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The T-Cells' Role in Antileukemic Reactions - Perspectives for Future Therapies'

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1. Introduction

1.1 Background (Schmid, Schmetzer)

Highly specialized and sensitive defence against infections as well as tumors is provided in great part by the adaptive, T-cell-mediated part of our immune system. Those T-cell specialists arise out of a cell pool of 25-100 million distinct naïve T-cell clones, after a very efficient priming phase by dendritic cells (DC), that present antigens in the context of major histocompatibility complexes (MHC), T-cell receptors (TCR) and costimulatory signals. It is well known, that T-cells are most important effectors of cellular tumor immunity and carry long lasting memory. Therefore recent tumor research has focused on the development and improvement of T-cell based immunotherapies (Barrett & Le Blanc, 2010; Smits et al., 2011). The T-lymphocyte pool includes naïve (T_{naive}), effector (T_{eff}), effector memory (T_{em}) and central memory (T_{cm}), either CD8 or CD4 T-cells. After an antigen driven TCR engagement T_{naive} proliferate and give rise to large numbers of T_{eff} . Most of them die after depleting target cells. Some 'memory T-cells' of either T_{cm} or T_{em} phenotype remain, persist and can differentiate to T_{eff} after a re-challenge with antigens. It has been demonstrated that memory T-cells are able to self-renewal and differentiation (Sallusto et al., 2005). However antitumor immunity is limited by regulatory T-cells (T_{reg}) that are in general responsible for the prevention of autoimmunity, regulation of inflammatory antimicrobial or antitumor reactions and are regarded as the mediators of tolerance (Vignali et al., 2008). T_{reg} reactions can be either mediated by inhibitory cytokines (IL-10, IL-35, TGF- β), Granzyme A/B dependent cytotoxicity, CD25 dependent IL-2 deprivation-mediated apoptosis, adenosine receptor mediated immunosuppression of DCs' maturation (Vignali et al., 2008). T_{reg} subtypes can be identified by their expression profiles and be subdivided in resting or activated T_{regs} of either CD4 or CD8 subtype (Miyora et al., 2009; Jordanova et al., 2008; Schick et al., 2011). Resting T_{regs} can convert to activated T_{regs} after proliferation. The functional repertoire of T-cells depends on their survival and their cooperation via cellular contact and the secretion of humoral factors. Improved knowledge of these properties should contribute to detect or select T-cells with defined markers or functional profiles for adoptive therapy.

In the past the potential of adoptively transferred T-cells to eradicate tumor cells has been intensively studied in patients with melanoma, EBV-associated tumors and especially in the context of a transplantation of allogeneic hematopoietic stem cells including immunocompetent cells in patients with lymphoid- or myeloid-derived malignant diseases (Kahl et al., 2007; Kolb et al., 2004; Schmid et al., 2011; Parmar et al., 2011; Moosmann et al., 2010).

In patients with acute myeloid leukemia (AML), a malignant clonal disease of the hematopoietic stem cells, conventional chemotherapy induces remission in 60- 80% of patients. However, relapse occurs in the majority of patients in the following two years (Buechner et al., 2003). Allogeneic stem cell transplantation (SCT) is considered as a curative therapy for patients with AML: Following engraftment of donor cells, the established hematopoietic chimerism persists even after discontinuation of immunosuppressive therapy, reflecting tolerance both in Graft-versus-Host and Host-versus-Graft direction (Kolb et al., 2004; Schmid et al., 2011). T-cells of the healthy donor mediate the 'graft versus leukemia' (GvL), effect which was perceived in principle as early as in the 1960ies. Clinical evidence for the efficacy of GvL reactions in AML came from the observation, that leukemia relapse after allogeneic SCT, the most frequent reason of treatment failure in AML, occurred most frequently in patients who had either been transplanted from a syngeneic twin, or had received a graft which had been depleted from donor T-cells prior to transfusion. In contrast, relapse incidence was lowest in patients developing acute or chronic Graft-versus-Host Disease (GvHD), which represents the second clinical manifestation of the allogeneic immune reaction mediated by donor T-cells (Hemmati et al., 2011; Schmid et al., 2007; Schmid et al., 2011; van den Brink et al., 2010). Based on these observations, the infusion of donor lymphocytes (DLI) was developed as a form of immunotherapy for relapsed disease after allogeneic SCT. However, up to now, not every patient, in particular those with rapidly proliferating AML, responds to or permanently benefits from those T-cell based immunotherapy.

1.2 Aims (Schmetzer)

The ability of T-cells to eliminate tumor cells and even to cure tumors has been demonstrated in experimental animal models. In man the development of effective T-cell therapies to treat human tumors remains still a challenge. Tumor antigens that elicit curative responses have been identified in animal models; in man tumor associated antigens are also known, but their use is limited due to HLA-restriction and limited duration of responses. This applies also to hematological malignancies as AML. In the last years several approaches have been made to further characterize T-cells and to explore the role of soluble and cellular factors on the regulation and mediation of antitumor reactions in AML-patients. Known tumor antigens may help to identify specific T-cells and their subtypes. This could be monitored in the course of treatment and they could be prepared for further treatment. However the majority of potential tumor antigens is unknown. Their presence may be deduced from T-cell reactions initiated and mediated by leukemia-derived dendritic cells (DC), presenting the whole antigenic repertoire of the leukemic cell. In analogy to T-cell reactions against known tumor antigens DC-stimulated T-cells reacting against unknown tumor antigens may be analysed against healthy cells before further use. In addition the identification of possible immune escape phenomena in cases without successful SCT or DLI or without antileukemic functions *ex vivo* could contribute to develop strategies to overcome those immunological barriers.

In this chapter we want to present experimental and clinical results of our group with a special focus on the following topics and discuss perspectives for future therapies:

- T-cells addressing known (leukemia-) specific antigens
- T-cells addressing unknown leukemia-specific antigens
- T-cell profiles to predict antileukemic reactions and prognosis
- Clinical use of donor T-cells for prevention and treatment of AML relapse after allogeneic SCT
- Perspectives for future therapies: adoptive transfer or *in vivo* activation of antileukemic T-cells?

2. Research methods (Schmetzer)

Cellular characterizations (especially of T-cells, leukemic blasts and dendritic cells) were performed by Flow Cytometric Analyses applying a panel of marker-specific, fluorochrome labelled monoclonal antibodies: **T-cells:** Naïve T-cells ($T_{naïve}$) CD45RO-CCR7⁺; non-naïve T-cells ($T_{non-naïve}$) CD45RO⁺; central memory T-cells (T_{cm}) CD45RO⁺CCR7⁺CD8⁺; Effector memory T-cells (T_{em}) CD45RO⁺CCR7-CD27⁺; Effector T-cells (T_{eff}) CD45RO⁺CCR7-CD27⁻; Regulatory T-cells ($CD8^{+}T_{reg}$) CD8⁺CD25⁺CD127_{low}; ($CD4^{+}T_{reg}$) CD4⁺CD25⁺CD127_{low}; ($T_{naïve reg}$) CD25⁺CD127_{low}CCR7⁺CD45RO⁻; ($T_{cm reg}$) CD25⁺CD127_{low}CCR7⁺CD45RO⁺; ($T_{eff/em reg}$) CD25⁺CD127_{low}CCR7-CD45RO⁺ (Liepert et al., 2010; Vogt et al., 2011; Schick et al., 2011). **Blasts:** Myeloid cells co-expressing patient-specific markers (e.g. CD34, CD117, CD56, CD65); **DC:** DC co-expressing DC-antigens; Mature DC: DC coexpressing CD83; DC_{leu} : DC coexpressing DC-antigens (e.g. CD80, CD86, CD40) with blast-markers; (Schmetzer et al., 2007; Kremser et al., 2010). Untouched or touched CD4⁺, CD8⁺ or CD3⁺ T-cells were isolated by Magnetic labelled cell sorting (MACS) (Schick et al., 2011; Vogt et al., 2011; Grabrucker et al., 2010), leukemia-antigen specific T-cells by Interferon gamma (IFN- γ) capture assay (Neudorfer et al., 2007) or by MHC-multimer (Streptamers) staining for several described leukemia associated antigens (Knabel et al., 2002). In some cases spectratyping analyses were performed to observe clonal restriction among T-cells characterized by defined T-cell receptor-V β chains (Schuster et al., 2008). The expression of leukemia-associated antigens (LAA; e.g. WT1, PRAME, PR1) was evaluated by PCR-technology (Steger et al., 2011). 'Taq man low density arrays' were used to study expressions of most of the known protein-coding Y-chromosome genes (Liu et al., 2005). To predict the HLA-A0201 binding potential of selected peptides a HLA-A0201 peptide binding assay was performed by using the HLA-A0201 positive TAP-deficient T2 cell line system (Nijman et al., 1993; Saller et al., 1985). Leukemic blasts were cultured in 'DC-media' containing a cocktail of immune-modulators and cytokines, (thereby converting blasts to leukemia-derived DC (DC_{leu}), theoretically presenting the whole leukemic antigen spectrum (Kremser et al., 2010; Schmetzer et al., 2007).

