Chapter 4, Discussion

The immune system is a very precise and efficient defence mechanism against parasites and viruses. It is trained not to act against body-own cells. Nevertheless, studies and clinical trials have shown that the immune system can also attack tumour cells, which are altered body-own cells. T cells have been shown to play an important role in tumour rejection. However, in most patients such tumour-specific T cells are only present in low numbers and they are often hampered in their effector functions. For this reason attempts have been undertaken to amplify tumour-reactive T cells *ex vivo* and to then re-infuse them into patients. But also this approach harbours difficulties, as it is often not possible to isolate tumour infiltrating lymphocytes (TIL) from tumour tissue, let alone amplify the T cells to therapeutic numbers (~10^{10} cells). The transfer of tumour-specificity to autologous primary blood lymphocytes (PBLs) offers a solution to this problem. A successfully combined gene therapeutic and immunotherapeutic anti-cancer approach is dependent on many different factors. With regard to gene therapy, the right gene transfer vehicle has to be chosen. Concerning the immunological side, the antigen receptor carrying the specificity and the performance of the modified cells are of crucial importance. In this thesis the feasibility of such an approach is shown for a renal cell carcinoma (RCC)-specific TIL.

4.1 MP71 is a retroviral vector optimised for gene transfer in T lymphocytes

Prerequisites for grafting T cells with a new antigen specific receptor for adoptive T cell therapy are high transduction rates and high and durable transgene expression. For many cells to date the only transfer system guaranteeing such demands are retroviral vectors (Mulligan, 1993; Somia and Verma, 2000; Kay *et al.*, 2001). Especially with regard to durable expression, the integration of the viral genome is of great advantage. However, also other vector systems allow long term expression through genome integration. These include lentivirus, foamy virus and adeno-associated virus (AAV) vectors (reviewed in Thomas *et al.*, 2003), and also non-viral systems such as the transposable element sleeping beauty (Ivics and Izsvak, 2004).

Nevertheless, the simple genomic structure of retroviruses, the absence of cellular immunity to later-generation retroviral vectors (Hildinger *et al.*, 1999; Yu *et al.*, 2000; Kondo *et al.*, 2005), the ease of use, and the broad knowledge based on *in vitro*, *in vivo* and clinical data makes them the first choice. Enhancing transgene expression levels and the duration of expression by retroviruses is still an aim of many researchers, showing the need but also the possibility of vector improvement (Wong *et al.*, 2002; Cooper *et al.*, 2004; Kraunus *et al.*, 2004).

In Chapter 2, the optimisation of a murine oncoretroviral vector for gene transfer in T cells is described. Emphasis has been laid on transgene expression, but transduction efficacy was also investigated. Concerning transduction of murine T lymphocytes it was clearly shown that ecotropic Moloney murine leukaemia virus (MoMLV) envelopes are superior to amphotropic pseudotypes. Transduction of human T cells was highest when employing the amphotropic 10A1 MLV pseudotype, which is in concordance with part of the literature (Farson *et al.*, 1999; Uckert *et al.*, 2000). Retroviruses pseudotyped with other envelope proteins, as from the wild type amphotropic MLV, the feline retrovirus RD114 or the vesicular stomatitis virus were shown to transduce human PBLs less effectively (Farson *et al.*, 1999; Uckert *et al.*, 2000). The gibbon ape leukaemia virus (GaLV) envelope protein is the most widely used pseudotype for human T cell transductions and has also been used in clinical trials (Bunnell *et al.*, 1995; Bolhuis *et al.*, 1998; Lamers *et al.*, 2002). Nevertheless, in our hands the 10A1 MLV pseudotyped virions perform better than the GaLV pseudotyped viruses when transducing human PBLs. This is a plausible finding with regard to the capability of the 10A1 MLV envelope protein to bind to two cell surface receptors, Pit1 and Pit2 (Miller and Miller, 1994; Uckert *et al.*, 2000).

4.1.1 The impact of promoter sequences on transgene expression

Analysis of *cis*-regulatory elements is a complex task, since many different elements exist and combinations and/or repeats of these elements are possible. On the one hand different promoters can be employed, which can be of cellular or viral origin. On the other hand elements influencing RNA nucleo-cytoplasmic export, stability or translation can be incorporated into retroviral vectors. In Chapters 2 and 3 the long terminal repeat (LTR) of MoMLV is compared to the myeloproliferative sarcoma virus (MPSV) LTR. The latter LTR was only analysed in the context of an improved 5’

untranslated region (UTR) (see 4.1.2). Using a cytoplasmic protein (green fluorescent protein, GFP) and a cell surface protein, which is part of a multi-protein complex (T cell receptor, TCR), it could clearly be shown that the MPSV LTR is superior to the MoMLV LTR. In primary human T lymphocytes a 20-fold increase in GFP expression was observed when transduced with MP71GPRE (MPSV LTR) compared to LGSN (MoMLV LTR), based on mean fluorescence intensity (MFI) of flow cytometry measurements. With regard to TCR, only the MP-tcr (MPSV LTR) vector showed clear and functional transgene expression. This data based on cell surface protein expression was verified by analysis of mRNA levels, in order to exclude post-translational effects. For both, GFP and TCR expression, more transcripts were detected for the MPSV LTR driven transgenes. A quantification of RT-PCR bands revealed a 4-fold increase in TCR-26 transcripts. It is difficult to judge whether this finding reflects the striking difference in TCR-26 surface expression by both vectors. Possible explanations for the relatively small difference in mRNA levels but striking differences in protein surface expression are (i) differing nucleo-cytoplasmic distribution that cannot be monitored by RT-PCR, but has an impact on translation, or (ii) the existence of a certain threshold for cytoplasmic TCR expression that has to be reached to obtain TCR surface expression. Further analysis of the transduced cells was performed by Southern blot (for GFP-vectors), DNA PCR and real-time PCR (for TCR-vectors). All three methods revealed similar integration copy numbers for the vectors used. This clearly indicates that viral supernatants of similar titres were used for transductions and led to similar numbers of transduced cells containing similar numbers of viral copies. The differences in transgene expression can therefore be attributed to the *cis*-regulatory elements. For the TCR, the difference in expression was so large that expression could hardly be detected in L-tcr-transduced cells. This clearly shows that the same elements driving expression of different transgenes can lead to differing evident expression, depending on the nature of the transgene. While monitoring the expression of the cytoplasmic protein GFP showed transgene expression at different levels, the multimeric surface proteins of the TCR cannot be detected when expressed at low levels. The latter finding indicates that an expression threshold for TCRs may exists, which has to be surpassed in order to obtain cell surface expression.

