Towards Effective TCR Gene Therapy: Preclinical requirements

Preklinische vereisten voor klinisch effectieve TCR gentherapie

Nadine Pouw

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Enjoy the little things, for one day you may look back
and realize they were the big things

Robert Brault

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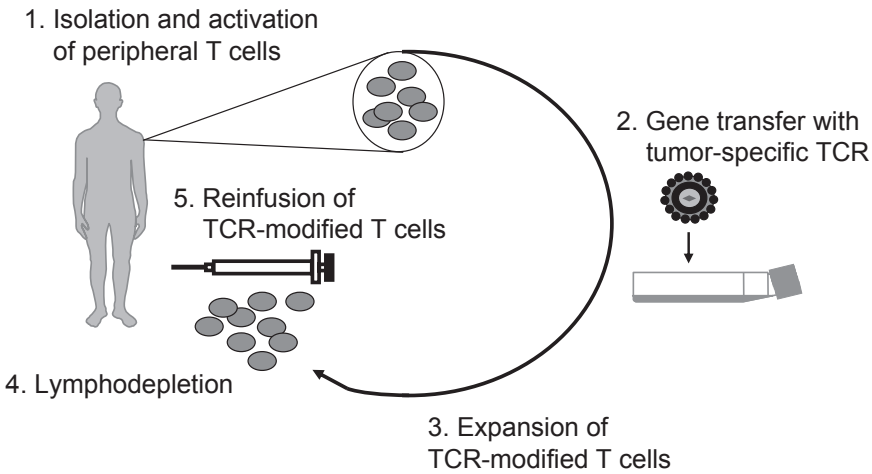
CHAPTER 1

General introduction

1.1 OVERVIEW OF T CELL RECEPTOR (TCR) GENE THERAPY OF SOLID TUMOURS

Adoptive T cell therapy (AT) entails transfer of autologous antigen-specific T cells that have been expanded to large numbers *ex vivo* into tumour-bearing patients. The potential ability of T cells to help in controlling tumour growth has been suggested by enhanced tumour prevalence observed in immunodeficient mice and humans (Goedert, 2000; Shankaran et al., 2001). In addition, the depletion of CD8+ T cells in both fibrosarcoma and lymphoma mouse models showed that these lymphocytes are critical for controlling tumours (Shankaran et al., 2001; Smyth et al., 2000). The first publication of adoptive therapy with immune cells in patients with solid tumours stems from 1966, in which Southam and colleagues described the inhibition of subcutaneous growth of tumour autografts on patients with advanced cancers by co-transfer of autologous leukocytes (Southam et al., 1966). The isolation of tumour infiltrating lymphocytes (TIL) from cancer patients provided a next step in the development of AT in the 1990's (Belldegrun et al., 1988; Brichard et al., 1993; van der Bruggen et al., 1991). TIL were isolated from tumour tissue, activated and expanded *ex vivo*, and re-infused into the patient. At those times TIL therapy, combined with high dose Interleukin-2 (IL-2), occasionally resulted in objective anti-tumour responses (Kawakami et al., 1994; Rosenberg et al., 1988). The therapeutic use of autologous tumour-specific (CD8+) cytotoxic T cell lymphocyte (CTL) clones isolated from metastatic melanoma patients and combined with IL-2 resulted in minor, mixed or stable responses (Yee et al., 2002). Over time studies in mice have led to the refinement of adoptive transfer techniques and the combination of this method of immunotherapy with other therapies has achieved a high degree of success in the treatment of tumours in preclinical models (Palmer et al., 2004; Rosenberg et al., 1986; Spiess et al., 1987; Wang et al., 2002). For example, the combination of adoptive T cell transfer with antigen vaccination plus IL-2 (Overwijk et al., 2003) and/or pre-conditioning with chemotherapy or radiation has shown preclinical success (Cheadle et al., 2009; de Witte et al., 2008; Gattinoni et al., 2005a). Similar results are observed in the clinical setting. Whereas clinical success of TIL infusions as monotherapy has remained limited (Figlin et al., 1999; Kradin et al., 1989; Rosenberg et al., 1994), the combination of TIL therapy with chemotherapy or irradiation and/or vaccination has improved clinical anti-tumour results dramatically. The most successful clinical trial thus far has been performed at the National Cancer Institure (NCI, USA), in which autologous TIL were infused into metastatic melanoma patients after intensive myeloablative host conditioning and additional use of IL-2. In this study a maximal objective response rate of 72% was observed (Dudley et al., 2008). Non-myeloablative conditioning with fludarabine and cyclophosphamide prior to adoptive T cell therapy has already shown 49% overall response rates in melanoma patients (Dudley et al., 2002; Dudley et al., 2005) and would represent a good treatment rationale without the risk of multiple serious toxicities associated with myeloablative regimes (Abraham et al., 1999; Chen et al., 2001; Diaconescu et al., 2004; Moreau et al., 2002; Sorror et al., 2004). A recent case report in a treatment-refractory metastatic melanoma patient demonstrated that although no



Therapy	Type of T cells	Target Antigen	Conditioning	Response	Reference
Conventional T cells	CTL clone	Mart-1/A2	IL-2	30% minor/mixed; 50% stabilization	(Yee et al., 2002)
	TIL	MART-1/A2; gp100/A2	Non-myeloablative (Cy+Flu) IL-2	49% ORR	(Dudley et al., 2002; Dudley et al., 2005)
	TIL		Myeloablative (200 cGy+HSC) IL-2	52% ORR	(Dudley et al., 2008)
	TIL		Myeloablative (1200 cGy+HSC) IL-2	72% ORR	(Dudley et al., 2008)
	TIL	gp100/A2	gp100 +vaccination	PR (case report)	(Smith et al., 2009)
TCR-engineered T cells	Primary T cells	Mart-1/A2	IL-2	13% ORR	(Morgan et al., 2006)
	Primary T cells	Mart-1/A2	Non-myeloablative (Cy+Flu) IL-2	30% ORR	(Johnson et al., 2009)
	Primary T cells	gp100/A2	Non-myeloablative (Cy+Flu) IL-2	19% ORR	(Johnson et al., 2009)

Figure 1. Adoptive T cell therapy in metastatic melanoma

MART-1 or gp100-specific T cells, either peripheral Cytotoxic T Lymphocyte (CTL) clones or Tumour Infiltrating Lymphocytes (TIL), are obtained from melanoma patients and expanded *ex vivo* to sufficient numbers. Prior to adoptive transfer of autologous TIL, melanoma patients were treated with non-myeloablative chemotherapy or with myeloablative Total Body Irradiation (TBI) in combination with Hematopoietic Stem Cell (HSC) transplantation. Objective response rates (ORR) indicated were determined according to Response Evaluation Criteria for Solid Tumours (RECIST; (Eisenhauer et al., 2009)).

clinical response was seen when TIL were administered alone, an objective clinical response to therapy was achieved with a combination of TIL therapy with a highly immunogenic fowlpox vaccine expressing a gp100 antigenic epitope (Smith et al., 2009). An overview of clinical adoptive T cell therapy trials in metastatic melanoma patients is given in Figure 1.

Despite significant clinical results obtained for AT in patients with advanced melanoma, it is recognized that for most human cancers the generation of large numbers of highly tumour-reactive TIL cultures from individual patients is laborious and has a limited success rate. The tolerant state of T cells recognizing self-antigens (which constitute the majority of tumour antigens), and the limited life span of T cells generated and expanded to large numbers *in vitro* are two factors that may account for this limited success. One way to enhance AT's applicability is to genetically provide patient T lymphocytes with anti-tumour specificity. Genetic modification of T cells as a means to improve anti-tumour effects is an attractive strategy that has proven successful in many settings (Ho et al., 2003). Dembic *et. al.* were the first to show that it is indeed possible, using a T cell line, to generate antigen specific T cells by the transfer of α and β TCR genes (Dembic et al., 1986). A number of *in vitro* studies subsequently demonstrated the feasibility to generate virus or tumour specificity into human T cell lines or primary lymphocytes via TCR gene transfer (Clay et al., 1999; Cooper et al., 2000; Orentas et al., 2001; Stanislawski et al., 2001; Willemsen et al., 2000). *In vivo*, the adoptive transfer of TCR modified T cells demonstrated the ability of these cells to expand upon viral vaccination and to induce regression of tumours expressing a model antigen (Kessels et al., 2001; Morris et al., 2005). The preservation of both the cytotoxic potency and peptide fine specificity of CTL following TCR gene transfer, which is crucial to the successful use of TCR genes for immunogene therapy, was shown by Schaft and colleagues in 2003 (Schaft et al., 2003). The first clinical trial using TCRαβ-engineered autologous T cells in AT of solid cancer patients who were pretreated with fludarabine and cyclophosphamide was published four years ago. This study used a MART-1/HLA-A2-specific TCRαβ (MART-1/A2 TCR) derived from a TIL clone isolated from a melanoma patient who demonstrated near complete tumour regression following TIL therapy) (Morgan et al., 2006). The overall response rate of MART-1/A2 TCR immunogene therapy was 12% (Morgan et al., 2006). Recently, another clinical trial in metastatic melanoma patients was conducted, in which autologous T cells were engineered to express highly selective and affine anti-melanoma antigen TCR's. Both a human TCR specific for MART-1/A2 (with a higher affinity than the original MART-1/A2 TCR) and a murine TCR specific for human gp100/A2 were used. Objective cancer regressions were seen in 30% and 19% of patients who received a human (MART-1) or mouse (gp100) TCR, respectively. However, patients treated in this 2nd TCR gene therapy trial exhibited destruction of normal melanocytes in the skin, eye, and ear, and sometimes required local steroid administration to treat uveitis and hearing loss. Clincal TCR gene therapy trials currently ongoing at the NCI include TCRs directed against human tumour antigens NY-ESO and CEA (M. Dudley, personal communication). Details on the steps involved in the generation of antigen-specific T cells by TCR gene transfer are described in Figure 2.