Alternatively LAA/HA1 or 'male specific' antigens were loaded as peptides or full length proteins on either unmanipulated or irradiated antigen presenting cells (APC; e.g. MNCs or CD4 cell depleted cell fraction (Adhikary et al., 2008), EBV-transformed B-cells 'mini-LCL' or DC (Moosmann et al., 2002) were irradiated with 45/80 Gray and used for T-cell stimulations as given in figure 3.1.3-1 (Steger et al., 2012; Bund et al., 2011). Antileukemic reactivity of T-effector cells was measured by chromium release-, IFN- γ ELISPOT assays or non-radioactive Fluorolysis assays (Bund et al., 2011; Kremser et al., 2010). Intracellular cytokine staining (ICS) and cytokine release profiles were performed by FACS (Cytometric Bead arrays (CBA)), ELISA or ELISPOT (Elbaz & Shaltout 2001; Schmittle 2000; Fischbacher

et al., 2011; Merle et al., 2011; Bund et al., 2011). In a dog model we performed in addition an *in vivo* immunisation with antigen positive cells (Bund et al., 2011).

Using these methods we could evaluate antigen expression profiles on T-cells, antigen presenting cells or blast cells as well as cytokine secretion profiles assigning these profiles to cellular subtypes and correlate them with antileukemic reaction profiles or the clinical response to immunotherapies. Moreover we could contribute and quantify reaction profiles of 'antigen stimulated' or '-unstimulated' as well as of (specifically) selected, enriched or cloned T-cells against blast targets. Statistical evaluations were performed with standard excel programmes or SPSS software.

3. Experimental key results

In the following chapter the most important experimental results generated by our group are summarized. Responsible co-workers and cooperation partners are given and their contributions listed below.

3.1 T-cells addressing known (leukemia-) specific antigens

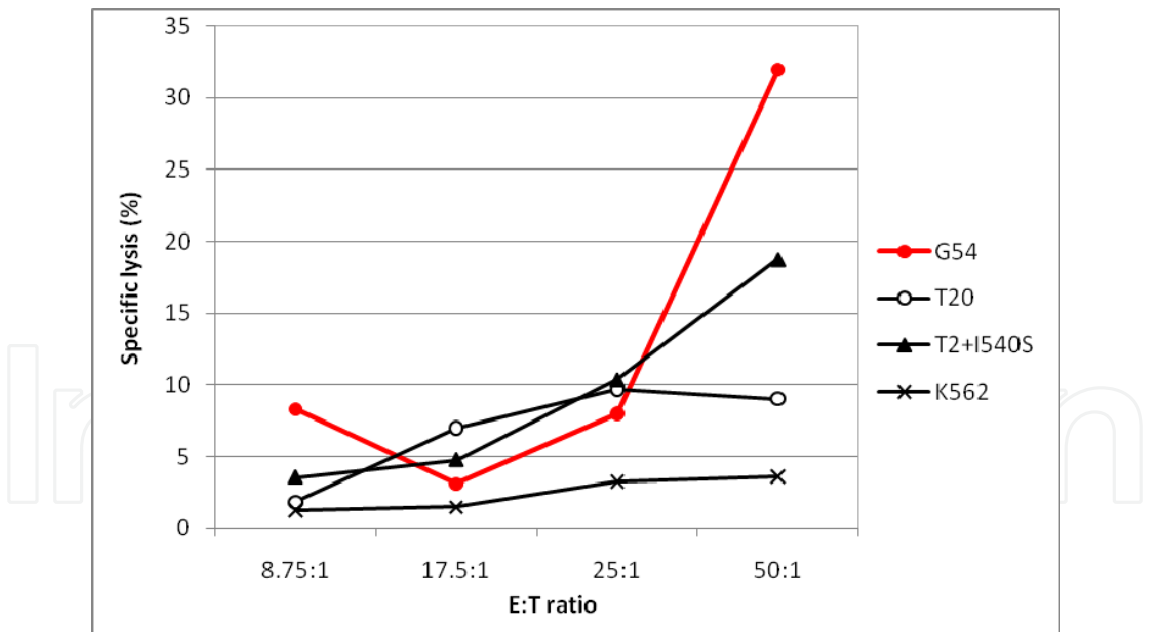
3.1.1 Y-chromosome encoded proteins overexpressed in acute myeloid leukemia and CD8+ T-cell reactions (Group leaders: Kolb, Adamski; Scientists: Bund, Gallo)

In haploidentical SCT we and others observed that female-donors (especially mothers) show a higher GvL reactivity against male-patients (particularly sons) compared to all other haploidentical donor-recipient combinations for AML patients (Stern et al., 2008). These effects could be due to Y-chromosome-encoded male minor histocompatibility antigens (minor-H-Ags, mHAs) recognized by female alloimmune effector (memory) T-cells, immunized during pregnancy, in the context of a GvL-reaction (Ofra et al., 2010). We studied the expression profiles of Y-chromosome genes in healthy male stem cell donors compared to male AML patients in order to possibly detect new AML-typical Y-restricted expression patterns. Blasts from male patients with acute (myelo) monocytic leukemia and monocytes from healthy male donors as the healthy control counterpart were used to determine and compare the expression profiles of Y-chromosome genes. We could detect several genes being up-regulated in male AML-cells. Among those, we focused on *PCDH11*, *VCY*, *TGIF2LY*, known to be expressed only in male tissues and its X-chromosome-encoded homolog *TGIF2LX* (Blanco-Arias et al., 2002; Skaletsky et al., 2003; Gallo et al., 2011). In a next step, we studied the immunological impact of the identified Y-encoded genes. We analyzed the proteins encoded by those four genes for the presence of nonameric peptides that could potentially bind HLA-A0201 molecules. Such analysis was performed with the help of publicly available peptide-motif scoring systems (http://bimas.dcrt.nih.gov/molbio/hla_bind/ and <http://www.syfpeithi.de>; Rammensee et al., 1995). High-scoring peptides were not found for *PCDH11*, whereas HLA-A0201-binding peptides could be identified *in silico* for the *VCY*, *TGIF2LY* and *TGIF2LX* genes. Their effective binding efficacy was determined in a standard HLA-A0201-T2 binding assay: Two peptides derived from *VCY* as well as from *TGIF2LX* and one derived from *TGIF2LY* were able to bind to the HLA-A0201-molecules of the T2 cells. Furthermore these peptides were tested for their ability to induce a CD8+ T-cell response: we selected CD3+ T-cells from female volunteers and cultured them in the presence of T2 cells loaded with the different Y-chromosome-encoded peptides for four weeks. We could show that the *VCY*-encoded peptide G54 stimulated female effector T-cells as shown by a specific lysis of G54-loaded T2 target cells in a chromium release assay

(Figure 3.1.1-1). Further research will focus on the immunogenic G54-peptid particularly with respect to our assumption that mothers (who had given birth to a son) bear CD8+ T-cells being reactive to male-specific antigens, leading to a strong GvL effect. Moreover, peptides restricted to other HLA-types will also be investigated.