Interestingly, also depending on the cell type transduced, different transgene expression levels can be detected. The performance of the MPSV LTR was best in T cell lines and primary T cells. A 10 to 75-fold increase in GFP MFI was detected in human T cells. In contrast, the increase was only 3-fold in the human fibrosarcoma cell line HT1080. Similar data were also obtained for mouse cells. This cell type dependence might be due to the influence of differentiation-specific transcription factors interacting with viral promoters, leading to differing transcription rates. However, *in silico* analysis did not reveal any striking difference in transcription factors binding to either LTR (data not shown).

The two different promoter/enhancer pairs analysed not only showed differences in transgene expression levels but also in duration and stability of expression. GFP- or TCR-26-retrovirus transduced human and mouse T cells were cultured for a period of 30 to 111 days. Both, T cell lines and primary T cells of either species showed a decrease in transgene expression when mediated by the LXSN vectors. Transgene expression by the MP71 vectors remained stable or showed only a slight decrease over time. This observation was more closely analysed for TCR-26-retrovirus transduced primary human T lymphocytes, where 111 days after transduction TCR-26 expression was 83% of initial TCR expression for MP-tcr-transduced cells and 26% for L-tcr-transduced cells. DNA PCR analysis proved similar numbers of viral genome integration for both vectors at several time points. This indicates a reduction in the initially low TCR translation and transcription by L-tcr, while the MP-tcr driven expression remained high. Silencing of MoMLV LTR dependent transgene expression has been previously reported, and is most probably related to DNA methylation of the promoter/enhancer regions (Jahner *et al.*, 1982; Challita and Kohn, 1994; Wang *et al.*, 1998; Swindle *et al.*, 2004). In contrast, several reports indicate that the MPSV LTR is less prone to methylation (Wang *et al.*, 1998; Swindle *et al.*, 2004). While the effect of enhanced transgene expression must be attributed to the combined promoter/enhancer and UTR sequences (see 4.1.2), the duration and stability of expression is mainly based on the nature of the promoter/enhancer.

An analysis of internal promoters has not been included in these studies. Although some publications describe a successful application of e.g. internal phosphoglycerate kinase (PGK) promoters (Cooper *et al.*, 2000), there is a body of literature reporting

promoter interference in such constructs. A negative impact on gene expression by two adjacent promoters has been reported for episomal plasmids (Proudfoot, 1986) and integrated vectors (Emerman and Temin, 1984; Cheng *et al.*, 1997). I have also observed such an effect using the LXSN vector. When the entire vector was used to express the TCR-26 in early experiments, no surface expression could be detected (data not shown). A deletion of the SV40 promoter and the aminoglycoside 3'-phosphotransferase (neomycin resistance) gene led to the L-tcr vector, which yielded very low but detectable TCR-26 expression. Retroviral constructs in which such internal promoters could effectively perform are self-inactivating retroviral vectors (SIN), where a defective 3' LTR inactivates the 5' LTR upon integration into the host genome (Yu *et al.*, 1986; Werner *et al.*, 2004).

4.1.2 The use of additional internal *cis*-regulatory elements

The impact of other *cis*-regulatory elements was further investigated using GFP as a reporter gene (Chapter 2). For this means the MoMLV vector was analysed with or without a fragment of the human CD2 locus control region (LCR; LCD2GSN and LGSN, respectively). The MPSV-based vector was equipped with the Woodchuck hepatitis virus post-transcriptional regulatory element (PRE; MP71G and MP71GPRE). Furthermore, the MP71 vectors contained an improved 5' untranslated leader sequence including a splice signal and mutated aberrant ATG start codons (Hildinger *et al.*, 1999; Schambach *et al.*, 2000). The impact of the different modules was evaluated based on the MFI of GFP expression.