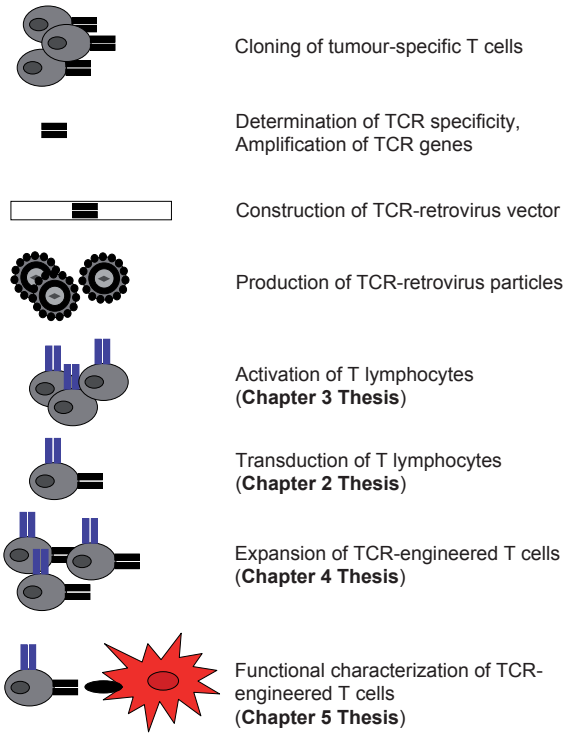


Figure 2. Generation of antigen-specific T cells by TCR gene transfer
Shown are the individual steps that contribute to the necessary laboratory cloning and characterization of an antigen-specific TCR. Activation of T cells, retroviral transduction and expansion, and functional characterization of TCR-engineered T cells are discussed in more detail in Chapters 3, 2, 4 and 5, respectively, of the current thesis. This figure has been adapted from (Engels and Uckert, 2007).

1.2 CHALLENGES OF TCR GENE THERAPY

Clinical results of TCR gene therapy stay behind the 49% overall response observed in patients treated with adoptively transferred TIL (Dudley et al., 2002; Dudley et al., 2005). Challenges of adoptive TCR gene therapy include (but are not limited to) (a) auto-reactivity, both on-target (Johnson et al., 2009; Lamers et al., 2006a) and potentially off-target (Bendle 2009 Nat Medicine, in press), (b) suboptimal functional T cell avidity, and (c) limited peripheral T cell persistence. In light of recent reviews on possible strategies to enhance adoptive T cell therapy (Coccoris et al.; Govers et al., 2010; June, 2007; Rosenberg, 2008; Rosenberg et al., 2008), I will not discuss all strategies to enhance AT in great depth but will rather focus on those strategies explored in the present thesis, schematically depicted in Figure 2. Challenges and potential solutions to address these challenges are presented in Table I and discussed below.

Table I: Challenges of TCR gene therapy and potential solutions to address these challenges

Challenges		Potential solution	References
TOXICITY	• On-target	• Target antigens with surface expression (highly) restricted to tumour cells	• (Coulie et al., 2002; Scanlan et al., 2002)
	• Off-target	• Genetic modification of TCR transgenes to improve preferential TCR pairing: - murinized TCR - cysteine modified TCR - TCR-ζ - TCRβ-2A-TCRα	• (Cohen et al., 2006; Kuball et al., 2007; Sebestyen et al., 2008)
SUBOPTIMAL FUNCTIONAL T CELL AVIDITY	• Limited surface expression of TCR	• - Optimal gammaretroviral vectors - Optimal TCR transgene cassettes - Codon optimization - Genetic modification of TCR transgenes to improve preferential TCR pairing (as mentioned above) - Optimized/enhanced gene transfer technologies	• (Engels et al., 2003; Jorritsma et al., 2007; Leisegang et al., 2008; Schambach et al., 2000; Scholten et al., 2006)
	• Limited T cell activation mediated by TCR transgenes	• - Affinity maturation of TCR-V domains - De-glycosylation of TCR-C domains - Genetic incorporation of optimal signalling domains into TCR transgenes - Combination of TCR gene therapy with chemotherapy, irradiation or vaccination	• (Burns et al., 2009; De Plaen et al., 1994; de Witte et al., 2008; Kuball et al., 2009; Li et al., 2005; Schaft et al., 2006; Smith et al., 2009; Varela-Rohena et al., 2008; Willemsen et al., 2008)
LIMITED PERIPHERAL T CELL PERSISTENCE		- *Ex vivo* treatment of T cells with common-γ cytokines - CD4 T cell help - Defined host T cells for TCR gene transfer: * less-differentiated T cells * CTL clones - Combination therapies (as described above)	(Berger et al., 2008; Hinrichs et al., 2009; Kuball et al., 2005; Morris et al., 2005; Willemsen et al., 2006)

1.2.1 TOXICITIES

Several AT studies using expanded T cell populations in mice (Bos et al., 2008; Overwijk et al., 2003; Palmer et al., 2008; Ugel et al., 2010) and men (Dudley et al., 2008) as well as two recent receptor gene therapy studies (Johnson et al., 2009; Lamers et al., 2006a) demonstrated the occurrence of toxicities towards healthy tissues expressing cognate antigen. In the NCI clinical trials (Johnson et al., 2009; Morgan et al., 2006), T cells used were directed at either gp100 or MART-1, both self-antigens presented by HLA-A2 and over-expressed by melanoma cells. Although vitiligo (observed in multiple patients) might be a manageable side effect, destruction of normal melanocytes in the skin, eye and ear (Johnson 2009) and even corticosteroid treatment refractory uveitis (which occurred in at least one patient, (Dudley et al., 2008)) compose more serious side effects. In an earlier clinical trial with renal cell carcinoma patients using autologous T cells transduced with an antibody-based receptor, severe liver toxicity was observed (Lamers et al., 2006a). Liver toxicity coincided with the expression of the targeted antigen by larger bile duct epithelial cells. Very recently, a case report presented

the unfortunate death of a colon carcinoma patient treated with T cells modified to express an ErbB2-specific CAR (Morgan et al., 2010). The patient died of total organ failure, which was anticipated to be a result of a cytokine storm produced by high numbers of CAR-redirected T cells recognizing low levels of ErbB2 expressed in normal lung epithelium shortly after infusion. These examples indicate that one should be careful when choosing self-antigens as target antigens for AT. Cancer testis antigens may provide a good alternative since these antigens are expressed in many tumours but not in normal adult tissues except male germline cells (which do not express MHC molecules) and thymic medullary epithelial cells (in which expression is only detected at the mRNA level) (Chomez et al., 2001; Gotter et al., 2004; Scanlan et al., 2002). Cytolytic T cell responses have been observed in cancer patients vaccinated with MAGE antigens, demonstrating the suitability of these antigens in anti-tumour immunotherapy (Coulie et al., 2002). In addition, mutated (and thus unique) antigens would also provide safe AT targets, yet their expression is limited to individual tumours, which likely hampers broad clinical applicability (Parmiani et al., 2007).

In addition to on-target toxicity, potential off-target toxicities, caused by TCR-mispairing, should be taken into account. TCR mis-pairing is a recognized phenomenon in the field of TCR gene therapy, which defines the incorrect pairing between a TCRα or β transgene and an endogenous TCRβ or α chain, respectively, and results in diluted surface expression of the therapeutic TCRαβ. Although currently there is no clinical evidence for TCR mis-pairing-induced autoreactivity, the generation of autoreactive TCRs upon TCR mis-pairing cannot be excluded. In fact, T cells expressing mis-paired TCRs and expanded under high IL-2 conditions (similar to the current clinical setting) were demonstrated to induce Graft-versus-Host Disease (termed TCR gene transfer-induced (TI-)GvHD) in a preclinical model (Bendle Nat Med in press). Strategies that increase preferential TCR pairing and counteract TCR mis-pairing are anticipated to avoid the generation of unknown TCR specificities and the development of potential off-target autoimmune reactivity and at the same time yield T cells with higher avidity for the antigen of interest. Strategies to induce preferential TCR pairing include murinization of TCR constant domains (Cohen et al., 2006; Thomas et al., 2007), inclusion of cysteine bond(s) (Cohen et al., 2007; Kuball et al., 2007; Thomas et al., 2007), incorporation of TCRζ domains (Sebestyen et al., 2008), and mutation of amino acid residues to induce a charged "hole-into-knob" configuration (Voss et al., 2008). In addition, equimolar expression of the two TCR chains via a TCRβ-2A-TCRα transgene cassette results in enhanced surface expression of TCR transgenes (see below) and has been shown to limit TCR gene transfer-induced GvHD (Bendle, Nat Med in press). Although some of the strategies to prevent TCR mis-pairing have shown promising first *in vitro* results, thorough evaluation of both function and long-term persistence of T cells gene-transduced with such a modified TCR in preclinical models is highly recommended. A more detailed description on strategies to enhance TCR pairing by modifying TCR formats can be found in a recent review article by Govers and colleagues (Govers et al., 2010).

1.2.2 SUBOPTIMAL FUNCTIONAL T CELL AVIDITY

An increased objective response rate (i.e., 30%) has been observed with a modified, high-affinity MART-1/A2 TCR by Johnson and colleagues (Johnson et al., 2009) when compared to an earlier study with TCR-engineered T cells (expressing the original, non-modified, MART-1 TCR) by Morgan and colleagues (i.e, 13%, Morgan et al., 2006). This suggests that increasing functional T cell avidity realistically enhances the efficacy of TCR gene therapy. Suboptimal T cell avidity, as often observed for TCR-engineered T cells, can be explained by limited TCR surface expression and/or TCR-mediated T cell activation.