In conclusion we identified three new potentially antileukemic, male-specific human genes being upregulated in blasts of male AML-patients. These might be valuable targets for T-cells.

As a rule Y-chromosome coded antigens are expressed in all cells of the organism. However some data have been reported, that differentially spliced forms of the Y-linked UTY (ubiquitously-transcribed tetratricopeptide-repeat-gene, Skaletsky et al., 2003) may be restricted in a tissue restricted fashion (Warren et al., 2000). UTY is highly conserved in man and animal species. We worked out in a dog model *in vitro* and *in vivo* that the Y-chromosome coded mHA UTY might be a promising candidate target-structure to improve GvL immune reactions after SCT: female T-cells were stimulated with either autologous (female) DCs, loaded with three different UTY-derived (male) peptides or with allogeneic, donor-matched male cells (PBMCs) endogenously expressing UTY. We identified 3 out of 15 identified UTY-encoded peptides bearing immunological potential to stimulate 'antimale'-(i.e. anti-UTY-) directed immune reactions. Amongst those, W248 showed highest immunogenic potential in both *in vitro* and *in vivo* settings: *In vitro* expanded CTLs specifically recognized mainly bone-marrow (BM) from DLA-identical male littermates in an MHC-I-restricted manner (Figure 3.1.1-2; *in vitro*). *In vivo*, comparable W248-(UTY-) specific reactivity against BM was also obtained after stimulation and immunization of a female dog with DLA-identical male PBMCs (Table 3.1.1-1; *in vivo* (Bund et al., 2001)).



T2-cells were loaded with the VCY-derived G54-peptide and incubated with female HLA-A0201+ T-cells for 4 weeks. Cytotoxic activity of the generated G54-specific CTLs were tested in Chromium release assays (E:T = 8.75:1 to 50:1). T-cells specifically lysed T2-cells loaded with the cognate peptides G54 (G54, ● red). T2-cells alone (T20, ○), T2-cells loaded with I540S (non-HLA-A0201 binding; HFLLWKLIA; T2+I540S, ▲) and K562-cells (NK-cell target, ×) were not recognized or only to a low extent

Fig. 3.1.1-1. Female T-cells stimulated with the Y-chromosomally encoded G54-peptid can specifically lyse T2-cells loaded with G54 *in vitro*.

		E:T*	male DLA-identical target cells	W248-specific spots/100,000 T-cells*
In vitro	80:1		BM	85
			BM + <anti MHC-I antibody>	24
In vivo	20:1		BM	45
			BM + <anti MHC-I antibody>	25
			BM + W248-peptide	35
			BM + W248-peptide + <anti MHC-I antibody>	15

BM bone marrow; <MHC-I>=<MHC-I>-antibody.

* E:T= Effector-to-target-ratio

**number of UTY-specific spots per 100,000 T-cells

T-cells from female dog(s) were expanded using autologous DCs pulsed with the UTY-encoded peptide W248 (in vitro; n=3/6) or male DLA-identical PBMCs (in vivo; n=1). Female UTY-specific T-cell recognition was determined in IFN-γ-ELISPOT-assays (day 21-28 (E:T = 80:1) and day 49 (E:T = 20:1), respectively). Female T-cells mainly recognized male DLA-identical BM in vitro and in vivo verifying the male-specific UTY-presentation. T-cells` MHC-I-restriction was shown by an anti-MHC-class-I-mAb.

Table 3.1.1-1 Female dog T-cells stimulated with UTY (W248)-peptides loaded on autologous DC *in vitro* or male DLA-identical PBMC *in vivo* specifically recognized ‘male’ target cells

Taken together we could demonstrate, that female dog effector T-cells could be specifically stimulated against male, *UTY*-gene product specific cells meaning in turns, that UTY seems to be a promising candidate antigen to improve GvL reactions after SCT.

3.1.2 LAA-specific CD8+ T-cells (Groupleaders: Busch, Borkhardt, Kolb; scientists: Doessinger, Steger, Schuster)

Between 60-90% of AML cases overexpress leukemia-associated antigens (LAA), that means antigens that are absent or only weakly expressed in normal tissues (e.g. WT1, PR1 or PRAME (Greiner et al., 2006; Steger et al., 2011)). Therefore T-cell based immunotherapeutic strategies addressing those LAA-expressing cells could be promising. Alternatively mHAs, preferentially expressed on hematopoietic cells, could qualify as T-cell targets in a GvL reaction (Mutis & Goulmy, 2002). Principally T-cell based strategies could be based on a vaccination with LAA/mHAs or by identification, selection and transfer of LAA/mHAs specific T-cells already present in the donor. LAA/mHAs specific T-cells can be found at a low frequency in normal persons implying a low level of immunity. Vice versa a long lasting immunity against leukemic cells overexpressing LAA or mHAs should imply the presence of specific T-cells. Therefore our experimental approach was to detect LAA-specific T-cells by MHC-multimer technology in AML patients after SCT. We constructed human HLA-A2 peptide multimers (Knabel et al., 2002) and tested CD8+ T-cells in 5 AML- and 2 MDS-patients after SCT for antigen specificity, as given in table 3.1.2-1

Patients (pt)	Dgn.	stage of the disease at T-cell aquisition	Cytogen. Marker at first dgn.	Blasts in PB at sample aquisition	IC blast phenotype (CD) in acute phases	IC mono-cytes (%)	IC B-cells (%)	IC T-cells (%)	IC NK-cells (%)	performed analyses
pt 1147	AML-M2	CR2 after SCT and DLI	+21,+21 , +21	0	7,33,34,117	nd	nd	nd	nd	MHC-multimer staining
pt 1148	AML-M2	CR after 3 rd SCT	inv (3qq)	0	13,33,34,117	nd	nd	nd	nd	MHC-multimer staining, ICS
pt 1149	AML-M4	CR after SCT and DLI	46, XX	0	13,15,33,34 , 117	7	6	14	6	MHC-multimer staining, ICS
pt 1150	MDS-RAEB I	CR after SCT and DLI	-5, +1 (at relapse)	0	13,33,34,117	11	6	13	7	MHC-multimer staining, ICS
pt 1151	AML-M4	CR after SCT	46, XX	5	15,33,7,65, 64,4	nd	nd	28	12	MHC-multimer staining, ICS
pt 1152	AML-M5	CR after SCT	46, XX	0	nd	nd	nd	nd	nd	MHC-multimer staining, Spectraty-ping
pt 1153	MDS-CMML	Pers after SCT and DLI	46, XX	8	nd	nd	nd	nd	nd	MHC-multimer staining, ICS, LAA, CD4+ exp.
pt 1154	MDS-RAEB II	CR after SCT and DLI	46, XY	0	nd	10	3	34	8	ICS, LAA, CD4+ exp.
pt 1155	MPS-atyp. CML	CR after SCT and DLI	t (8;22)	0	nd	nd	nd	nd	nd	ICS, LAA, CD4+ exp.

nd not done; CR complete remission; Rel. relapse; MDS myelodysplastic syndrome; RAEB Refractory Anaemia with Excess Blasts; AML-M3 acute myeloid leukemia FAB M2; CMML Chronic Myelomonocytic Leukemia; LAA leukemia associated antigen, overexpression analyses compared to healthy controls, detected by the $RQ=2^{-\Delta\Delta ct}$ method in MNCs; dgn. diagnosis; ICS intracellular staining; CD4+ exp. CD4+ experiments