The impact of a 1 kbp fragment of the CD2 LCR (Greaves *et al.*, 1989; Kowolik *et al.*, 2001), which confers T cell-specific enhancer activity but no LCR effects, was analysed in the context of the LGSN vector, which contained a 210 bp deletion of the viral enhancer of the MoMLV LTR (Indraccolo *et al.*, 2001). The cellular enhancer could not compensate for the deleted viral enhancer and led to no increase in GFP expression in mouse and human T cells (0.2 to 0.7-fold MFI compared to LGSN for primary human and mouse T lymphocytes, respectively). Surprisingly, the T cell specific enhancer performed best in the human fibrosarcoma cell line HT1080 (1.2-fold increase compared to LGSN). Previous studies on the application of the CD2 LCR in retroviral vectors deliver a unclear picture. The first study by Kaptein *et. al.* (1998) describes a non functional LCR in context of a modified MoMLV vector. Later

studies by Indraccolo *et. al*. (2001 and 2002) and Kowolik *et. al*. (2001) show CD2 LCR function in the context of MoMLV and human immunodeficiency virus (HIV) vectors. The full length LCR mediated LCR function as seen by reduced position effect variegation and a stabilisation of *in vivo* transgene expression. The truncated 1 kbp enhancer fragment only partially compensated for the deleted viral enhancer in human T-lymphoid JM and Molt-3 cells. However, reduced viral titres and slightly reduced MFI compared to the standard LGSN were observed. Our findings give rise to the conclusion that the application of the CD2 LCR enhancer in context of MoMLV vectors with or without truncated LTRs is not feasible for gene transfer in T lymphocytes. The insertion of the full length CD2 LCR into HIV vectors seems more promising. A successful application of a LCR has been shown for the β-globin LCR, in combination with the β-globin enhancer, both incorporated into a HIV vector (May *et al.*, 2000).

The PRE element (Donello *et al.*, 1998; Schambach *et al.*, 2000) was studied in the context of the MP71G vector. It led to a 1.2 to 4.5-fold increase of MFI in primary mouse T cells and the human T cell line HuT78, respectively. The increase detected in primary human T cells was 1.5-fold. These results are difficult to compare to findings described in the literature. Although the PRE has been extensively studied in MoMLV, HIV, SIN, AAV and adenovirus vectors (Schambach *et al.*, 2000; Zufferey *et al.*, 1999; Werner *et al.*, 2004; Loeb *et al.*, 1999; Glover *et al.*, 2002), the enhancing effect on transgene expression varies strongly. It ranges from negative effects observed in haematopoietic cells *in vivo* using an SIN with the Spleen Focus Forming virus promoter (Werner *et al.*, 2004) to an observed 10-fold increase in transgene expression in 293 cells using an internal cytomegalovirus (CMV) promoter in an AAV vector (Loeb *et al.*, 1999). It is clear however, that the PRE exerts an enhancing effect on transgene expression in mouse and human T cells in the context of the MP71 vector. This effect is most probably based on the enhancing effect on polyadenylation, mRNA nuclear export, and maybe translation initiation (Donello *et al.*, 1998; Huang *et al.*, 1999). The rather small increase in transgene expression in our hands might be due to the application of the PRE in the context of a splice signal. Both elements directly or indirectly promote mRNA export and translation (Donello *et al.*, 1998; Matsumoto *et al.*, 1998; Huang *et al.*, 1999; Luo and Reed, 1999). Therefore, their functions are partially redundant, and what is more, the PRE inhibits

splicing per se. Another reason for the only modest increase in transgene expression by PRE could be the localisation of the element with respect to the transgene. In the woodchuck hepatitis virus the PRE is part of the coding region of the viral polymerase and X protein (Donello *et al.*, 1998). In the retroviral setting the element is placed downstream of the transgene. In such a case, the distance between the PRE and the transgene influences the functional activity of the element (V. Patzel, personal communication).

Apart from its effect on gene expression, the PRE also leads to an increase in viral titres from producer cell lines (Schambach *et al.*, 2000; Werner *et al.*, 2004). I have also observed this effect using the MP71 vector in both GFP and TCR-26 transgene settings. The generation of high titre producer cells was more difficult for the GFP and TCR-26 vectors lacking PRE (data not shown). Interestingly, the MP-tcr vector lacking PRE did not show a detectable decrease in TCR expression, but clearly in virus titre. This finding can be explained by the PRE dependent inhibition of splicing on the one hand and enhanced nuclear export of non-spliced mRNA on the other (Huang *et al.*, 1999). Both effects lead to higher virus production, but lower translation efficacy.

As mentioned earlier, the PRE was analysed in the context of an optimised leader sequence, which is based on the mouse embryonic stem cell virus (MESV) (Grez *et al.*, 1990). An artificial splice acceptor site complementing the retroviral splice donor site has been included into the 5’ UTR. In addition, all start codons have been removed, and consequently, the ATG of the transgene is the first start codon 3’ of the promoter (Hildinger *et al.*, 1999). The promotion of RNA export and translation by splice signals has been shown before (Matsumoto *et al.*, 1998; Luo and Reed, 1999; Hildinger *et al.*, 1999). It is mediated by the formation of the spliceosome but also simply by removing a stretch of 5’ UTR. The shortened distance between the cap and the initial ATG reduces the extent of ribosomal scanning, which is an ineffective and a potentially error-prone process (Berlioz and Darlix, 1995). For additional optimisation, also regarding non-spliced RNA, aberrant ATG codons were removed from within the 5’ UTR (Hildinger *et al.*, 1999). The impact of these single modifications on enhanced transgene expression could not be dissected, as they

were only employed in the context of the MPSV LTR (see 4.1.1). Nevertheless, the combination of the MPSV LTR and the MESV leader proved to be very effective.

There is still, however, the need and room for optimisation of retroviral vectors. One example is the oscillation of transgene expression in T cells depending on their activation status (data not shown; Cooper *et al.*, 2004). One solution to this problem could be the integration of scaffold attachment regions (SAR) that stabilise transgene expression (Cooper *et al.*, 2004). However, it remains to be shown how high receptor expression in resting or memory T cells has to be, in order to trigger activation. Expression levels as seen for MP-tcr in resting T cells might be high enough to trigger a response.