Strategies to counteract limited surface expression of TCR transgenes

First, TCR transgene expression is enhanced by use of optimized gamma-retroviral vectors. Most TCR gene transfer studies published to date have made use of gammaretroviral vectors for transgene delivery. Clinically, there is experience with gammaretroviral MFG vectors or MFG-derived vectors (such as pSTITCH), vectors that contain Moloney Murine Leukemia Virus (moMLV) or Murine Stem Cell Virus (MSCV) Long Terminal Repeats (LTRs), and vectors that contain optimized splicing and start codons (Johnson et al., 2009; Kershaw et al., 2006; Lamers et al., 2006a; Morgan et al., 2006). There is now accumulated preclinical evidence that transduction efficiencies differ substantially between different vectors, with a major role for the viral origin of the LTRs and splice and start sequences. In this respect it of interest to mention that the pMP71 vector, which has a Myeloproliferative Sarcoma Virus (MPSV) LTR and optimal 5' sequences, demonstrated highly improved TCRαβ transduction efficiencies (Engels et al., 2003; Schambach et al., 2000).

Second, optimal TCR transgene cassettes enhance TCR surface expression, and hence the functional avidity of T cells. Retroviral transduction of TCRα and TCRβ separately, although facilitating high transduction efficiencies by a relatively small 'genetic baggage' per vector, is associated with possible differences in transduction efficiency and thus surface expression of the two TCR chains. Moreover, the use of two TCR vectors will potentially result in additional sites of gene integration. To circumvent these issues, single constructs containing both the TCRα and β chain, separated by either an internal ribosomal entry site (IRES) or 2A peptide sequence, have been made. IRES, but not 2A, may result in lowered expression of the gene downstream relative to the one upstream of IRES (de Felipe et al., 1999; Klump et al., 2001; Mizuguchi et al., 2000). In clinical studies, both IRES and 2A sequences proved valid to separate TCRα and β genes (Johnson et al., 2009). Placing the TCRβ chain in front of the TCRα chain, especially when separated by a 2A sequence, demonstrated optimal functional TCR expression levels for most TCRs tested (Leisegang et al., 2008).

A **third** option to increase TCR transgene expression is the optimization of the TCR genetic code such as to generate a maximal amount of transgenic protein. Notably, recent data show that codon optimization of TCR genes has a beneficial effect not only on surface expression but also on *in vitro* and *in vivo* function of TCR-engineered T cells (Jorritsma et al., 2007; Scholten et al., 2006).

Fourth, several strategies to induce preferential pairing of TCRα and β, such as those mentioned in section 1.2.1, also result in enhanced surface expression of the introduced TCRα and β chains.

Fifth, enhanced TCR surface expression is achieved by optimizing gene transfer technologies, which are described in more detail in chapter 1.3.

Strategies to counteract limited T cell activation mediated by TCR transgenes

First, affinity maturation of TCR-V Complementary Determining Regions (CDR) will lower the antigen-specific threshold for T cell activation (Chames et al., 2002; Varela-Rohena et al., 2008). It is reasonable to expect that T cells with high affinity TCRs for self-antigens will be absent from the normal T cell repertoire due to negative selection in the thymus. As a result, the cloning of genes of high-affinity TCR from T cells from healthy volunteers or cancer patients is difficult. Various phage display techniques have been used to facilitate the generation of high affinity TCRs (up to $x10^3$ fold the affinity of a natural TCR) directed against the Cancer Testis Antigens NY-ESO and MAGE, and other antigens (De Plaen et al., 1994; Li et al., 2005b; Varela-Rohena et al., 2008; Willemsen et al., 2008). The phage-display methodology can be applied for the *in vitro* affinity evolution of CDR-mutated TCRs. For example, HIV-1-specific TCRs have recently been selected with ligand-binding affinities for which dissociation constants improved from the nM range to a high affinity pM interaction and provided T cells with HIV-1-specific and highly-avid responsiveness (Varela-Rohena et al., 2008). In addition to phage display technology, mRNA-based screenings have proven useful for the selection of mutated TCRs specific for MART-1/A2 and NY-ESO-1/A2 (Robbins et al., 2008). Also, high affinity TCRs can be cloned from T cells generated in human HLA-transgenic mice (Kuball et al., 2005; Stanislawski et al., 2001).

A **second** strategy to enhance T cell activation mediated by TCR transgenes is by de-glycosylation of TCR-C domains. Decreased N-glycosylation of the TCR has been reported to enhance TCR-pMHC interactions and to result in improved functional T cell avidity as measured by cytokine release and lytic activity (Kuball et al., 2009). Glycosylation of T cell proteins, such as the TCR may lead to an increased threshold for T cell activation and de-glycosylation of the TCR-C domain is anticipated to decrease this threshold possibly as a consequence of improved membrane movement or multimerization of the TCR. This de-glycosylation technique was found to be effective for multiple TCRs without evidence for self-reactivity and may be widely applicable in the field of TCR gene transfer.

Third, genetic incorporation of optimal T cell signalling domains into TCR transgenes enhances T cell activation. The CD28 co-stimulatory domain has been incorporated into TCR:ζ and TCR-like antibodies, which resulted in enhanced tumour-specific cytokine production of receptor-transduced T cells (Schaft et al., 2006b; Willemsen et al., 2005b). In addition to CD28, the incorporation of other TNF receptor super family members, such as CD134 and CD137, into TCR molecules may further optimize T cell functions as suggested by scFv receptors

that incorporate such co-stimulatory molecules (Hombach and Abken, 2007). For example, a recent publication demonstrated that the incorporation of CD28 and CD137 signalling domains in scFv leads to potent anti-tumour activity and prolonged *in vivo* persistence (Carpenito et al., 2009).

Fourth, activation of TCR-engineered T cells is enhanced by a combination of TCR gene transfer and chemotherapy, irradiation or vaccination. A more detailed discussion on a combination of treatment rationales can be found in chapter 1.2.4.

1.2.3 COMPROMISED PERIPHERAL PERSISTENCE OF ANTI-TUMOUR T CELLS

A clinical study in advanced melanoma patients demonstrated that although tumour-specific T cell clones were present at high frequencies in the blood of patients 2-3 days after adoptive transfer, these T cells were undetectable by 2-3 weeks after AT. This lack of peripheral T cell persistence correlated with a lack of clinical response (Robbins et al., 2004). These data extended findings of an earlier trial using adoptively transferred TIL, in which most responses were transient and in most patients transferred T cells persisted only for a limited period of time (Rosenberg et al., 1994). In metastatic melanoma patients treated with autologous T cells transduced with a MART-1/A2 TCR, less than 10% of transferred TCR-transduced T cells persisted for 30 days and less than 2% of TCR-transduced T cells persisted beyond 50 days (Morgan et al., 2006). When using a more affine TCR to gene-engineer T cells, 20% tetramer-positive T cells (i.e., 20% of total CD3+ T cells) were observed in blood from metastatic melanoma patients one month after infusion (Johnson et al., 2009), but no data on persistence beyond one month after infusion were presented. The observed limited persistence of transferred T cells may have several causes. First, transferred T cells may not become sufficiently activated *in vivo* (Burns et al., 2009), for example as a result of low T cell avidity. Tumour cell down-regulation of MHC molecules (Garcia-Lora et al., 2003) and absence of sufficient antigen readily available to TCR transduced T cells in the circulation and in tumour tissue might be of importance for this lack of *in vivo* T cell activation. In addition, inhibition of T cell activation may result from a suppressive tumour microenvironment caused by mechanisms such as hypoxia, induction and recruitment of suppressor cells (e.g., regulatory T-cells (T-regs), myeloid-derived suppressor cells (MDSCs) and immature dendritic cells), appearance of immunoregulatory immune complexes, oxidative stress, enhanced proteolytic activity and the secretion of pro-tumour cytokines such as IL-6 and IL-23 (Kortylewski and Yu, 2008; Langowski et al., 2006; Wang et al., 2009). In a suppressive environment effector T cells typically do not become activated, and may even acquire suppressive properties (Huang et al., 2006). Second, humoral and/or cellular immunity against the introduced receptor have been found to be associated with limited *in vivo* persistence of gene-engineered T cells. For example, in studies by Lamers et al. ((Lamers et al., 2006a) and manuscript submitted), antibodies directed against a chimeric antibody-based receptor (CAR) and T cells directed against the murine CDR parts of the scFv transgene were detected in peripheral blood of all patients after treatment. Third, infused T

cells may have suboptimal phenotypical and functional characteristics that may not allow peripheral persistence for prolonged periods post-transfer. Until recently IFNγ production upon *in vitro* antigen stimulation was one of the key criteria to select T cells for adoptive transfer. CD8+ T cells that mainly produce IFNγ have generally differentiated into effector memory T cells (Hamann et al., 1997), which suggests that those T cells used in *in vivo* experiments and clinical trials were of a terminally differentiated effector CD8 T cell phenotype (Dudley and Rosenberg, 2003; Yee et al., 2002). In melanoma patients, adoptive transfer of T cell clones following extensive proliferation *in vitro* did not result in long-term persistence of transferred T cells and clinical responses were not observed (Dudley et al., 2001; Powell et al., 2006). In fact, T cells that did persist for a longer period of time following AT, expressed co-stimulatory molecules CD27 and CD28, characteristics of less differentiated T cells (Powell et al., 2005). Current evidence from murine models and clinical trials suggests that a less differentiated T cell phenotype correlates with better anti-tumour protection (Gattinoni et al., 2005b; Klebanoff et al., 2005a; Shen et al., 2007; Zhou et al., 2005). Prolonged antigen stimulation during *in vitro* culture, such as often required for transfer of high numbers of tumour reactive TIL, can cause the generation of exhausted T cells with a limited proliferative capacity (Lee et al., 1999; Moskophidis et al., 1993; Zajac et al., 1998). However, less differentiate lymphocytes, next to more fit, also contain certain phenotypical characteristics that make them more suitable for use in immunotherapy. For example, less differentiated T cells have been shown to express higher levels of lymphoid homing molecules such as CD62L and CCR7, secrete higher levels of T cell growth factor IL-2, and confer superior anti-tumour immunity when transferred to tumour bearing mice (Gattinoni et al., 2005b; Kaech et al., 2003; Klebanoff et al., 2005a; Wherry et al., 2003). Furthermore, CD8-enriched younger TIL may show clinical advantage in the treatment of metastatic melanoma (Dudley, personal communication).

