dose-dependent adverse effect, that led to a rapid decline in cell counts of bead-exposed T cells. Adverse effects of bead-stimulation on T cell functionality have been reported in the literature, however currently no publications are available that could provide a well-founded explanation for the effect in AML-reactive T cells. Despite literature data suggesting that CD137 might be a promising candidate for enrichment [33, 119] and expansion [116] of AML-reactive T cells, the overall data on CD137-isolation as well as on bead-stimulation indicate that in this specific setup CD137 may be an unsuitable activation marker and costimulatory ligand.

6.1 Zusammenfassung

Bei stammzelltransplantierten Patienten, die ein Rezidiv ihrer Leukämie erleiden, kann eine Donor-Lymphozyten-Infusion (DLI) dauerhafte vollständige Leukämieremissionen induzieren. T-Zellen in der DLI vermitteln sowohl den potentiell kurativen Graft-versus-Leukaemia (GVL) Effekt, als auch die potentiell lebensbedrohliche Graft-versus-Host Disease (GVHD). Hingegen könnte die Infusion von leukämiereaktiven T-Zellen einen selektiven GVL Effekt und einen Langzeitschutz vor Rezidiven durch eine spezifisch gegen die Leukämie gerichtete Immunantwort und Immunität vermitteln. Unsere Arbeitsgruppe hat Protokolle zur *in vitro* Generierung leukämiereaktiver T-Zellen entwickelt, die hohe zytotoxische Aktivität gegen akute myeloische Leukämie-Blasten (AML) bei minimaler Reaktion auf mögliche GVHD Zielstrukturen zeigen.

Für die klinische Anwendung sind diese Protokolle jedoch zu aufwändig, wobei vor allem eine erhebliche Verkürzung der Kulturzeit auf wenige Wochen erforderlich ist. Diese

Verkürzung der *in vitro* Kulturzeit könnte das Wachstum von T-Zellen vom *central memory* oder frühen *effector memory* Phänotyp fördern, für die eine bessere *in vivo* Effektorfunktion und längere Persistenz im Rezipienten verglichen mit T-Zellen aus Langzeitkultur gezeigt werden konnte. Der Aktivierungsmarker und Kostimulations-Rezeptor CD137 kann zur Erkennung und Isolation antigenspezifischer T-Zellen genutzt werden, ohne dass dafür das von den T-Zellen erkannte Peptidepitop bekannt sein muss. Eine CD137-vermittelte Anreicherung mit Hilfe von *clinical grade* Materialien könnte verwendet werden, um DLI-Produkte mit leukämiespezifischen T-Zellen herzustellen, die sich sowohl durch eine effizientere T-Zell Generierung durch *in vitro* Selektion und Kostimulation, als auch durch eine verbesserte Spezifität des T-Zell-Produkts auszeichnen.

Lymphozyten-Leukämie Cokulturen (*mixed lymphocyte leukaemia cultures*) wurden mit CD8 T-Zellen gesunder Spender und HLA-identischen oder einzel-HLA-*mismatch* AML-Blasten angesetzt und wöchentlich restimuliert. Nach zwei Wochen wurden die T-Zellen 12 Stunden nach Restimulation über den Marker CD137 positiv isoliert und anschließend separat weiterkultiviert. Die isolierten Fraktionen und unseparierten Kontrollen wurden im ELISPOT-Assay und im Chrom-Freisetzungstest an Tag 5 nach der Restimulation getestet. Es wurden keine konsistent nachweisbaren Vorteile im Hinblick auf Wachstum und Funktion der isolierten CD137-positiv Fraktion im Vergleich zur unseparierten Kontrolle gefunden. Verschiedene Isolationsmethoden, Patient-Spender-Systeme, Methoden zur Restimulation, Temperaturbedingungen, Zytokinkombinationen und Methoden der Zytokinzugabe sowie zusätzliche *Feeder*-Zellen oder AML-Blasten konnten Wachstum, funktionelle Daten und die deutlichen Zellverluste während der Isolation nicht entscheidend beeinflussen. Vitalfärbungen zeigten, dass aktivierungsinduzierter Zelltod CD137-positiver Zellen zu diesen Ergebnissen beitragen könnte. Im Gegensatz zur Stimulation mit AML-Blasten wurden erfolgreiche CD137-Anreicherungen für peptidstimulierte T-Zellen publiziert. Unterschiedliche CD137-Expressionskinetiken, aktivierungsinduzierter Zelltod und regulatorische T-Zellen sind mögliche Faktoren aufgrund derer die CD137-Anreicherung in diesem spezifischen Kontext ungeeinet sein könnte.

Der stimulatorische Effekt eines CD137-Signals auf AML-reaktive CD8 T-Zellen wurde mit Hilfe von CD3/CD28 und CD3/CD28/CD137 Antikörper-beschichteten magnetischen *beads* untersucht. Für Nierenzellkarzinom-reaktive T-Zellen war die Stimulation mit CD3/CD28/CD137 *beads* genauso effektiv wie mit Tumorzellen und effektiver als mit CD3/CD28 *beads*. Beide Arten von *beads* waren für eine Stimulation während der ersten Wochen der Zellkultur geeignet, sodass ein zusätzliches CD137-Signal für die länger anhaltende Expansion tumorreaktiver T-Zellen zur klinischen Anwendung nützlich sein könnte. Die *bead*-Expansion veränderte die IFN-γ-Sekretion im ELISPOT nicht, aber verursachte eine mäßige Verschlechterung der Zytotoxizität im Chrom-Freisetzungstest.

Im Gegensatz dazu zeigten bei AML-reaktiven T-Zellen beide Arten von *beads* einen nicht apoptosevermittelten, dosisabhängigen zellschädigenden Effekt, der zu einer raschen Abnahme der Zellzahl in Kulturen mit *beads* führte. Unerwünschte Effekte auf die T-Zell-Funktionalität durch *bead*-Stimulation sind in der Literatur beschrieben, dennoch gibt es aktuell keine Veröffentlichungen, die eine fundierte Erklärung für den Effekt auf AML-reaktive T-Zellen bieten könnten. Abgesehen von Literaturdaten, die darauf hindeuten, dass CD137 ein vielversprechendes Kandidatenmolekül für die Anreicherung [33, 119] und Expansion [116] von AML-reaktiven T-Zellen sein könnte, zeigen die eigenen Daten sowohl zur CD137-Isolation als auch zur *bead*-Stimulation, dass für diese spezielle Anwendung CD137 ein ungeeigneter Aktivierungsmarker und Kostimulations-Ligand ist.

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