Taken together, the combination of the optimised MESV leader sequence, the PRE and the MPSV LTR guarantees a strong and long lasting transgene expression. Further comparisons on GFP expression by retroviral vectors used for TCR and chimeric antigen receptor (CAR) transfer by other groups (Yang *et al.*, 2002; Weijtens *et al.*, 1998) confirmed the high efficacy of transgene expression by the MP71 vector (our own unpublished data).

4.1.3 TCR expression and T cell avidity

The avidity of a T cell is dependent on both the affinity of the TCR and the number of TCRs on the cell surface. In several studies the direct correlation of T cell avitidy and T cell effector functions has been shown. All studies indicate the importance of high TCR expression rates, leading to avidities that mediate recognition of physiologic antigen levels. *In vivo* studies with TCR transgenic mice showed a direct correlation of TCR expression/T cell avidity and T cell function (Derby *et al.*, 2001; Labrecque *et al.*, 2001). In a mouse model using *in vitro* generated CTL lines against tumour-associated antigens (TAAs), Zeh and colleagues showed a strong link between avidity and *in vivo* anti-tumour effects (Zeh *et al.*, 1999). In addition, a study with human CTL lines showed that high functional avidity correlated with tumour recognition *in vitro* (Dudley *et al.*, 1999). The same relation of function and avidity has also been demonstrated for receptor grafted T cells. A comparison of TCR-grafted T cell clones indicated that reduced TCR expression correlates with diminished lytic activity (Cooper *et al.*, 2000; Heemskerk *et al.*, 2003). A similar effect was also

observed for cytokine secretion by Epstein-Barr virus (EBV)-specific (Chapter 5 in Schaft, 2003) and MAGE-1-specific (Willemsen *et al.*, 2001) CAR expressing T cells and by a tyrosinase-specific TCR grafted mouse lymphoma cell line (Roszkowski *et al.*, 2003). Altogether these data argue for the use of high transgene-expressing vectors for the redirection of T cells for adoptive transfer. The data shown in this thesis demonstrates that the MP71 vector is suited for this application.

4.1.4 Safety of retroviral vectors in gene therapy

The safety of retroviruses has become a big issue over the last years. The potential risk of insertional mutagenesis through retroviral integration has been neglected in the gene therapy field for a long time. Since three patients of an otherwise very successful severe combined immunodeficiency (SCID)-trial have developed leukaemia due to retroviral integration more attention is being paid to safety aspects of retroviral gene transfer (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002; Li *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2003; Bonini *et al.*, 2003; Check, 2005). It has to be noted though that the specific combination of treating young patients, the transduction of haematopoietic stem cells, and a very strong growth advantage for the transduced cells make the SCID-trial a special case. Upon stem cell transfer the transduced cells proliferated very strongly due to the inserted stimulatory transgene (common γ-chain of interleukin receptors) and the homeostatic proliferation. The risk of a gene therapy application with differentiated T cells, as described in this thesis, remains to be thoroughly investigated. Nevertheless, several approaches to increase the safety of retroviral vectors are already being analysed. They include for example retroviral transductions with the lowest possible multiplicity of infection (MOI), as in several studies the direct correlation of MOI and number of insertions into the cellular genome has been elucidated (Kustikova *et al.*, 2003; Fehse *et al.*, 2004b; Modlich *et al.*, 2005). A prerequisite for transductions at low MOI are vectors with high transgene expression levels, as described in Chapter 2. Another approach is the employment of SIN vectors (Kraunus *et al.*, 2004). These vectors strongly reduce the problematic promoter activity of the 3' LTR that may lead to the expression of downstream oncogenes, as observed in the SCID-trial. However, most SIN vectors currently used are still suboptimal in terms of viral titre and transgene expression. Further effort is needed to reduce the risk of adverse effects

linked to retroviral gene transfer without loosing transgene transfer capabilities. However, it should be noted that insertional mutagenesis remains a risk as long as vectors that integrate randomly are used. Strategies to obtain site specific vector integration have so far not been fruitful.

4.2 Transfer of renal cell carcinoma specificity

4.2.1 Redirection of PBLs with TCR-26

In the second part of this thesis the retroviral transfer of renal cell carcinoma (RCC) specificity was investigated. For this means a model composed of a TIL, a RCC tumour cell line, a transformed normal kidney cell line (NKC) and a B-lymphoblastoid cell line (LCL) from one RCC patient were used. The TCR genes isolated from TIL-26 were cloned into two retroviral vectors. As discussed before, only the MP-tcr vector was able to express the TCR-26 at levels high enough to guarantee avidities that are sufficient to exert RCC-26-specific effector functions by transduced PBL of healthy donors. The comparison of the TCR-26 expressing PBLs and TIL-26 showed no functional alterations with regard to peptide-specificity and HLA-A2 restriction. Furthermore, the transduced cells and the TIL showed cytokine release and cytotoxicity of similar magnitude, when compared at a cell-to-cell basis. This was demonstrated in interferon-γ and tumour necrosis factor-α release assays using cell numbers of transduced PBLs and TIL-26 equilibrated for vβ22-positive cells and ^{51}Cr-release assays with identical cell numbers of vβ22-enriched transduced PBLs and TIL-26. Both assays indicated similar reactivity of redirected PBLs and TILs. Also the unsorted bulk culture of up to 52% TCR-26 expressing PBLs demonstrated anti-tumour reactivity in both assays. An important observation, as many previous publications on TCR gene transfer investigated the transduced cells as cloned or enriched cultures, possibly due to lower TCR expression levels (Clay *et al.*, 1999; Cooper *et al.*, 2000; Heemskerk *et al.*, 2003). Only Morgan (Morgan *et al.*, 2003; Zhao *et al.*, 2005; Hughes *et al.*, 2005), Schaft (Schaft *et al.*, 2003b), and Roszkowski (Roszkowski *et al.*, 2005) and colleagues have shown transferred specificity and anti-tumour reactivity for transduced PBL bulk cultures. Notably, all reports on human TCR transfer for anti-cancer therapies describe the transfer of melanoma- or leukaemia-specific TCRs (Clay *et al.*, 1999; Schaft *et al.*, 2003b; Morgan *et al.*, 2003; Heemskerk *et al.*, 2003). Only in a recent study the transfer of specificity for the

broad TAA NY-ESO-1 was shown (Zhao *et al.*, 2005). In this thesis the feasibility of specificity transfer for RCC has been shown for the first time.