Potential strategies to increase peripheral persistence of TCR-engineered T cells

First, less differentiated TCR-engineered T cells can be obtained either by *ex vivo* exposure of TCR-engineered T cells to common-γ cytokines, or by using less differentiated T cells as host cells for TCR gene transfer, such as haematopoietic stem cells. For a detailed description of *ex vivo* exposure of TCR-engineered T cells to common-γ cytokines, see chapter 1.4. With respect to the use of less differentiated T cell populations as recipient cells for gene transfer, a study in macaques demonstrated prolonged persistence and effective memory after the adoptive transfer of central memory T cells, possibly making them ideal candidates for TCR gene therapy studies (Berger et al., 2008). Recently, Hinrichs and colleagues demonstrated improved anti-tumour activity of naive T cells when compared to central memory T cells (Hinrichs et al., 2009). Furthermore, hematopoietic stem cells have been used as recipient cells for TCRαβ genes, and were shown to mature when challenged with antigen following either AT in a mouse model or when *in vitro* co-cultured with OP9 stromal cells expressing a human Notch-1 ligand (van Lent et al., 2007; Yang and Baltimore, 2005; Zhao et al., 2007).

Second, peripheral persistence of TCR-engineered T cells is enhanced by the administration of CD4 T helper cells concurrently with CD8 T cells. This strategy has been shown to prevent exhaustion of infused CD8 T cells (Hunziker et al., 2002; Marzo et al., 2000) and to result in effective anti-tumour T cell responses (Antony et al., 2005). It has been well documented that CD4 T cells activate professional antigen presenting cells, which leads to priming of antigen-specific CD8 CTL function (Schoenberger et al., 1998). Moreover, adoptive transfer of CD4 T cells results in *de novo* generation of antigen-specific CD8+ T cells, activation of CD8 T cells that are already present at the site of the tumour, activation of other immune cells, and induction of an anti-tumour humoral response. In addition, CD4 T cells are a major source of IFN-γ (Hunziker et al., 2002; Ibe et al., 2001; Surman et al., 2000). Building on the therapeutic need for high antigen-specific IFN-γ production, CD4 T cells have been studied as recipient T cells for MHC class I-restricted TCRαβ in combination with CD8α genes. Not only can CD4 T cells be functionally endowed with MHC I-restricted TCRαβ via gene transfer (Kuball et al., 2005; Willemsen et al., 2005a), genetic co-introduction of CD8α skews TCR-engineered T cells towards an antigen-specific Th1-type T cell response (Willemsen et al., 2006). However, the *in vivo* proliferation and anti-tumour responses of adoptively transferred CD4 T cells that produce high levels of IFN-γ, such as TCR/CD8α-engineered CD4 T cells, appears compromised when compared to IFN-γ low CD4 T cells (Morris et al., 2005). A recent but as yet incompletely understood role for Th17 CD4+ T cells in tumour-specific IFNγ production is suggested by the observation that these cells can evolve into IFNγ-producing Th1-like cells capable of tumour destruction (Muranski et al., 2008).

Third, introducing TCR transgenes into a T-cell population with a restricted TCR usage, such as virus-specific T cells, improves peripheral persistence of T cells due to ongoing stimulation via the endogenous TCR (Rossig et al., 2002). In fact, treatment of neuroblastoma patients with EBV-specific CTL expressing a CAR directed against diasialoganglioside GD2 showed enhanced T cell survival and tumour regression in four out of eleven patients treated (Bos et al., 2008).

1.2.4 COMBINATION THERAPIES TO ENHANCE THE ANTI-TUMOUR EFFICACY OF TCR-ENGINEERED T CELLS

The efficacy of adoptive transfer is enhanced by combining this treatment with chemotherapy and radiation (Chakraborty et al., 2003; Ganss et al., 2002; Lake and Robinson, 2005). Recent studies indicate that the choice of chemotherapy may be more important than was previously realized, as some cytotoxic agents render tumour cells more immunogenic than others, independent of the fractional killing effect of the drug (Machiels et al., 2001; Obeid et al., 2007). In addition to direct effects on tumour cells, cytotoxic therapies also improve anti-tumour effects of transferred T cells by several indirect mechanisms. First, myeloablative conditioning decreases competition of endogenous lymphocytes for homeostatic cytokines such as IL-7 and IL-15 (Gattinoni et al., 2005a). Second, cytotoxic therapies result in the

elimination of suppressor cells (Antony et al., 2005) and enhanced activation of antigen presenting cells (Klebanoff et al., 2005b). Third, myeoablative conditioning can delay or limit a potential adverse immune response towards TCR-engineered T cells. Indeed, in a non-human primate model it was shown that non-myeloablative pre-conditioning delayed the induction of a cellular immune response towards gene-engineered T cells and prolonged their *in vivo* persistence (Berger et al., 2001). In extension, in immune-suppressed human lymphoma patients, Till and colleagues have shown that treatment with T cells electroporated with a CD20-specific CAR did neither result in clear humoral nor cellular anti-scFv responses (Till et al., 2008). In addition to chemotherapy and irradiation, adoptive T cell therapy can be enhanced by immunotherapeutic approaches such as vaccination (de Witte et al., 2008; Jiang et al., 2006; Overwijk et al., 2003) and cytokine administration (Cheever et al., 1982). In a recent clinical study, vaccination of melanoma patients with recombinant fowlpox expressing human gp100 increased the efficacy of adoptively transferred T cells, whereas T cells in non-vaccinated patients did not elicit any objective response (Smith et al., 2009). As with other forms of immunotherapy, it is likely that the most effective clinical application of adoptive T cell transfer will employ combinatorial approaches (Pardoll and Allison, 2004).

1.3 GENE TRANSFER METHODOLOGY

Essential to the success of genetic engineering of T cell specificity are improved gene transfer technologies, taking into account the use of virus or non-virus transfer vehicles, (in case of viruses) type of packaging cells, and the actual gene transfer protocol (Finer et al., 1994; Grignani et al., 1998; Pollok et al., 1998; Riviere et al., 2000; Weber et al., 2001; Willemsen et al., 2000).

1.3.1 NON-VIRAL GENE TRANSFER

Non-viral gene transfer methods include RNA electroporation and the use of transposon systems. RNA electroporation of TCRαβ transgenes has been exploited by several groups (Schaft et al., 2006a; Zhao et al., 2006). Although this method is suitable to rapidly screen TCR-transduced T cells, the transient expression of TCR genes due to RNA partitioning during subsequent cell divisions make it currently less attractive as a clinical tool. In addition, transposon-based gene transfer systems are being evaluated in T lymphocytes (Mates et al., 2009). The use of transposons, however, like RNA electroporation, may be limited to preclinical screening and not suitable for long-term *in vivo* TCR gene transfer purposes.

1.3.2 VIRAL VECTORS FOR GENE TRANSFER

Gammaretroviruses are widely used for the stable transfer of genes to somatic cells, including tumour cells and immune cells (Weber et al., 2001). Most retroviruses only infect proliferating

cells (Hajihosseini et al., 1993; Miller et al., 1990; Roe et al., 1993) and as a consequence, immune cells have to be activated *in vitro* prior to gene transduction. Procedures for retroviral gene transfer have currently been optimized in our laboratory for transduction of both murine T cells (Pouw et al., 2007) and human T cells, the latter at GMP-level (Lamers et al., 2006b). In addition to retroviral transduction, other viral gene transfer methods are employed in T cell retargeting, and lentiviral vectors might be of particular interest in the setting of TCR gene therapy. Lentiviral vectors may have a number of potential advantages in comparison to gammaretroviral vectors, including their capacity to carry a larger genetic payload, the ability to transduce minimally stimulated immune cells and a potentially safer site preference for gene integration (Cavalieri et al., 2003; Montini et al., 2006). It is noteworthy that although strong pre-stimulation of T cells is not required for lentiviral transduction, a low level of T cell activation is still needed for integration of the transgene, since lentiviral infection of totally quiescent cells is blocked (Cavalieri et al., 2003; Unutmaz et al., 1999). In addition, *ex vivo* T cell expansion following lentiviral transduction is in most cases needed to obtain a sufficient number of T cells for adoptive transfer. Consequently, activation of T cells after lentiviral transduction is often still needed. Lentiviral vectors have proven their value with respect to transduction of both anti-tumour reactive CTL (Zhou et al., 2003) and minimally stimulated PBL (Cavalieri et al., 2003; Joseph et al., 2008; Tsuji et al., 2005; Yang et al., 2008). Recently, generation of antigen-specific human T-cells by lentiviral TCR gene transfer in combination with cytokine stimulation (i.e., IL-15+IL-21), but not anti-CD3 mAb stimulation, has been demonstrated by Perro and colleagues (Perro et al.). To date the clinical use of lentivirally transduced T cells in humans has been restricted to the setting of HIV-1 infection (Levine et al., 2006). High levels of *ex vivo* transduction were reported along with short-term persistence of the transferred T cells with no adverse advents attributed to the lentiviral vector gene transfer system.