Table 3.1.2-1 Patients' characteristics I:

In all of these 7 cases we could detect LAA-specific CD8+ T-cells (that means more than 0.1% T-cells with LAA specificity) –although not directed against all given LAA, suggesting the persistence of T-cells with antileukemic potential (table 3.1.2-1 and fig 3.1.2-2). Four of five cases with two different types of LAA- specific CD8+ T-cells (pt 1149, 1151, 1152, 1153) were characterized by long-lasting clinical remissions for more than 2 years. One patient with two different types of LAA-specific CD8+ T-cells relapsed after 9 months (pt 1151) and in addition the patient with only one type of LAA-specific T-cells, who relapsed after one

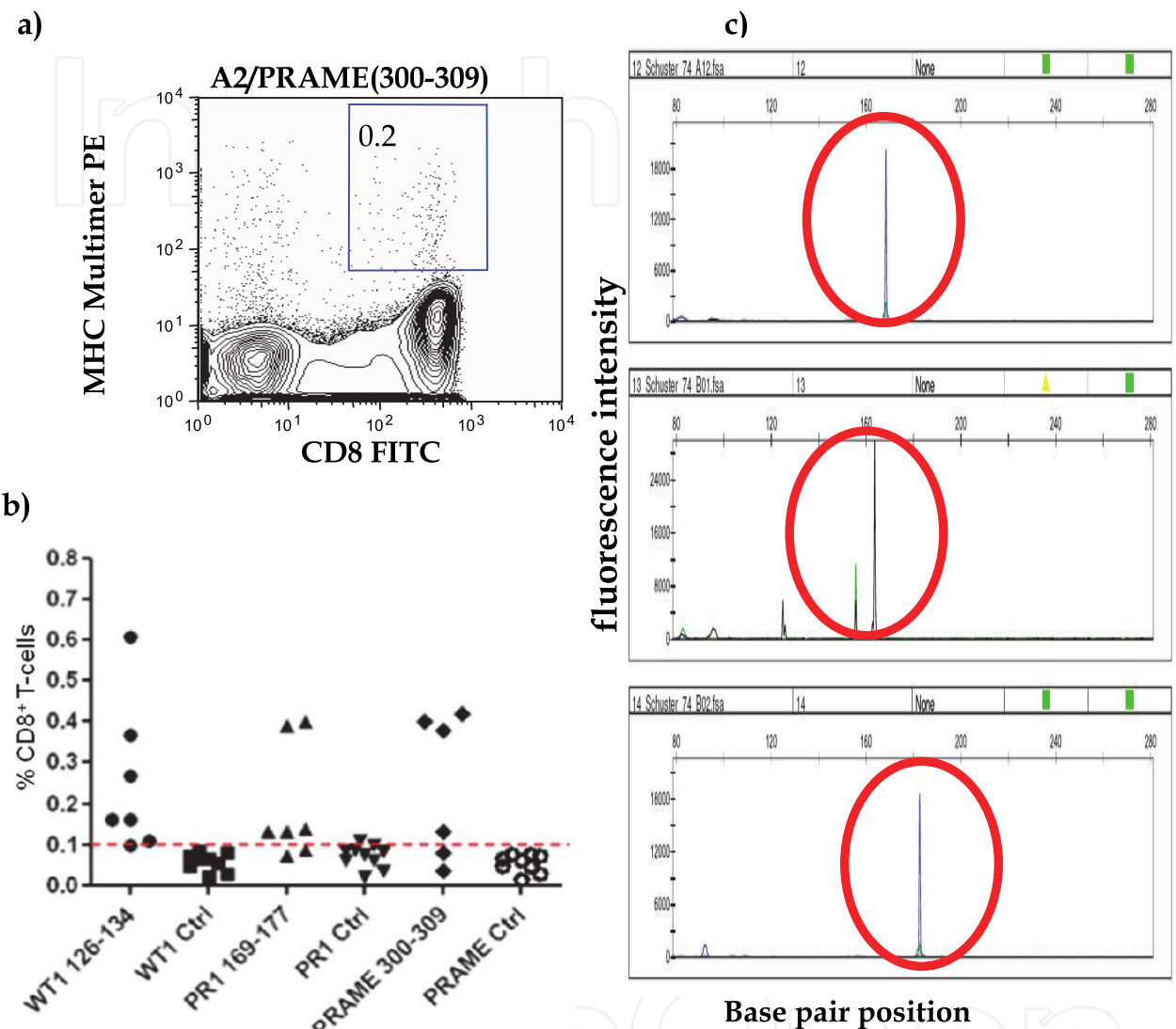
year (pt 1147). One patient could not be analysed under a clinical point of view since he died one month after sample acquisition of an infect (pt 1148, table 3.1.2-2, Steger et al., 2012).

Patients (pt)	LAA over-expression in MNCs (at sample preparation)	Peak response MHC-Multimer staining (% of specific CD8+ T-cells)	Cytokine profile (IFN- γ , IL-2) after LAA-stimulation	Time to relapse or last follow up
			CD 4+/C8+ T-cells	
pt 1147	nd	A2/WT1 0.11 A2/PR1 0.13 A2/PRAME 0.034	NR/NR	04/07 local rel.; 08/09 systemic rel., 05/11 CR after 2 nd SCT, DLI and 3 th rel.
pt 1148	nd	A2/WT1 0.37 A2/PR1 0.4 A2/PRAME nd	NR/NR	03/06 death with infect in CR after SCT
pt 1149	nd	A2/WT1 0.61 A2/PR1 0.39 A2/PRAME 0.4	NR/NR	until 02/11 in CR after SCT
pt 1150	nd	A2/WT1 0.27 A2/PR1 0.13 A2/PRAME 0.081	NR/NR	until 03/11 in CR after SCT
pt 1151	nd	A2/WT1 0.16 A2/PR1 0.088 A2/PRAME 0.42	NR/NR	11/06 rel; 04/07 death after 3 th DLI, rel. and 2 nd SCT
pt 1152*	nd	A2/WT1 0.16 A2/PR1 0.074 A2/PRAME 0.38	NR/NR	until 04/11 in CR after SCT
pt 1153	WT1 (-), PRAME (+), PR1 (-)	A2/WT1 0.1 A2/PR1 0.14 A2/PRAME 0.13	PRAME 0.32/NR	until 02/11 in CR after SCT
pt 1154	WT1 (-), PRAME (-), PR1 (-)	nd	NR/NR	until 07/11 in CR after SCT
pt 1155	WT1 (-), PRAME (-), PR1 (-)	nd	NR/NR	until 07/11 in CR after SCT

No LAA overexpression (-), 1-10x LAA overexpression (+), compared to healthy controls, analysed by the $RQ=2^{-\Delta\Delta Ct}$ method; nd not done
Bold: > 0.1% CD8+ T-cells defined as ‘LAA-specific T-cells present’; * restricted T-cells by spectratyping in PRAME- MHC multimer selected T-cells detectable

Table 3.1.2-2 Anti-LAA-peptide reactive T-cells are detectable by MHC-Multimer staining in all given PB samples from AML/MDS patients after allogeneic SCT

In one case (pt 1152) we performed spectratyping in T-cells selected for PRAME-specificity and could demonstrate a highly restricted TCR-repertoire. This points to a specific clonal expansion of CD8+ cells after (*in vivo*) PRAME challenge by residual PRAME overexpressing blasts (Figure 3.1.2-1).