4.2.2 The antigen recognised by TIL-26 is unknown

To date the antigen recognised by TCR-26 has not been identified. Attempts to screen a peptide library and a RCC-26 expression library with TIL-26 did not lead to any result (E. Nößner, personal communication). Similar approaches are often unsuccessful, as the cultivation of TILs is problematic and not always possible. The transfer of TCR genes into T cells circumvents this problem and presents a tool to identify antigens of such TILs. By this means the specificity of isolated TILs can be saved, further analysed, and even used for therapy.

Additional screening experiments with TCR-26 expressing PBLs will hopefully identify the antigen recognised by the RCC-specific TIL-26. Another approach to identify antigens recognised by TCRs is the employment of indicator cell lines that induce marker gene expression upon specific stimulation. Such lines are mostly based on T cell lymphoma lines and express β-galactosidase (lacZ) or luciferase under the control of a nuclear factor for activated T cells (NFAT)-responsive promoter (Kerschbaum *et al.*, 1997; Aarnoudse *et al.*, 2002; Schaft *et al.*, 2003a). Transducing a T cell line with TCR retroviruses has the advantage of obtaining large numbers of transduced cells, easy cell culture conditions, and furthermore, the marker genes allow high throughput screening experiments.

Knowing the identity of an antigen recognised by an isolated, potentially therapeutic TIL is mandatory. It is necessary to evaluate the risk of autoimmune reactions, especially when a TAA is targeted. The question about the therapeutic value of TCR-26 has not yet been cleared. So far TIL-26 has been reported to recognise two RCC lines apart from the autologous tumour cell line (Schendel *et al.*, 1993; Schendel *et al.*, 1997). However, many other RCC lines and also tumours of different origin have not been recognised (Chapter 3; Schendel *et al.*, 1993; Schendel *et al.*, 1997). This contradiction could be solved by identifying the antigen. This knowledge could also be applied in the context of the ongoing RCC-26 vaccination trial (Pohla *et al.*, 2000; Schendel *et al.*, 2000). With the help of the antigen the induction of specific T cells

could be more precisely monitored. Finally, only few antigens expressed by RCC cells have been described so far (Chapter 1 Tab. 2; Zhou *et al.*, 2005), and references therein). Therefore the identification of further RCC antigens is of great interest. Especially, as the only shared RCC-associated antigen known is G250/MN/CA IX the antigen recognised by the G250 monoclonal antibody (Grabmaier *et al.*, 2000).

4.3 Engineering of T cell specificity and adoptive immunotherapy

4.3.1 Possibilities and limitations of adoptive T cell therapy

The potential of adoptive T cell therapy has been shown in mouse experiments and several clinical trials (Spiess *et al.*, 1987; Hanson *et al.*, 2000; Kolb *et al.*, 1990; Walter *et al.*, 1995; Rooney *et al.*, 1995). However, successful trials have been limited to the treatment of chronic myelogenous leukaemia (CML), CMV infections and EBV-associated tumours. Studies with other tumour entities, namely melanoma, have only been partially successful (Rosenberg *et al.*, 1994; Yee *et al.*, 2002; Dudley *et al.*, 2002; Dudley *et al.*, 2005). With the introduction of retroviral redirection of T cell specificity, successful applications of adoptive T cell therapy can hopefully also be achieved for other tumour entities. First clinical trials have shown that the transfer of retrovirally redirected HIV-specific T cells is safe, and that CD4/ζ receptor grafted T cells migrate and persist in patients (Walker *et al.*, 2000; Mitsuyasu *et al.*, 2000). However anti-virus effects were only minor. Further *in vitro* and mouse experiments, as well as clinical trials have been conducted to investigate the efficacy of transferred T cells to exert anti-tumour functions. Several methods have been developed to increase the anti-tumour effects of transferred cells. Mouse studies have demonstrated the expansion of transferred redirected T cells *in vivo*, homing to effector sites (Tahara *et al.*, 2003) and also tumour rejection (Kessels *et al.*, 2001; Brentjens *et al.*, 2003; Chamoto *et al.*, 2004; Morris *et al.*, 2005).

One major problem of adoptive T cell transfer is the short persistence of donor cells. It has been shown in animal models that an adoptive transfer of T cells into an “empty” host prolongs *in vivo* persistence of the transferred cells (Berger *et al.*, 2001), probably due to homeostatic expansion upon transfer. Furthermore, nonmyeloablative conditioning of the host allows durable engraftment even with