An important safety issue concerning retroviral gene transfer is linked to insertional mutagenesis, as evidenced by reports of leukaemia as a result of treating X-SCID patients with CD34+ progenitor cells transduced with common γ-chain (Hacein-Bey-Abina et al., 2003). It is of note that mature T cells in contrast to haematopoietic progenitor cells are resistant to oncogenic transformation when followed up long-term in a preclinical transplant setting (Newrzela et al., 2008). Also clinically, there are currently more than 100 patients treated with high numbers of retrovirally transduced T cells providing no evidence of adverse effects of retroviral gene transfer into mature T cells. As mentioned before, lentiviral vectors are generally believed to constitute a safer alternative to gammaretroviral vectors with respect to insertial mutagenesis, since integration appears to be less biased towards transcription start sites (Mitchell et al., 2004). The development of novel self-inactivating lentiviral vectors to efficiently transduce human primary T cells with TCRαβ transgenes (Chen et al., 2009) is expected to increase the safe use of these vectors in anti-cancer immunotherapy, although

current experimental data are still limited. In addition, although SIN vectors are believed to disturb expression of endogenous genes in the vicinity of integration sites at a lower level than non-SIN vectors, a recent case indicates that (relative) clonal dominance can still occur, as demonstrated in a patient with β-thalassemia major, two years after receiving hematopoietic stem cells genetically modified with a SIN lentiviral vector (press release of NIH office of Biotechnology Activities, June 10, 2009).

1.3.3 T CELL RETROVIRAL TRANSDUCTION PROTOCOLS

Retroviral transduction efficiency is influenced by many factors, including (but not limited to) (1) T cell activation conditions, (2) viral pseudotype, (3) the type of (stable) packaging cell lines used to generate virus particles, and (4) the use of supporting materials such as Retronectin™ (fibronectin). These four parameters will be shortly described below and in more detail in chapters 2 and 3.

1.3.3.1 T cell activation conditions

T cell activation prior to gene transfer can be provided by either mitogenes or antigenes. In preclinical studies, T cells are mostly activated with lectins, such as Phytohaemagglutinin (PHA) or ConcanavalinA (ConA) (Fujio et al., 2000; Kessels et al., 2001; Kolen et al., 2002; Pouw et al., 2007; Zhang et al., 2004), or anti-CD3 mAb with or without anti-CD28 mAbs (Bondanza et al., 2006; Hollatz et al., 2008; Hori et al., 2003; Rubinstein et al., 2003; Sauce et al., 2002). T cell activation not only influences the induction of cell cycling, and consequently retroviral transduction efficiencies, but also T cell phenotype and function.

1.3.3.2 Viral pseudotype

The transduction efficiency of primary T cells depends on the viral envelope used for vector pseudotyping (i.e., use of viral envelope proteins of defined virus species to modify viral tropism). Retroviruses commonly used for vector pseudotyping include glycoprotein G of the Vesicular Stomatitis Virus (VSV-G), gibbon ape leukaemia virus (GALV), murine moloney leukemia virus (MoMLV)-10A1, amphotropic MLV (MLV-A), ecotropic MLV (MLV-E), murine sarcoma virus (MSV) and lymphocytic choriomeningitis virus (LCMV). Whereas GALV or MoMLV-10A1 pseudotypes have been shown to confer highest transduction efficiency to human T lymphocytes (Bauer et al., 1995; Bunnell et al., 1995; Lam et al., 1996; Lamers et al., 2006b; Uckert et al., 2000), no data are available on a direct comparison of different viral pseudotypes for the transduction of primary murine T cells.

1.3.3.3 Type of (stable) packaging cell lines

Generation of a receptor-positive clone of packaging cells, often necessary to obtain a high-titer, helper-free retroviral stock, is laborious as it involves the screening of tens to hundreds of clones, and is only successful in cases where permanent expression of the transgene does

not adversely affect the growth of the producer cell line. An alternative method involves the use of a co-culture of different packaging cell lines, described as "ping-pong" system, and has been demonstrated to improve retroviral transduction efficiency (Eshhar et al., 2001; Kotani et al., 1994; Kozak and Kabat, 1990).

1.3.3.4 Other factors

Other factors influencing retroviral transduction efficiency are low-speed centrifugation and the use of fibronectin during gene transfer. The combination of these two factors has been shown to increase transduction efficiency by up to several fold (Uckert et al., 2000; von Kalle et al., 1994; Zhou et al., 2001). The most probable mechanism behind enhanced gene transfer is the co-localization of retroviral particles and target cells on the fibronectin molecules (Hanenberg et al., 1996).

1.4 T CELL TREATMENT WITH COMMON-γ CYTOKINES: IL-2, IL-15 AND IL-21

Historically, T cell expansion *in vitro* and *in vivo* is supported by IL-2 (Kim et al., 2006; Morgan et al., 1976). Currently, clinical protocols are based on a standard rapid expansion protocol for tumour-specific TIL using anti-CD3 antibodies, a limited number of expansion cycles on allogeneic feeders cells, and high dose IL-2 (Dudley et al., 2005). However, IL-2 has been demonstrated to decrease the expression of lymphnode homing molecules and to promote the terminal differentiation of T cells, predisposing them to activation-induced cell death (AICD) (Gattinoni et al., 2005b; Refaeli et al., 1998). In addition, *in vivo* use of high dose IL-2 is associated with serious toxicity such as vascular leak syndrome (Rosenstein et al., 1986). Other cytokines from the common-γ cytokine receptor family (i.e. common-γ chain (γc) cytokines) can be used to expand T cells both *in vitro* and *in vivo*. Below we discuss differences between three commonly used common-γ cytokines, representing a focus of the current thesis: IL-2, IL-15 and IL-21, with respect to (1) receptor use and signalling, (2) biological effects, in particular those on T cells, and (3) potential in anti-tumour therapy.

1.4.1 COMMON-γ CYTOKINE RECEPTORS

IL-2 binds a heterotrimeric receptor composed of the IL-2Rα (CD25), the common-β chain (i.e., IL-2Rβ or CD122, which is shared with the IL-15 heterotrimeric complex) and the γc chain (CD132) (Grabstein et al., 1994; Taniguchi and Minami, 1993; Waldmann, 1991). IL-15 binds both the IL-2Rβ and γc chain, as well as its own unique IL-15Rα chain (Waldmann et al., 2001). For both IL-2 and IL-15, heterotrimeric receptors containing the α,β and γ subunits confer high ligand-binding affinity, whereas receptors that contain only the β and γ subunits show intermediate ligand-binding affinity (Fehniger and Caligiuri, 2001; Waldmann, 1991). IL-21 binds a heterodimeric receptor consisting of a specific receptor subunit, the IL-21R, and the γc (Leonard and Spolski, 2005). A schematic representation of the receptors for IL-2, IL-15 and IL-21 is depicted in Figure 3. In addition to receptor chains, IL-2, IL-15 and IL-21 share Janus kinases (Jaks)

and Signal Transducers and Activators of Transcription (STATs), representing a rapid cytosol-to-nuclear signalling pathway (Parrish-Novak et al., 2000; Waldmann, 2006; Waldmann et al., 2001). Similarities and differences in JAK and STAT activation between IL-2, IL-15 and IL-21 receptors are also summarized in Figure 3. The expression of different common-γ cytokine receptor subunits on naive, effector and memory T cells (Tn, Teff and Tm, respectively) is summarized in Table II.

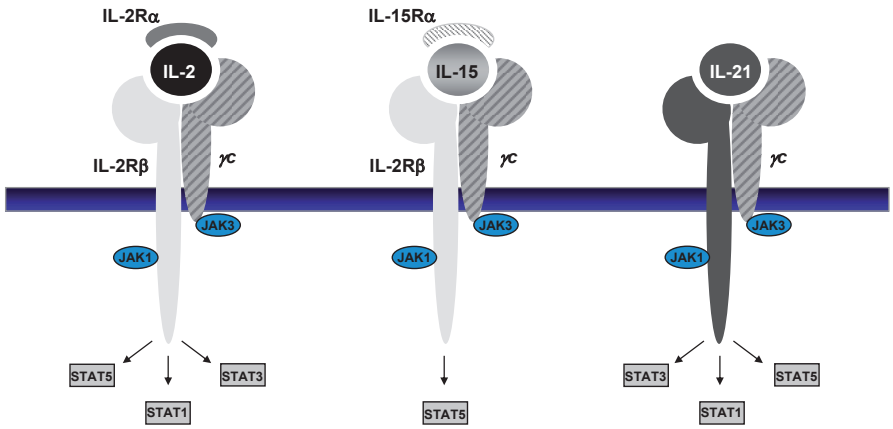


Figure 3. Common-γ cytokines and their receptors

IL-2, IL-15 and IL-21 and their respective receptors are shown. Receptors for all three cytokines contain the common-γ receptor subunit (γc). The receptors for IL-2 and IL-15 contain three receptor chains (α, β and γc), whereas IL-21 receptor consists of only two chains (α and γc). The receptor for each cytokine activates Janus Kinases (JAK) 1 and 3. The main signal transducer and activator of transcription (STAT) proteins that are activated by these cytokine receptors are shown in bold. STAT5 refers to both STAT5A and STAT5B. This figure has been adapted from (Rochman et al., 2009).