a) representative FACS-plot shows MHC-Multimer staining from peripheral blood of an AML-patient after SCT.
b) LAA-specific T-cells from PBMCs from AML-pts after SCT were labelled with MHC multimers binding T-cells, that recognize frequently overexpressed LAA antigens. PBMCs were analyzed by FACS. Healthy donors served as controls and multimer-staining revealed a threshold value of 0.11% multimer-positive cells in the CD8 cell fraction.
c) MHC-multimer positive CD8+ T-cells from peripheral blood of an AML-patient after SCT were enriched by FACS sorting. RNA was extracted and Spectratyping of the Vβ-composition reveals strong clonal restriction among TCR-species (1rd row: Vβ3-Jβ1.5; 2nd row: Vβ3-Jβ2.1; 3rd row: Vβ3-Jβ2.5)

Fig. 3.1.2-1. LAA-specific T-cells can be isolated from AML-patients after SCT by MHC multimer technique. Selected T-cells are highly Vβ restricted

Upon in vitro peptide-challenge we could not detect secretion of IFN- γ or IL-2 in T-cells by intracellular cytokine staining in 7 out of 8 patients tested (table 3.1.2-2). This however does not exclude a cytokine-independent functionality of multimer positive T-cells. Interestingly we could detect an IFN- γ response in CD4+ T-cells in one case (pt 1153) with PRAME overexpressing leukemic blasts detectable during persisting relapse after SCT. This is very surprising as the peptide triggering this response is MHC class I restricted and could indicate a CD8 coreceptor independent binding of a MHC I directed TCR and an unusual involvement of CD4+ T-cells in the leukemia directed immune response.

In summary that means, that in general LAA-specific, HLA-A2-restricted CD8+ T-cells can be prepared by MHC multimer technology from most of the patients after SCT at various time points. Possibly the simultaneous detection of two different LAA-specific CD8+ T-cells correlates with a higher chance of longlasting remissions. Optimal time points have to be evaluated for the preparation of (sufficient) LAA-specific CD8+ T-cells that could be used for adoptive transfer and concerning minimal amounts needed for maintenance of remission.

3.1.3 LAA-specific CD4+ T-cells (Groupleaders: Buhmann, Milosevic, Kolb, Schmetzer; scientists: Steger)

CD8+ T-cells recognize HLA-class I restricted peptides, therefore they can mediate strong cytotoxic, antileukemic reactions, but also severe graft versus host (GvH) disease. In contrast CD4+ T-cells recognize HLA class II restricted peptides that are mainly expressed by cells of the haematopoietic system and absent from other organs. In order to further analyse the antileukemic function of CD4+ T-cells we prepared (untouched) CD4+ T-cells from 6 patients, as given in table 3.1.3-1, after SCT or DLI immunotherapies and stimulated them with LAA-proteins (WT1, PRAME, PR1 and the mHA HA-1), that were loaded on the CD4 depleted cell fraction (containing monocytes and DC as antigen presenting cells (APCs)) or on 'mini-LCL'.

During the stimulation phase the cells lost their naïve (and central memory) T-cell phenotype and gained an effector memory or effector cell phenotype (data not shown).

As already shown for CD8 selected T-cells cytokine release assays for IFN- γ (ELISPOT, ICS) or GM-CSF (ELISA) did not reveal clear and specific cytokine release profiles of LAA or HA-1 stimulated, expanded or cloned CD4+ T-cells (data not shown).

Therefore we performed a functional Fluorolysis assay in case pt 1158: *blast cells* of the patient, that were characterized by a overexpression of WT1, PRAME and PR1 at first diagnosis served as leukemic target and *fibroblasts, effector cells* or *non-blast cells* of the patient as negative controls. *Effector cells* (E) used for these assays were: unstimulated MNC, CD3+ or CD4+ T-cells obtained in different stages of the disease, LAA or HA-1 stimulated CD4+ T-cells in different stimulation phases, enriched proliferating and CD40L+ CD4+ cells before or after single cell cloning (table 3.1.3-2).

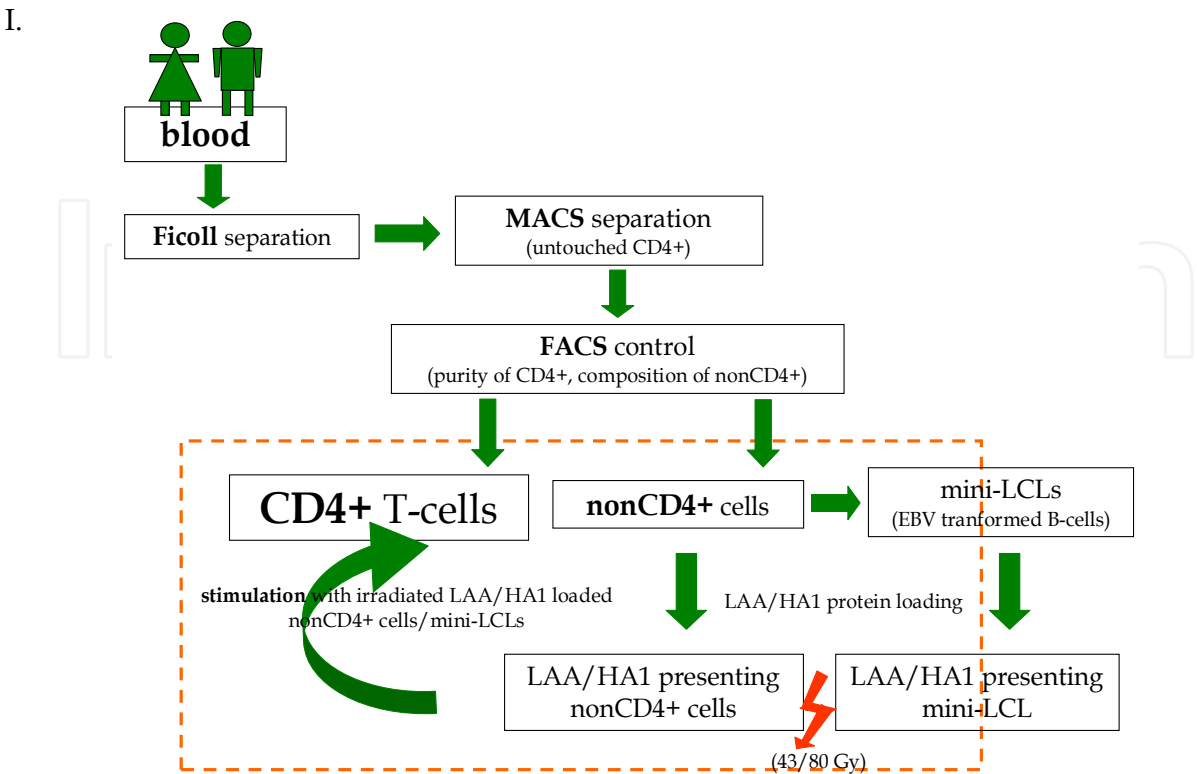
We could demonstrate that most of the CD4+ T-cell containing effector cell fractions (except MNCs before SCT, CD4+ T-cells after 16 stimulation rounds (E11) and two of the CD4+ T-cell clones (E13, E15)) were able to regularly and specifically lyse leukemic blasts, but not fibroblasts or non-blast-cells of the patient. Highest antileukemic activity was demonstrated for enriched proliferating and CD40L+CD4+ T-cells followed by one of the three tested clones. Unfortunately despite of provable (CD4+) immunity against leukemic cells the patient died two years after SCT in hematological CR from a myocardial chloroma.