MHC-incompatible cells (Luznik *et al.*, 2001). First clinical trials have shown an enhanced persistence and stronger anti-tumour effects of transferred T cells when patients underwent a nonmyeloablative therapy prior to the adoptive transfer (Dudley *et al.*, 2002). This effect is thought to be mediated by the elimination of suppressor T cells (Antony *et al.*, 2005) and by decreased competition with endogenous lymphocytes for homeostatic regulatory cytokines as IL-7 and IL-15 (Klebanoff *et al.*, 2004). A different approach to prolong the persistence and enhance anti-tumour effects of transferred cells is to optimise stimulation of the cells *in vitro* prior to transfer, as well as *in vivo* in the patient. Gattinoni *et. al.* have demonstrated that the stimulation of T cells with IL-15 was superior to using IL-2 (Gattinoni *et al.*, 2005), judged by subsequent *in vivo* anti-tumour function. Brentjens and colleagues have shown a positive effect when adding IL-15 and CD80 during *in vitro* stimulation (Brentjens *et al.*, 2003). They additionally show an enhanced persistence of CAR-expressing T cells when artificial APCs are co-administered, thereby guaranteeing co-stimulation in the host. As proper stimulation of the transferred T cells *in vivo* seems to be crucial for long persistence and strong effector functions, many different stimuli have been investigated. They include co-administration of anti-CD40 mAb (Tuma *et al.*, 2002), anti-4-1BB mAB (May *et al.*, 2002), *in vivo* treatment with IL-15 (Klebanoff *et al.*, 2004), and also extended IL-2 treatment, as conducted with melanoma patients (Rosenberg *et al.*, 1994; Yee *et al.*, 2002). These data show the importance of embedding the transferred T cells into a functioning immune reaction, because transferred CD8 cells cannot alone maintain a sustained anti-tumour response.

4.3.2 The importance of CD4 and CD8 T cells in anti-tumour reactions

Important counterparts of CD8 cells are the cognate CD4 cells. Many studies have shown the importance of the interplay of both T cell subsets for proper anti-cancer immune responses (reviewed in Pardoll and Topalian, 1998 and Ho *et al.*, 2002). The role of CD4 cells is however not reduced to providing help to CD8 cells, but they are also necessary to generate and maintain CD8 memory cells (Shedlock and Shen, 2003; Janssen *et al.*, 2003; Sun *et al.*, 2004). Furthermore, it has been shown that tumour-specific CD4 cells prolong the persistence of co-transferred tumour-specific CD8 cells (Walter *et al.*, 1995; Walker *et al.*, 2000; Morris *et al.*, 2005), and they can even induce *de novo* generation of tumour-reactive CD8 cells (Surman *et al.*, 2000).

Finally, CD4 cells can orchestrate a more general immune response against cancer, also activating eosinophils and macrophages (Hung *et al.*, 1998; Corthay *et al.*, 2005). Hence it seems necessary to not only transfer specific cytolytic T lymphocytes (CTLs) but also cognate CD4 cells. With regards to adoptive T cell therapy this is problematic, as to date only few tumour-specific CD4 cells have been identified and cloned. Several strategies have therefore been developed to circumvent this problem. Simply grafting CD4 cells with a MHC class I-restricted TCR generates T cells that would not be reactive, because of the lacking CD8-MHC interaction. Only the dual interaction of TCR-peptide/MHC-I and CD8-MHC-I guarantees an avidity high enough to stimulate T cells (Holler and Kranz, 2003). One possible approach is to introduce a CD8 chain into the TCR-grafted CD4 cells, which then enhances the T cell's avidity (Schaft *et al.*, 2003a; Willemsen *et al.*, 2005a). Another strategy is the generation of high affinity TCRs that are CD8 independent. Such TCRs have been obtained *in vitro* using phage display (Holler *et al.*, 2000), *in vivo* with A2.1 transgenic mice (Kuball *et al.*, 2005), from influenza virus infected mice (Morris *et al.*, 2005), and they have also been identified in a melanoma patient (Nishimura *et al.*, 1999; Roszkowski *et al.*, 2003). Either strategy shows that the identification of a high-affinity $CD8^+$ tumour-reactive CTL clone is enough to develop both tumour-specific helper and cytolytic T cells. A different approach makes use of CARs that display very high affinity and are not dependent on CD4 or CD8 co-receptors. Grafting PBLs with the same CAR also leads to helper and cytolytic T cells that positively interact (Walker *et al.*, 2000). Furthermore, these chimeric receptors may include the lck-binding domains of CD4 or CD8 chains. Thereby CARs not only compensate for reduced co-receptor-dependent avidity, but also for the co-signalling function of these co-receptors.

4.3.3 Risks related to the adoptive transfer of redirected T cells

Although the adoptive transfer of T cells redirected towards tumour-specificity is a very promising strategy for cancer immunotherapy, it harbours several risks that are important to discuss. Firstly there is the risk of insertional mutagenesis, as discussed above (4.1.4). Second, the introduction of an additional pair of TCR genes into a T cell represents a certain danger itself (reviewed in Schumacher, 2002). On the one hand, there is the formation of hybrid TCRs, which contain an exogenous and an endogenous TCR chain. Flow cytometry data using antibodies and tetramers in