Table II: Expression of receptors for γc family cytokines on CD8+ T cell subsets

Receptor chain	Level of expression		
	Naive T cells (Tn)	Effector T cells (Teff)	Memory T cells (Tm)
γ_c (CD132)	Intermediate	Intermediate	Intermediate
IL-2Rα (CD25)	None	High	None
IL-2Rβ (CD122)	Low	High	High*
IL-15Rα	Low	High	High
IL-21R	Low	High	ND

* Sustained at a high level on CD8+ memory T cells; ND: not determined.

Adapted from (Rochman et al., 2009).

1.4.2 BIOLOGICAL EFFECTS, IN PARTICULAR THOSE ON T CELLS

In light of common receptor components and signalling pathways, IL-2, IL-15 and IL-21 are expected to share biological effects on immune cells. Indeed, both IL-2 and IL-15 co-stimulate TCR-mediated proliferation of T cells, induce the generation of CTLs, facilitate the proliferation of B cells and the synthesis of immunoglobulin molecules, and induce the generation and persistence of natural killer (NK) cells (Carson et al., 1997; Fehniger and Caligiuri, 2001; Waldmann et al., 2001; Waldmann and Tagaya, 1999). However, in many adaptive immune responses, IL-2 and IL-15 have distinct, and often competing roles (Fehniger et al., 2002; Ku et al., 2000; Marks-Konczalik et al., 2000). In example, IL-2, through its unique role in AICD and its participation in the maintenance of peripheral CD4+CD25+ regulatory T cells (Tregs), is involved in the elimination of self-reactive T cells (Fontenot et al., 2005; Lenardo, 1991; Lenardo, 1996; Maloy and Powrie, 2005; Shevach, 2000). In addition, IL-2 has been shown to inhibit memory CD8+ T cell proliferation and survival (Ku et al., 2000). In contrast, IL-15 supports the survival of CD8+ memory T cells and maintains long-lasting, high-avidity T cell responses to invading pathogens (Ku et al., 2000; Marks-Konczalik et al., 2000; Zhang et al., 1998). In fact, in IL-15-transgenic mice, IL-2 induced AICD is inhibited (Marks-Konczalik et al., 2000). Another property of IL-15 that is in contrast to IL-2 and important towards the current thesis is its suppressive action on the differentiation of naive T cells into effector T cells following antigen priming (Gattinoni et al., 2005b).

In analogy to IL-2 and IL-15, IL-21 acts as a co-mitogen for antigen-activated effector CD8+ T cells (Moroz et al., 2004; Parrish-Novak et al., 2000; Spolski and Leonard, 2008b). However, in contrast to IL-2, IL-21 does not induce proliferation of Tregs (Peluso et al., 2007), but rather results in long-term sustainment of CD8+ T cell numbers (Moroz et al., 2004) and accumulation of memory CD8+ T cells (Allard et al., 2007). In addition, antigen priming of CD8+ T cells in the presence of IL-21 suppresses differentiation of naive T cells into effector T cells to a greater extent as is observed for IL-15 (Hinrichs et al., 2008). Consequently, *ex vivo* T cell expansion with IL-21 results in the least differentiated phenotype when compared to T cell expansion with IL-15 or IL-2 (Alves et al., 2005; Gattinoni et al., 2005b; Hinrichs et al., 2008). The effects of IL-21 are not limited to CD8+ T cells. For instance, IL-21 influences the growth and functional activity of B and NK cells and many other lymphocytes (Collins et al., 2003; Ebert, 2009; Kasaian et al., 2002; Leonard and Spolski, 2005). A summary of the effects of IL-2, IL-15 and IL-21 on T cell proliferation, homeostasis and differentiation is depicted in Figure 4.

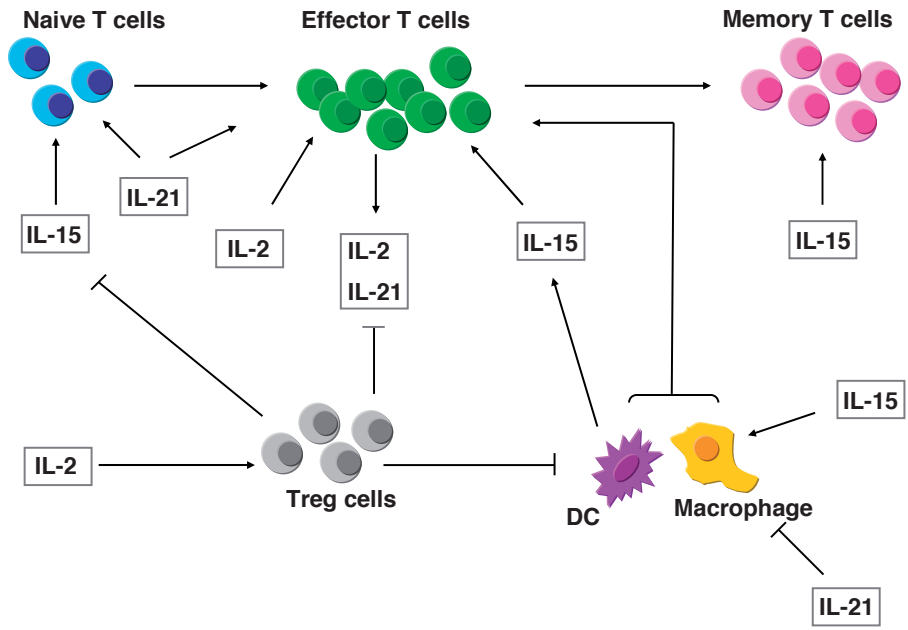


Figure 4. Direct and indirect effects of IL-2, IL-15 and IL-21 on CD8 T cell proliferation, homeostasis and differentiation

IL-2, IL-15 and IL-21 can directly influence the activation, proliferation and differentiation of T cells, as well as indirectly affect these processes through effects on dendritic cells (DCs), macrophages and regulatory T cells (Tregs). Although IL-15 is a crucial factor for memory CD8+ T cell homeostasis, it is also responsible for the recovery of naive CD8+ T cells in lymphopenic conditions. In the absence of IL-7, IL-15 has important effects on the homeostasis of memory CD4+ T cells. IL-2, IL-15 and IL-21 have essential roles in T cell differentiation. In addition, IL-2 and IL-15 can increase the proliferation of T cells after antigen stimulation. See text in 1.4.2 for more details and references. This figure has been adapted from (Rochman et al., 2009).

1.4.3 POTENTIAL IN ANTI-TUMOUR THERAPY

The functional differences between IL-2, IL-15 and IL-21, as described above, have considerable implications for the use of these cytokines to augment T cell responses for the treatment of cancer (Rosenberg, 2000; Waldmann et al., 2001). IL-2 accelerates differentiation of naive T cells into memory effector CD8 T cells which may adversely affect T cell function *in vivo* and as such compromise clinical responses of IL-2 treated adoptively transferred T cells (Evans et al., 1995; Gattinoni et al., 2005b; Hinrichs et al., 2008; Wherry et al., 2003). Notably, clinical anti-tumour efficacy of T cells, irrespective of gene-modification, appears to be directly related to T cell persistence (Ciceri et al., 2007; Kershaw et al., 2006; Robbins et al., 2004), which in turn is reported to be associated with the differentiation state and replicative history of transferred T cells (Huang et al., 2005). Interestingly, IL-15 supports the growth of anti-tumour T cells similar to IL-2, but does not induce the detrimental T cell differentiation and apoptosis that are observed with IL-2 (Gattinoni et al., 2005b; Hsu et al., 2005). T cell populations that have been expanded in the presence of IL-15 were shown to have a superior ability to elicit tumour regression *in vivo* after adoptive cell transfer to tumour-bearing mice when compared with T cell populations that have been expanded in the presence of IL-2 (Gattinoni et al., 2005b; Klebanoff et al., 2004; Klebanoff et al., 2005a; Mueller et al., 2008). In example, human T cells modified with an anti-CD19 CAR and cultured in the presence of IL-15 were capable of long-term persistence in SCID Beige mice and could eradicate disseminated intra-medullary tumours (Brentjens et al., 2003). *In vivo* administration of recombinant hIL-15 showed better anti-tumour efficacy than rhIL-2 at the same dose level, which was accompanied by enhanced activities of both CTL and NK cells (Tang et al., 2008). Today, *in vivo* use of IL-15 has been limited to rodents and non-human primates (Berger et al., 2009), in which it has shown strong therapeutic activity in multiple settings, including mouse models of human cancer (Klebanoff et al., 2004). Furthermore, Teague and colleagues (Teague et al., 2006) demonstrated that IL-15 is able to break tolerance of anti-tumour T cells. Finally, IL-21 has been shown to enhance the tumour-infiltrating ability and anti-tumour activity of CD8+ T cells (Di Carlo et al., 2004; Sondergaard et al., 2007). In fact, IL-21 turns out to be superior to IL-2 and IL-15 in inducing anti-tumour responses of endogenous as well as adoptively transferred CD8+ T cells, and achieving long-term tumour control (Moroz et al., 2004). Clinical application of rIL-21 shows good tolerance and anti-tumour activity in patients with metastatic renal cell carcinoma and melanoma (Thompson et al., 2008), which was most likely associated with *in vivo* activation of CD8+ T cells and NK cells (Frederiksen et al., 2008). To further enhance anti-tumour efficacy of IL-21, it might be combined with IL-15. In fact, Zeng and colleagues (Zeng et al., 2005), reported enhanced CD8+ T cell expansion and anti-melanoma responses upon *in vivo* administration of both IL-21 and IL-15 relative to either cytokine separately.