Patients (pt)	Dgn.	T-cell source (stage of disease)	Blasts in PB at sample aquisition	Cell source	Analyses performed in addition to CD4+experiments
pt 1153	MDS-CMML	Pers after SCT	8	CD4+, CD8+, MNCs	ICS, IFN-γ ELISPOT, MHC-Multimer staining, LAA analyses
pt 1154	MDS-RAEB II	CR after SCT	0	CD4+, CD8+, MNCs	ICS, IFN-γ ELISPOT, LAA analyses
pt 1155a and 1155 b	MPS-atyp. CML	CR after SCT	0	CD4+, CD8+, MNCs	ICS, IFN-γ ELISPOT, GM-CSF ELISA, LAA analyses
pt 1156	Biphen. ALLL/AML	CR after SCT	0	CD4+	IFN-γ ELISPOT
pt 1157	AML-M4	CR after SCT	0	CD4+	IFN-γ ELISPOT
pt 1158 a ('E1')	AML-M4	CR before SCT	0	MNCs	LAA analyses, Fluorolysis assay
pt 1158 b ('E2')	AML-M4	CR after 1 st SCT and 1 st DLI	0	CD4+	Fluorolysis assay
pt 1158 c ('E3') pt 1158 d ('E12': after P5) pt 1158 e ('E11': after P16) pt 1158 f ('E6': after P17,FACS-Sort, P14) pt 1158 g, h, i ('E13, 14, 15' clones, after FACS-Sort, P14)	AML-M4	CR after 1 st SCT and 1 st DLI	0	CD4+	ICS, IFN-γ ELISPOT, GM-CSF ELISA , Fluorolysis assay
pt 1158 p ('blast targets')	AML-M4	Rel. after 1 st SCT and 2 nd DLI	34	CD3 depleted blasts	Fluorolysis assay
pt 1158 j ('E4')	AML-M4	Rel. after 1 st SCT and 2 nd DLI	nd	CD4+	Fluorolysis assay
pt 1158 k ('E5')	AML-M4	CR after 2 nd SCT and 4 th DLI	0	CD4+	Fluorolysis assay
pt 1158 l ('E16')	AML-M4	CR after 2 nd SCT and 4 th DLI	0	CD4+	Fluorolysis assay
pt 1158 m, n, o ('E7, 8, 9')	AML-M4	CR after 2 nd SCT and 4 th DLI	0	MNCs, CD3+, CD4+	LAA analyses, Fluorolysis assay

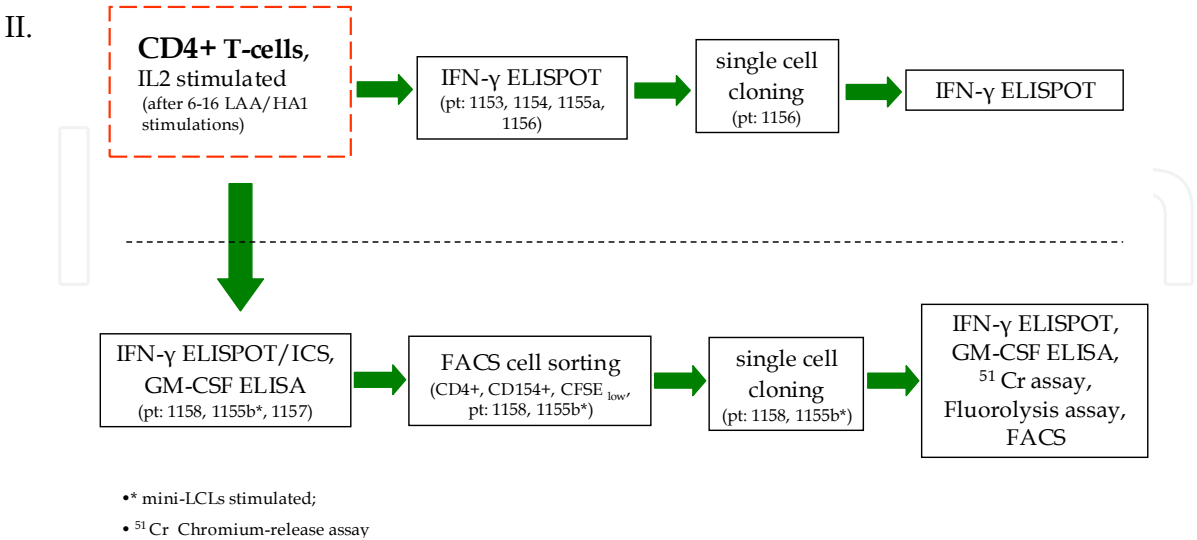
Dgn. diagnosis; Pers persistant disease; Rel. relapse; CR complete remission; MDS myelodysplastic syndrome; RAEB Refractory Anaemia with Excess Blasts; AML-M3 acute myeloid leukemia FAB M3; CMML Chronic Myelomonocytic Leukemia; LAA leukemia associated antigen, overexpression analyses compared to healthy controls, detected by the $RQ=2^{-\Delta\Delta Ct}$ method in MNCs; E effector cells prepared at different time points in the course of the disease or after stimulation with CD4 depleted LAA/HA1 protein loaded APCs; P passage after x stimulations with CD4 depleted LAA/HA1 protein loaded APCs, nd not done;

Table 3.1.3-1 Patients´ characteristics II:

A flow chart with the methodological strategy to create LAA-presenting nonCD4+ cells or mini-LCL is given in figure 3.1.3-1. We could demonstrate that in general it is possible to stimulate untouched CD4+ T-cells using the CD4 depleted fraction or ‘mini-LCL’ as stimulator fraction. Furthermore we enriched LAA-stimulated cells from these stimulation settings by either enrichment of proliferating, CD40L+CD4+ T-cells or by T-cell single-cell cloning after repeated stimulation with LAA- and HA-1- loaded stimulator cells. Resulting cells were characterized by proliferation, CD40L upregulation and cellular expansion.



I. Untouched CD4+ T-cells prepared from AML patients’ PBMNC after SCT were stimulated (6-16 stimulations with 20 U IL2 twice a month) with irradiated (43 Gy, 80 Gy by mini-LCLs), LAA/HA1 protein loaded nonCD4 cells (as APCs) for 24h. Alternative stimulation of the nonCD4+ cell fraction with EBV transformed B-cells as APCs.



II. Stimulated CD4+ T-cells were characterized by ELISPOTS, ELISAS or ICS for secretion of IFN-γ or GM-CSF before or after single cell cloning or sorting of CD40L+ CFSE_{low} cells. Moreover some cases were tested for antileukemic activity in a chromium-release or fluorolysis assay.

Fig. 3.1.3-1. Preparation, stimulation (I) and characterisation (II) of (untouched) CD4+T-cell

Patient 1158	T-cell source (stage of disease)	Cell source of the effectors	Proportion of blasts lysed by different effector cells (%)	Patient	T-cell source (stage of disease)	Cell source of the effectors	Proportion of blasts lysed by different effector cells (%)
pt 1158 a ('E1')	CR before SCT	MNCs	0	pt 1158 f ('E6')	CR after 1 st SCT and 3 weeks after 1 st DLI	CD4+ (after P17 and FACS Sort P14)	87
pt 1158 b ('E2')	CR after 1 st SCT and 3 weeks after 1 st DLI	CD4+	51	pt 1158 e ('E11')	CR after 1 st SCT and 3 weeks after 1 st DLI	CD4+ (after P16)	0
pt 1158 c ('E3')	CR after 1 st SCT and 5 weeks after 1 st DLI	CD4+	42	pt 1158 d ('E12')		CD4+ (after P5)	9
pt 1158 j ('E4')	Rel. after 1 st SCT and 2 nd DLI	CD4+	61	pt 1158 g ('E13')		CD4+ T-cell clones (after FACS Sort P14)	0
pt 1158 k ('E5')	CR after 2 nd SCT and 4 th DLI	CD4+	60	pt 1158 h ('E14')			16
pt 1158 m ('E7')	CR after 2 nd SCT and 4 th DLI	MNCs	22	pt 1158 i ('E15')			0
pt 1158 n ('E8')		CD3+	19 (38, 4h)	pt 1158 l ('E16')	CR after 2 nd SCT and 4 th DLI	CD4+	86
pt 1158 o ('E9')		CD4+	9	pt 1158 q	† Myocardial Chloroma		

E effector cells prepared at different time points in the course of the disease or after stimulation with CD4 depleted LAA/ HA1 protein loaded APCs (table 3.1.3-1); P passage after x stimulations with CD4 depleted LAA/ HA1 protein loaded APCs; † Exitus letalis

Table 3.1.3-2 Antileukemic functionality (demonstrated by fluorolysis assay) of different CD4+ effector cells prepared before or after specific stimulation, enrichment or sorting

In summary this means that the cytokine release of IFN-γ or GM-CSF is no reliable tool to detect specificity in T-cell fractions. We could demonstrate however, that CD4+ T-cells in general can mediate cytotoxic reactions and that certain enriched CD4+ subtypes might be even more promising candidates for GvL- without GvH reactions.