parallel suggest that these hybrid TCRs are formed (Stanislawski *et al.*, 2001; Schaft *et al.*, 2003b). Furthermore, hybrid TCRs were indirectly detected by flow cytometry by M. Gladow and D. Sommermeyer (personal communication). Such hybrid TCRs reduce the number of receptors with the desired, transferred specificity. More importantly, they give rise to new, potentially auto-reactive specificities. In order to exclude the formation of hybrid TCRs, mutations of the constant regions, introduction of leucine zippers, generation of chimeric TCRs containing CD3 ζ-chains for dimerisation (Willemsen *et al.*, 2000), the construction of single chain TCRs (Willemsen *et al.*, 2000), and the use of CARs seem feasible. However, mouse studies using TCR-redirected T cells do not show any sign of autoimmunity (Kessels *et al.*, 2001). Another autoimmune hazard arises when a tumour-specific TCR is introduced into tolerant, self-reactive T cells. The tolerant cells can be activated via the exogenous TCR, and then exert effector functions via the endogenous, auto-reactive TCR. It has been shown that T cells of a certain specificity retain their specificity even when grafted with a second functional TCR (Heemskerk *et al.*, 2004). Furthermore, a mouse model with genetically generated dual-TCR T cells shows the ability of these T cells to be activated by one TCR and react through the other (Gladow *et al.*, 2004). A very promising approach to circumvent the activation of such potentially auto-aggressive T cells is the preselection of T cells with a certain specificity. These cells are then transduced with receptor encoding retroviruses (Kershaw *et al.*, 2002; Rossig *et al.*, 2002; Heemskerk *et al.*, 2004). Such a strategy not only excludes the transduction of auto-reactive T cells, but it also reduces the probability of obtaining auto-reactive hybrid TCRs, as the repertoire of transduced cells is very limited. Furthermore, the preselected specificity could be used to boost the redirected cells through vaccination. However, starting from a reduced number of T cells will again pose the difficulty of obtaining sufficient numbers of T lymphocytes for adoptive transfer. Precursor frequencies are 0.1 to 10% for the very frequent CMV-specific T lymphocytes (Wills *et al.*, 1996; Khan *et al.*, 2004), and would therefore demand for several rounds of expansion *in vitro* prior to transfer.

A totally different hazard of autoimmunity lies in the choice of the antigenic receptor. Be it a TCR or CAR, when TAAs are targeted, there is always the risk of also damaging normal tissue, which expresses the TAA at low levels. A harmless example is the occurrence of vitiligo accompanying anti-melanoma therapy (Yee *et al.*, 2000;

Dudley *et al.*, 2002). But TAA expression in other tissues might cause serious harm. For this reason targeting tumour-specific antigens or strict cancer-testis or carcinoembryonic antigens would be a better choice for adoptive T cell therapy. Yet another potential autoimmune hazard lies in the use of very high-affinity TCRs or CARs, which tend to lose their antigen specificity and can be easily activated in an antigen-independent manner (Holler *et al.*, 2003; Willemsen *et al.*, 2005b).

Taken together, it seems worthwhile to investigate safety mechanisms that would allow the elimination of transferred cells in case of lymphoma formation due to insertional mutagenesis or the occurrence of severe autoimmunity due to auto-reactive transferred T cells. Examples for such safety approaches are the use of suicide genes, as the herpes simplex virus thymidine kinase or cytosine deaminase, the introduction of a tag into the antigen receptor, by which an antibody depletion of the redirected cells could be carried out, and the use of chimeric apoptosis inducers. However, also these approaches bear difficulties and disadvantages. Concerning suicide genes, the escape of transformed T lymphocytes to suicide gene therapy through genetic and epigenetic instability should be mentioned (Frank *et al.*, 2004). There is also the risk of patients developing an anti-suicide gene immune reaction (Fehse *et al.*, 2004a). This would lead to the eradication of the transduced T cells shortly after transfer. Approaches using an inserted tag for depletion of transduced cells are technically challenging: (i) The tag must not interfere with antigen recognition. (ii) It must be well exposed on the surface in order to be accessible for the depleting antibody. (iii) Antibodies with a depleting potential must be available for the tag. Recently, an approach using an inducible caspase 9 showed promising results. Transduced T cells were effectively eradicated *in vitro* and *in vivo* by apoptosis through induced dimerisation of a chimeric caspase 9 (Straathof *et al.*, 2005). All in all there is the need for further investigations on the safety aspects concerning adoptive transfer of gene engineered T lymphocytes.

4.3.4 Limitations and potentials of CARs

Another important part of T cell redirection is the transfer of CARs. The design of artificial chimeric receptors offers solutions to some problems connected to TCR transfer, but it also creates new difficulties, mainly due to the artificial nature of the receptor. One of the problems of TCR gene transfer is the reported instability of some

TCR α-chains (Chung *et al.*, 1994; Debets *et al.*, 2002), which leads to low surface expression of the exogenous TCR. The use of CARs or single chain TCR receptors circumvents this potential stability problem. A further limitation of TCR-grafted T lymphocytes is their dependency on peptide presentation by MHC molecules. In some cases tumour cells down regulate MHC-I expression, which protects them from T cell recognition and lysis. Furthermore, a TCR with a certain specificity can only be used if its HLA restriction is complementary to the patient's haplotype. This is why mainly HLA-A2 restricted TCRs have been described so far. The HLA-A2 haplotype is expressed at high frequencies in all ethnic groups, for example in half of the Caucasian population. Last but not least, only peptides can be presented by MHC molecules. Carbohydrate and glycolipid antigens, two important categories of tumour antigens, cannot be targeted by the TCR approach. Most of these limitations can be circumvented by using CARs to transfer specificity. Antigen recognition by these antibody receptors is not restricted by MHC presentation, and not only peptides but also other molecules can be recognised. Another advantage of CARs is that they do not depend on additional interactions of co-receptors, as already discussed above (4.3.2). CARs offer a great variability in combining and optimising different signalling and co-signalling domains (reviewed in Hombach *et al.*, 2002 and Sadelain *et al.*, 2003). This allows one to select and design the intensity and nature of receptor signalling. Finally, a great advantage of CARs is the easier generation of new specificities compared to TCRs, which are mainly derived from isolated TILs. Many different monoclonal antibodies against TAA have been already described, offering a big pool for the generation of CARs. Additionally, it is possible to create antibodies with new specificities (Rossig *et al.*, 2001). Other interesting possibilities are the *in vitro* generation of MHC-dependent antibody receptors (Chames *et al.*, 2000; Willemsen *et al.*, 2001) and the *in vitro* affinity maturation of CARs (Chames *et al.*, 2002).