1.5 SCOPE OF THIS THESIS

The aim of the research presented in the current thesis was to determine optimal *in vitro* conditions to obtain highly functionally avid and not fully differentiated primary murine T cells following TCR gene transfer. In **chapter 2**, we have developed a fast and effective procedure to transfer TCRαβ genes into primary T cells. For this, we compared different (combinations of) packaging cells, including Phoenix-A, Phoenix-E and 293T cells, and viral envelopes, including VSV-G, MLV-A, MLV-E and GALV. In addition, we tested other parameters, such as cell density and the use of fibronectin (Retronectin™), for their effects on T cell transduction efficiencies. In **chapter 3**, we tested T cell activation with ConA or anti-CD3/CD28 mAbs, either soluble or immobilized to polystyrene or latex beads, and T cell treatment with either IL-2 or a combination of IL-15 and IL-21 with respect to *in vitro* phenotype and function of TCR-engineered T cells. **Chapter 4** describes the use of IL-2, IL-15 and/or IL-21 for their effects on primary T lymphocytes during short-term cultures. Cytokine cultured T cells, and their subsets based on T cell differentiation markers, were evaluated for proliferation and expansion, apoptosis, cytokine receptor expression and gene expression profile. The use of different common-γ cytokines in the setting of TCR gene therapy was further investigated in **chapter 5**. IL-2, IL-15 and IL-21 were used to *ex vivo* expand TCR-transduced primary murine T lymphocytes and compare these T cells for the surface expression of the introduced TCR, as well as tumour-specific cytotoxicity and cytokine expression. In addition, we evaluated the presence of different lymphocyte subsets. In **chapter 6** we reflect our main findings, discuss our findings in view of the current literature, and speculate on the implications of our results for future clinical trial designs.

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CHAPTER 2

Gene transfer of human TCR in primary murine T cells is improved by pseudotyping with amphotropic and ecotropic envelopes

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ABSTRACT

TCR gene therapy represents an attractive anti-cancer treatment but requires further optimization of its efficacy and safety in clinically relevant models, such as those using a tumour antigen and TCR of human origin. Currently, however, there is no consensus as to what protocol is most optimal for retroviral human TCR gene transfer into primary murine T cells, most notably with respect to virus pseudotype. Primary murine T cells were transduced, expanded and subsequently tested for transgene expression, proliferation and antigen-specific function. To this end, MLV retroviruses were produced upon transfection of various packaging cells with genes encoding either GFP or TCRαβ specific for human melanoma antigen $gp100_{280-288}$ and the helper elements GAG/POL and ENV. Next to viral pseudotyping, the following parameters were studied: T cell densities; T cell activation; the amounts of IL-2 and the source of serum used to supplement medium. The pseudotype of virus produced by packaging cells critically determines T cell transduction efficiencies. In fact, MLV-A and MLV-E pseudotyped viruses derived from a co-culture of Phoenix A and 293T cells resulted in T cell transduction efficiencies that were two-fold higher than those based on retroviruses expressing either VSV-G, GALV, MLV-A or MLV-E envelopes. In addition, T cell densities during transduction were inversely related to transduction efficiencies. Further optimization resulted in transduction efficiencies of over 90% for GFP, and 68% for both a murine and a human (i.e. murinized) TCR. Importantly, TCR-transduced T cells proliferate (i.e. showing a log increase in cell number in a few days) and show antigen-specific function. We set up a quick and versatile method to genetically modify primary murine T cells based on transient production of TCRαβ-positive retroviruses, and show that retroviral gene transfer of a human TCR into primary murine T cells is critically improved by viral pseudo-typing with both MLV-A and MLV-E envelopes.

INTRODUCTION

Adoptive transfer of antigen-specific T lymphocytes has recently shown clinical success in the treatment of viral infections and tumours (Dudley et al., 2002; Dudley et al., 2005; Rosenberg and Dudley, 2004; Rosenberg et al., 1994; Walter et al., 1995; Yee et al., 2002). We and others have demonstrated that transfer of TCRαβ genes into T cells (i.e., genetic T cell retargeting) represents a feasible and attractive alternative to provide tumour-specific immunity (Clay et al., 1999; Schaft et al., 2003; Willemsen et al., 2000). Currently, gene transduction and T cell expansion procedures have been optimized, meet good clinical practice (GCP) criteria, and are implemented in phase I trials (Kershaw et al., 2006; Lamers et al., 2006a; Lamers et al., 2006b; Morgan et al., 2006).

To further optimize efficacy and safety of TCR gene transfer, it is important to analyze the in vivo behavior of adoptively transferred gene-modified T cells in clinically relevant mouse models. In an effort to enhance the translational value of such models, we have chosen a tumour antigen and TCR of human origin. At the moment, however, there is no consensus as to what protocol is most optimal for retroviral transduction of primary murine T cells. For example, primary murine T cells are activated by either anti-CD3 mAb (alone or in combination with anti-CD28 mAb) or Concanavalin A (ConA) for 24h or 48h. T cells are activated and expanded in different volumes and at different cell densities, and in the presence of various amounts of IL-2 and/or other common-γ cytokines. Transductions are performed in the presence or absence of fibronectin fragments CH-296 (Retronectin™) or polybrene in a supernatant or co-culture system. In addition, there is no consensus as to what retrovirus production system is most optimal to transduce primary murine T cells. Various packaging cells, including Phoenix-E (Ph-E), are used for retrovirus production, following either one or multiple rounds of infection (Beisner et al., 2003; Chamoto et al., 2004; Fujio et al., 2004; Hagani et al., 1999; Haynes et al., 2002; Hori et al., 2003; Kessels et al., 2001; Rubinstein et al., 2003; Zhang et al., 2003). In the present study, we optimized retroviral gene transfer into primary murine T cells using co-cultures of 293T and Phoenix cells as a transient source of MLV retrovirus positive for GFP or TCRαβ specific for the human melanoma antigen $gp100_{280-288}$. We observed that the pseudotype of the produced virus critically determines T cell transduction efficiencies. Viral pseudo-typing with both MLV-A and MLV-E envelopes resulted in T cell transduction efficiencies that were two-fold higher than those based on retroviruses expressing either MLV-A, VSV-G, GALV, or MLV-E envelopes. In addition, we observed an inverse relationship between T cell densities during transduction and actual transduction efficiencies. Additional optimization with respect to T cell activation prior to gene transfer; the amounts of IL-2; the use of fibronectin fragments CH-296 (Retronectin™); and source of medium during T cell activation, transduction and expansion resulted in transduction efficiencies of over 90% for GFP, and 68% for a murine TCR and a human TCR (i.e. murinized TCR). Importantly, TCR-transduced T cells proliferate (i.e. showing a log increase in cell number in a few days) and show antigen-specific function.

Taken together, we set up a quick protocol that only takes 5 days from transfection to flow cytometric analysis of transduced T cells, and that is versatile with respect to the receptor of interest since there is no prior need to generate a clone of receptor-positive packaging cells. A novel observation of the present paper, as a consequence of a direct comparison of differently pseudotyped retroviruses, is that gene transfer of a human TCR into primary murine T cells is critically improved by the use of retroviruses pseudotyped with both MLV-A and MLV-E envelopes.

MATERIALS AND METHODS

Cells and reagents

B16 wildtype (B16F10, kindly provided by dr. G. Adema, Nijmegen, The Netherlands), and B16 transfected with AAD (human HLA-A2 containing the α3 loop of murine H2-Kd) (B16AAD) (kindly provided by dr. V. Engelhard, Charlottesville, USA) were cultured in DMEM (Cambrex, Verviers, Belgium) supplemented with 200 nM L-Glutamine, 10% Fetal Bovine Serum (Greiner Bio-one, Alphen a/d Rijn, the Netherlands), 1% MEM non-essential amino acids, 100 µg/ml streptomycin and 100 U/ml penicillin (DMEM complete). B16AAD was grown under selection of 1 mg/ml G418. Expression of HLA-A2 molecules was verified by flow cytometry (mAb clone BB7.2, BD Biosciences, San Jose, CA, USA). The packaging cell lines 293T (kindly provided by dr. Y.Soneoka, Oxford, UK), Ph-A and Ph-E (both kindly provided by dr. G. Nolan, Boston, MA, USA) were also cultured in DMEM complete. Ph-A and Ph-E were validated by flow cytometry for expression of GAG/POL (anti-Ly-2, clone 53-6.7, BD Biosciences) and ENV (83A25 hybridoma supernatant, kindly provided by dr. Evans, Hamilton, Montana, USA) every 20 passages following 1 week of selection with 300 µg/ml of Hygromycin (GAG/POL) and 1 µg/ml of Diphteria Toxin (ENV) (Calbiochem, La Jolla, CA, USA).

Other mAbs used for flow cytometry were: PE-conjugated anti-human TCRVβ14 mAb (clone CAS1.1.3, Beckman Coulter, Marseille, France); PE-conjugated anti-mouse TCRVβ6 mAb (clone RR4-7); (BD Biosciences); and PE-conjugated anti-human HLA class I mAb (clone w6/32, ITK, Uithoorn, The Netherlands). Other reagents used in this study were rat IFNγ (Peprotech, Rocky Hill, NY, USA), human rIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands), and gp100 wild type peptide (gp100 wt) (YLEPGPVTA, (Schaft et al., 2003)).