3.2 T-cells addressing unknown leukemia-specific antigens

3.2.1 Establishment and maintenance of protective immunity by dendritic cells derived from leukemic blasts (Group leaders: Borkhardt, Buhmann, Schmetzer; Scientists: Fischbacher, Freudenreich, Grabrucker, Liepert, Merle, Reuther, Schick, Schuster, Vogt)

Although technically possible the preparation of LAA-specific T-cells is cumbersome, in case of MHC-Multimer technology HLA-A2 restricted and moreover restricted to defined LAAs. Therefore we wanted to study the antileukemic activity of T-cells stimulated with DC derived from leukemic blasts (DC_{leu}), as those DC bear the advantage of potentially presenting the whole (including as yet unknown) and patient-typical antigen pool of the leukemic cells. We have already established the methods to generate sufficient amounts of DC and especially of DC_{leu} in every given case with AML: in a minimalized assay we cultured DC in three different media and choose the DC generation method with the quantitatively highest DC/DC_{leu} counts (Kremser et al., 2010; Schmetzer et al., 2007). In a next step we study the functional profiles of DC/DC_{leu}-stimulated T-cells. We could demonstrate, that isolated *unstimulated* T-cells were able to lyse blasts in 47% of cases, whereas only 26% of those T-cells showed antileukemic activity after a 10 days culture with blasts – pointing to an establishment of a T-cell inhibitory microenvironment in the presence of blasts. However, stimulation of T-cells with blasts after their conversion to DC_{leu} resulted in a blast lytic activity in 58% of all cases. These data suggest, that the inhibitory (blast-induced) atmosphere could be abolished after blast-conversion to DC_{leu}, although not completely: even a DC/DC_{leu} stimulation of T-cells was not effective to induce antileukemic T-cells in every case (Schmetzer et al., 2011; Grabrucker et al., 2010). Therefore we analysed potential conditions and factors being responsible for these impaired immune reactions. We could demonstrate that the *quality of DC* – especially with respect to proportions of mature DC and DC_{leu} – is predictive for their activation capacity for antileukemic T-cells. Those DC/DC_{leu} induce an ‘antileukemically effective’ T-cell composition of DC-stimulated T-cells, characterized by higher proportions of CD4+ and non-naïve T-cells (Grabrucker et al., 2010; Liepert et al., 2010). A detailed analysis of T-cells’ compositions in cases *with* compared to those *without* antileukemic activity revealed significantly higher proportions of naïve (T_{naïve}) and central memory T-cells (T_{cm}) and lower proportions of effector memory regulatory (T_{eff/em reg}) as well as CD8+ regulatory T-cells (CD8+ T_{reg}) (Schick et al., 2011; Vogt et al., 2011). Moreover we could define soluble factors that are predictive for the mediation of antileukemic activity of DC/DC_{leu}-stimulated T-cells: A higher release of ‘inflammatory’ chemokines (CXCL8, CCL2) in DC culture supernatants or of ‘T-cell-promoting’ cytokines (IFN- γ , IL-6) in mixed lymphocyte culture (MLC) supernatants of T-cells with DC clearly correlated with antileukemic activity of DC-stimulated T-cells (Fischbacher et al., 2011; Merle et al., 2011; Schmetzer et al., 2011).

Detailed studies of T-cell subsets via spectratyping analysis could verify that especially after DC-stimulation CD4+ as well as CD8+ T-cells were characterized by a highly restricted V β T-cell-receptor (TCR) repertoire (Schuster et al., 2008). Interestingly, in one patient studied comprehensively *in vitro* stimulation with DC/DC_{leu} resulted into an identical TCR β chain restriction pattern which could be identified *in vivo* in the patient’s T-cells 3 months after allo-SCT (Reuther et al., 2011).

In summary, DC_{leu} are promising candidates to stimulate and enrich antileukemic T-cells without knowledge of defined antigen targets which is attended with the creation of an ‘antileukemic cellular microenvironment’ and could contribute to develop strategies to

overcome immunological resistances. With our experimental *in vitro* models combining culture methods and functional flow cytometry with spectratyping we can moreover provide explanations for clinical observations and might provide predictive information about T-cellular response patterns *in vivo*. Further studies of selected T-cells (e.g. by their V β type) for their phenotype and function will allow to understand clinical responses and to prepare T-cells for treatment.

3.2.2 Antileukemic T-cell profiles to predict antileukemic reactions of DC/DC_{leu} stimulated T-cells and prognosis of patients (Group leaders: Schmetzer; Scientists: Fischbacher, Freudenreich, Grabrucker, Liepert, Merle, Schick, Vogt)

Since not every AML patient responds to immunotherapy (SCT, DLI) *in vivo* and since not every *ex vivo* T-cell stimulation with DC_{leu} results in antileukemia effector T-cells we wanted to elucidate responsible cells or soluble factors. We could evaluate 'cut-off values' for DC- or T-cell subtypes in the cellular settings and in addition amounts of soluble factors that allow a prediction of the antileukemic function of T-cells in this cellular or microenvironmental context or a prediction of the clinical response to immunotherapy. We could demonstrate, that T-cells stimulated with DC_{leu} in a 'favorable cellular and soluble chemokine/cytokine context' (with >45% mature DC and >65% DC_{leu} with a release of >200pg CXCL8, >100pg CCL2, >10pg IFN- γ or >15pg IL-6, resulting in >65% CD4+, T_{non-naive} and <60% CD8+ T-cells and especially >3% T_{naive}, >11% T_{cm} and low proportions of CD8+ T_{reg} after the DC stimulation) had a more than 75% chance to gain antileukemic *ex vivo* activity. In addition we could demonstrate, that clinical responders to immunotherapy were characterized by a higher *ex vivo* generability of DC_{leu} and mature DC, a better *ex vivo* T-cell proliferation and CD4:CD8 and T_{non-naive} : T_{naive} ratios >1 and in addition by a high release of CCL2, IFN- γ and IL-6 (Liepert et al., 2010; Schmetzer et al., 2011; Fischbacher et al., 2011; Merle et al., 2011; Grabrucker et al., 2010).

That means, that we can not only associate cellular subtypes of T-cells and DC or cytokine/chemokine release patterns with antileukemic functions of T-cells in *ex vivo* settings and in the context of a clinical response to immunotherapies, but in addition define predictive 'cut-off values', that means proportions of cells with certain cellular subtypes or concentrations of soluble factors, that allow a correlation with cellular responses or the clinical course of the disease after immunotherapy.