There are, however, also drawbacks of CAR therapy. Firstly, the risk of anti-CAR immunogenicity in the host exists. The antigen recognition domains are mostly based on mouse monoclonal antibodies, which are recognised as "foreign" by the human immune system (Lamers *et al.*, 1995), a problem faced with any foreign protein used for gene therapy (Riddell *et al.*, 1996). Some groups have addressed this point and have humanised CARs, using as little murine sequence as possible by fusing it to

human protein domains (Hombach *et al.*, 1999; Beecham *et al.*, 2000). However, the joining regions of the different domains are still potentially immunogenic. A further disadvantage of CARs is that only antigens on the cell surface are accessible by these receptors. Furthermore, antigens cannot distinguish between membrane bound and shed antigen. Shed TAA, which are often found in sera of cancer patients, are ideal ligands for CARs. They can block the receptor and also cause activation induced cell death (AICD) through strong and long lasting stimulation of the grafted T cells (Bitton *et al.*, 1999; Hombach *et al.*, 1999). A main concern is the creation of a novel receptor, whose properties are often not fully understood. The exact mechanisms that govern CAR signalling have not yet been deciphered. This includes the affinity for the antigen, the interactions with other cell-surface proteins, and the formation of the immunological synapse, all important factors for T cell activation and regulation. The generation of receptors with high affinities combined with strong signalling and co-signalling functions might lead to T cells that can be activated easily, and consequently loose antigen specificity (Willemsen *et al.*, 2005b). Finally, high affinities could lead to strong associations with target cells, thereby inducing AICD in the effector cells (Krammer, 2000).

Few studies describe the use of chimeric receptors based on neither the TCR nor antibody domains. These receptors are based on extracellular domains as CD4 or the vascular endothelial growth factor (VEGF) fused to signalling domains as the CD3 ζ-chain (Mitsuyasu *et al.*, 2000; Walker *et al.*, 2000; Niederman *et al.*, 2002). A similar approach has been chosen by Jyothi and colleagues, who have developed peptide-MHC receptors fused to a signalling domain, capable of killing auto-reactive T cells (Jyothi *et al.*, 2002). However, studies using chimeric TCR CD3 ζ-chain receptors show that such artificial proteins are often not as efficient as their natural counterparts and need extensive optimisation (Schaft *et al.*, 2003a; Chapter 5 in Schaft, 2003). Further studies with CAR-grafted T cells indicate that TCR-grafted PBLs are often more reactive (Patel *et al.*, 2000; Rossig *et al.*, 2002; Willemsen *et al.*, 2005a).

4.4 Future prospects

The successful transfer of anti-RCC specificity to primary T cells of healthy donors proves the possibility of combining retroviral TCR gene-transfer and adoptive T cell therapy for a RCC therapy. I have employed the TCR originating from TIL-26 and the cognate RCC-26 tumour cell line to prove the functional redirection of PBLs *in vitro*. The next step in the analysis of adoptive transfer of TCR-grafted primary T lymphocytes would be the establishment of a mouse model. Previous approaches to inoculate immunocompromised mice with RCC-26 tumours have, however, not been successful (E. Nößner, personal communication). Furthermore, the identity of the antigen recognised by TCR-26 is unknown and experiments in our laboratory and in the laboratory of D. Schendel (Institute of Molecular Immunology, GSF-National Research Center for Environment and Health, Munich, Germany) suggest that the antigen is tumour-specific, thus also patient specific. For these reasons, the emphasis of further studies will be laid on the recently isolated TIL-53, another RCC-specific CTL clone that, in contrast to TIL-26, shows a broad recognition of RCC lines and also HLA-$A2^+$ cell lines of other tumour entities. Preliminary experiments show a functional redirection of T cells and also the growth of RCC-53 cells in immunocompromised mice. This enables us to establish an model in which we can analyse the performance of TCR-grafted T cells *in vivo*. In these studies the stimulation of the T cells for retroviral transduction and the *in vitro* expansion can be optimised with regard to *in vivo* anti-tumour reactivity (see 4.3.1). Also the importance of the interplay of $CD8^+$, $CD4^+$, and other cells can be analysed in this model.

Nevertheless, the TCR-26 will be used for further experiments. In order to accompany the ongoing RCC-26 vaccination trial (Pohla *et al.*, 2000; Schendel *et al.*, 2000), cell lines grafted with the TCR-26 will be used to generate an anti-idiotype antibody. This will make the detection of potential T cells with the same specificity as TIL-26 in the vaccinated patients much easier. Also the identification of the antigen recognised by TIL-26 will hopefully be possible using TCR-26 grafted indicator cell lines (see 4.2.2). The same holds true for the identification of the antigen recognised by TCR-53, a potentially clinically relevant antigen. Both grafted cell lines will be used to screen peptide libraries obtained from eluates of MHC molecules of either RCC-26 or -53 cells.

Finally, an established retroviral TCR-gene transfer system is readily applicable to different cloned TCRs, giving the possibility of grafting PBLs with any specificity. The MP71 retroviral vector system is an ideal vehicle to transfer genes into primary T cells due to its high and durable transgene expression properties and high transduction efficacy. Although TCR chains seem to have different properties regarding stability and pairing behaviour, the transfer of most TCRs should be possible with the MP71 vector. Ongoing experiments in our laboratory show successful transfer of murine and human TCR genes, enabling us to follow both basic biological questions and potential clinical applications.