4. Clinical key results

4.1 Clinical use of donor T-cells for prevention and treatment of AML relapse after allogeneic SCT (Schmetzer, Schmid)

Donor lymphocyte infusion (DLI) for treatment of leukemic relapse after allogeneic hematopoietic stem cell transplantation (SCT) has been introduced in the early nineties (Kolb et al., 1990). Being extremely effective in chronic myeloid leukemia, the procedure was less successful in AML, although remissions were observed in selected cases (Kolb et al., 1995; Collins et al., 1997). Therefore, on behalf of the Acute leukemia Working Party of the European Group of Blood and Marrow Transplantation (EBMT) our group performed a retrospective analysis of patients who had been transplanted for AML in complete remission, and had suffered from leukemia relapse post SCT (Schmid et al., 2007). The analysis was based on the EBMT transplant registry, and included 399 adult patients who had received (n=171) or not (n=228) DLI as part of their treatment. With a median follow up

of 27 and 40 months in the both groups, overall survival (OS) at two years was $21 \pm 3\%$ for patients receiving, and $9 \pm 2\%$ for patients not receiving DLI. After adjustment for differences between the groups, better outcome was associated with younger age ($p = 0.008$), remission duration >5 months after SCT ($p < 0.0001$), and use of DLI ($p = 0.04$). Among DLI recipients, a lower tumour burden at relapse ($<35\%$ of bone marrow blasts; $p = 0.006$), female gender ($p = 0.02$), favorable cytogenetics ($p = 0.004$) and remission at time of DLI ($p < 0.0001$) were predictive for survival in a multivariate analysis. Two-year survival was $56 \pm 10\%$, if DLI was performed in remission or with favourable karyotype, and $15 \pm 3\%$ if DLI was given in aplasia or with active disease. Therefore, an algorithm for the clinical use of DLI in the treatment of relapsed AML after allogeneic SCT was developed, comprising the sequence of cytoreductive chemotherapy for disease control or induction of complete remission, followed by DLI for long term control of the leukemia based on cellular immune effects (Schmid et al., 2011).

In an approach to increase the antileukemic efficacy of donor T-cells against myeloid leukemias, systemic application of GM-CSF was studied after DLI for relapse of AML or MDS after SCT (Schmid et al., 2004). GM-CSF was chosen due to its capacity to contribute *in vitro* to the generation of antigen-presenting cells (APC) from leukemic blasts (Woiciechowsky et al., 2001; Kufner et al., 2005; Kremser et al., 2010; Dreyssig et al., 2011). As described above, blasts from myeloid leukemias should have the full genetic repertoire for effective antigen presentation, but might be ineffective stimulators or even induce specific anergy, due to inferior or aberrant expression of co-stimulatory molecules, such as CD80 or CD86. GM-CSF has been shown to induce up-regulation of these molecules on the surface of leukemia blasts and to improve cytotoxic efficacy of autologous and allogeneic T-cells. In a clinical pilot trial for AML relapse after SCT, mild chemotherapy with low dose AraC, infusion of donor T-cells together with stem cells for reconstitution of haematopoiesis, and s.c. or i.v. application of GM-CSF, was studied. Overall response rate was 67% among evaluable patients', overall survival at 2 years was 29%. Long term survival was associated with longer remission post transplant, disease control by low dose AraC and development of chronic GvHD. These results confirm the proposed strategy of initial cytoreduction by chemotherapy and induction of a GvL reaction for long term disease control. Systemic application of GM-CSF was safe in this setting, however, its clinical efficacy remains to be evaluated in randomised studies. Nevertheless, in accompanying *ex vivo* experiments we could demonstrate, that cases in which DC could be generated *ex vivo* using GM-CSF-based protocols showed a more favourable outcome after *in vivo* immunotherapy (Freudenreich et al., 2011).

Since overall, the outcome of patients with AML who relapse after allogeneic SCT is poor, strategies to prevent occurrence of overt haematological relapse are of increasing interest. Intensive monitoring of minimal residual disease and donor chimerism in different cellular compartments (CD34+, CD3+) of bone marrow or peripheral blood has gained in importance by allowing early interventions (chemotherapy, DLI, second SCT) *before* haematological relapse has occurred (Bornhauser et al., 2009). Our group has developed a protocol for the use of prophylactic or preemptive DLI (pDLI) for patients with high-risk AML. Starting from day +120 after SCT, patients in haematologic remission, free of immunosuppression for at least 30 days without clinically evident GvHD, and free of infections receive up to 3 courses of DLI in 4 weeks' intervals, using an escalating cell dose schedule. Patients receiving prophylactic DLI have been compared to a control group of high-risk AML patients, who were treated according to the same transplant protocol, would

have fulfilled the criteria for pDLI, but did not receive the cells since their transplant centres did not take part at this part of the study (Schmid et al., 2005; Schleuning et al., unpublished results). Hence, patients receiving pDLI showed a significantly lower incidence of relapse and achieved a longer overall survival as compared to controls without pDLI. The treatment was also safe and induction of severe GvHD was a rare event in this setting.

In summary, although less effective as in CML, the clinical use of DLI for AML patients after SCT is an effective therapeutic tool for prevention or as part of treatment of relapses after SCT in AML.

5. Perspectives for future therapies: Adoptive transfer or *in vivo* activation of antileukemic T-cells? (Schmetzer, Schmid)

We could clearly demonstrate, that it is possible to *detect and monitor* leukemia specific T-cells by LAA-peptid specific (HLA-A2 restricted) MHC-Multimer analyses or LAA-protein specific CD4+ T-cells, especially if combined with spectratyping and cellular subtype analyses. Concerning 'known' (leukemia-) specific antigens we can conclude from our data, that LAA-peptide specific CD8+ T-cells can be prepared by MHC multimer-technology, LAA-protein specific CD4+ T-cells by preparation of (enriched) proliferating CD40L+ or cloned CD4+ cells and both cell types could be used for adoptive therapies. Moreover we could work out *in vitro* as well as in a dog model, that male specific antigens might be promising candidate antigens for immunotherapies. In addition we could show, that (enriched) T-cells addressing known as well as unknown leukemia-associated antigens, as demonstrated after DC/DC_{leu}-stimulation, can mediate cytotoxic reactions. Those cells could be promising candidates for *adoptive immunotherapies* in selected patients.

Since the manipulation and selection of antigen-specific T-cells is not only an oncological challenge, but has to be approved by special committees before a clinical application another strategy circumventing T-cell manipulations could be more promising: *applying immune-modulators* and cytokines like GM-CSF or IFN- α *in vivo* could possibly induce the conversion of (residual) blasts in patients to DC_{leu}. In a small patients' cohort we could already show, that patients receiving GM-CSF in the context of a DLI-relapse therapy had a better chance to respond to this relapse therapy compared to patients without additionally applied GM-CSF (Freudenreich et al., 2011). Moreover we could show, that the convertibility of blasts to DC_{leu} in *ex vivo* settings correlated with the clinical response and outcome to immunotherapies, what can be interpreted by an '*ex vivo* simulation' of the DC-generating potential out of blasts. We could even demonstrate, that cases, in that higher proportions of DC_{leu} could be generated *ex vivo* had a longer overall survival compared to cases with lower DC_{leu} proportions.

Our *ex vivo* focus in the future will therefore be to thoroughly investigate and optimize *in vivo* strategies with allo SCT applying different donor transplants or *ex vivo* 'manipulated' grafts. We further want to develop and test different 'immune modulating cocktails' (Ansprenger et al., 2011; Deen et al., 2011) that can be applied to patients with the aim to induce leukemia-derived DC *in vivo* and in consequence to stimulate the generation of leukemia-specific T-cells *in vivo*. In parallel we want to further enlighten the role of different (enriched, selected) effector cells - e.g. CD4+, CD8+, NK, NK-T- cells) in the mediation of antileukemic reactions in order to find promising candidates for adoptive T-cell transfer.

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This book documents the increased number of stem cell-related research, clinical applications, and views for the future. The book covers a wide range of issues in cell-based therapy and regenerative medicine, and includes clinical and preclinical chapters from the respected authors involved with stem cell studies and research from around the world. It complements and extends the basics of stem cell physiology, hematopoietic stem cells, issues related to clinical problems, tissue typing, cryopreservation, dendritic cells, mesenchymal cells, neuroscience, endovascular cells and other tissues. In addition, tissue engineering that employs novel methods with stem cells is explored. Clearly, the continued use of biomedical engineering will depend heavily on stem cells, and this book is well positioned to provide comprehensive coverage of these developments.

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