Preparation of splenocyte suspensions, T cell activation and expansion

C57BL/6 mouse spleens (Erasmus Dierexperimenteel Centrum, Erasmus MC, Rotterdam, The Netherlands) were dissected and homogenized into a single-cell suspension using the rubber end of a 2 ml syringe and a 70-µm filter. An ammonium chloride solution (150 mM NH_4Cl, 1 mM $KHCO_3$, 10 µM EDTA) was used for red blood cell lysing, after which remaining cells were washed twice with complete mouse medium (CMM). CMM consists of RPMI 1640 medium

supplemented with 25 mM HEPES, 200 nM L-Glutamine, 10% Fetal Bovine Serum, 1% MEM non-essential amino acids, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 100 µg/ml streptomycin and 100 U/ml penicillin. Single cell suspensions ($5x10^6$/ml) were activated with Concanavalin A (ConA, range: 0.5-10 µg/ml, Sigma, St-Louis, USA) and human rIL-2 (range: 10-200 U/ml) in horizontally placed T25 flasks for 24-48 hours. After activation, T cells were retrovirally transduced and subsequently cultured in CMM supplemented with 10-200 U/ml rIL-2 at 0.15-$1x10^6$ cells/ml in 24 well plates. ConA is compared to a combination of soluble anti-CD3 and CD28 mAbs (both at 30 ng/ml final) to pre-activate T cells prior to gene transfer. Anti-CD3 (clone 145-2C11) and anti-CD28 mAbs (clone 37.51) are kindly provided by dr. A. Mondino (Milan, Italy).

Retroviral gene transfer into primary murine T lymphocytes

Packaging cells were transiently transfected with the retroviral vector pSTITCH (Weijtens et al., 1998) containing a GFP construct, or with the retroviral vector pBullet (Willemsen et al., 2000) containing TCRαβ transgenes, via $CaPO_4$ (Life Technologies). The following packaging cells and co-cultures of packaging cells were used for retrovirus production: a mono-culture of Ph-E cells; a co-culture of 293T and Ph-A cells (293T/PhA); or a co-culture of 293T and Ph-E cells (293T/PhE). 293T/Ph co-cultures were always transfected with transgene DNA and pHIT60, a plasmid containing the MLV GAG/POL (kindly provided by dr. Y. Soneoka, Oxford, UK), and either one of the following ENV plasmids: pHIT456 (MLV-A, kindly provided by dr. Y. Soneoka), pCOLT-GALV (Weijtens et al, 1998), VSV-G (pVPack-VSV-G, Stratagene, La Jolla, CA, USA) or pHIT123 (MLV-E, kindly provided by dr. Y. Soneoka). Non-tissue treated 24 well culture plates were coated with 6 µg/well RetroNectin™ (human fibronectin fragments CH-296, Takara Shuzo Co. Ltd., Otsu, Japan) and pre-treated with 2 ml/well of virus supernatant (harvested 48h after transfection of packaging cells) by centrifugation at 1000xg for 1h at room temperature. Next, ConA-activated primary murine T lymphocytes were centrifuged in 2 ml of fresh virus-supernatant in pre-treated wells ($1x10^4$-$1x10^6$ cells/well), and subsequently cultured for 4-5 h at 37ºC/5% CO_2. T cells were allowed to recover in CMM suplemented with rIL-2 (range: 10-200 U/ml) overnight prior to a second transduction cycle, after which T cells were harvested and transferred to fresh 24 well culture plates. After T cell expansion (0.15-$1x10^6$ cells/ml), cells were analyzed for transgene expression by flow cytometry and used in functional assays.

TCR transgenes

Gp100/HLA-A2-specific (gp100/A2) human TCRαβ, gp100/A2 hu:mo TCRαβ (Schaft et al., 2003), and HDM2/HLA-A2 (HDM2/A2) mouse TCRαβ (Stanislawski et al., 2001) were introduced into pBullet (Willemsen et al., 2000) via NcoI and XhoI. Human full-length TCRα and β cDNAs were derived from $gp100_{280-288}$/HLA-A2-specific CTL-296 (Zarour et al., 1996). The hu:mo TCRαβ was constructed by overlap PCR. In short, human V domains were amplified

from gp100/A2 TCRαβ cDNA with the following primers: gp100/A2 TCRα fwd 5'-CTC TCC ATG GCA TCC ATT CGA GCT GTA TTT-3', moCα:gp100/A2 TCRα rev 5'-AGC AGG TTC TGG GTT CTG GAT GTT TGG ATG GAC AGT CAA GAT GGT-3', gp100/A2 TCRβ fwd 5'-CTC TCC ATG GGC CCC CAG CTC CTT GGC TAT G-3', and moCβ2:gp100/A2 TCRβ rev 5'-TGG AGT CAC ATT TCT CAG ATC CTC TGT GAC CGT GAG CCT GGT GCC-3'. Murine Cα and β domains were amplified from HDM2/A2 TCRαβ cDNA (a kind gift of dr. M. Theobald, Mainz, Germany) with the following primers: gp100/A2:moCα fwd 5'-ACC ATC TTG ACT GTC CAT CCA AAC ATC CAG AAC CCA GAA CCT GCT-3', moCα XhoI rev 5'-ATT CGC CTC GAG TCA ACT GGA CCA CAG CCT CAG CGT-3', gp100/A2 TCRβ:moCβ2 fwd 5'-GGC ACC AGG CTC ACG GTC ACA GAG GAT CTG AGA AAT GTG ACT CCA-3', and moCβ2 XhoI rev 5'-ATT CGC CTC GAG TCA GGA ATT TTT TTT CTT GAC CAT AGC CAT CAG CAC CAG GCC ACT-3'. Prior to using HDM2/A2 TCR cDNA for amplification of the murine Cα and β domains, internal NcoI sites were removed via a QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Flow cytometry of transduced T lymphocytes

GFP- or TCR-transduced T cells were analyzed for transgene expression by flow cytometry, with GFP expression directly monitored in the FL-1 channel, and TCR expression (following staining with PE-labelled mAbs) in the FL-2 channel using a Cytomic FC500 (Beckman Coulter, Miami, Fl, USA). Transduced T cells were stained for TCR transgene expression using PE-conjugated anti-human TCR-Vβ14 mAb (recognizing gp100/A2 TCRβ) or PE-conjugated anti-mouse TCR-Vβ6 mAb (recognizing HDM2/A2 TCRβ). For immunostaining, 0.5-1x10^6 transduced T cells were washed with PBS and incubated at 4°C with mAbs for 30 min. Upon completion of the immuno-stainings, cells were washed again, fixed with 1% paraformaldehyde and analyzed.

Cytotoxicity assay

Cytotoxic activity of primary murine T cells retrovirally transduced with TCRαβ genes was measured in a standard 4h ^{51}Cr-release assay. B16F10 and B16AAD target cells were labelled with 100 µCi $Na_2{}^{51}CrO_4$/10^6 cells for 2h at 37°C and 5%CO_2. In case of gp100-specific reactivity, B16AAD cells were pulsed with human gp100 peptide (final concentration of 10 µM) for 15 min at 37°C and 5%CO_2 prior to co-cultivation with effector T cells. Percentage of specific cytolysis, i.e. specific ^{51}Cr-release, was calculated as described previously (Weijtens et al., 1998).

Cell growth

TCR-transduced T cells were counted every 2nd or 3rd day up to 2 weeks post-transduction. Cellular viability was determined by trypan blue exclusion, and viable cells were counted microscopically (Leitz Laburlux 12, Leica Geosystems BV, Rijswijk, The Netherlands) using Bürker counting chambers. Cell growth is expressed as cumulative fold increase in cell number.

Statistical analyses

Statistical analysis of transduction efficiencies (upon the use of differently pseudotyped retroviruses) was performed with a student's t-test with statistical significance indicated by p values.

RESULTS

Virus pseudo-typing with both MLV-A and MLV-E envelopes facilitates transduction of primary murine T cells

Various packaging cells and ENV genes were used to transiently produce GFP-positive retroviruses, to study the effect of virus pseudotype on transduction efficiency of primary murine T cells. We tested the following MLV packaging sytems and thereby different virus envelopes: (i) a monoculture of Ph-E cells; (ii) co-cultures of 293T and Ph-E cells transfected with DNAs encoding MLV-GAG/POL and either VSV-G, GALV, MLV-A or MLV-E ENV; or (iii) a co-culture of 293T and Ph-A cells transfected with DNAs encoding MLV-GAG/POL and MLV-E ENV. Figure 1A shows a schematic overview of the different packaging systems. Figure 1B shows that 293T/Ph-E cells, in which viruses were pseudotyped with different envelopes, did only slightly improve transduction efficiencies of murine T cells (range of mean transduction efficiencies for the various ENV genes: 40-47% GFP^+ T cells, MFI: 39-40, n=6) when compared to a monoculture of Ph-E cells (mean: 32 % GFP^+ T cells, MFI: 34, n=4). The use of VSV-pseudotyped viruses led to slightly higher transduction efficiencies, when compared to GALV- or MLV-A-pseudotyped viruses. Importantly, 293T/Ph-A cells that produce viruses with both MLV-A and MLV-E envelopes perform significantly better than all the other packaging systems tested (74% GFP+ T cells, MFI: 73, n=4). Differences in transduction efficiencies were not due to differences in transfection efficiencies between different packaging cells (range of mean transfection efficiencies: 94-99% GFP^+ packaging cells, n=5). Flow cytometry data corresponding to the above-mentioned transductions are shown in Figure 1C.

Low T cell densities improve retroviral transduction efficiency

Next, we optimized the virus particle to T cell ratio in our transductions. We started at 1 million cells per well in a 24-well plate and titrated down the amount of cells per well using the same volume of virus-containing supernatants. Figure 2 shows that the transduction efficiency negatively correlates with T cell numbers per well. Decreasing cell number to 10^4 cells per well increased transduction efficiency up to 76% GFP^+ cells (data not shown).

In addition to virus pseudotype and T cell densities during transduction, we also optimized the following parameters: amount of ConA to activate primary T cells; time of activation prior to gene transfer; amount of rIL-2 and source of serum used during T cell activation, transduction, and expansion; the use of fibronectin fragments CH-296 (Retronectin™) as a

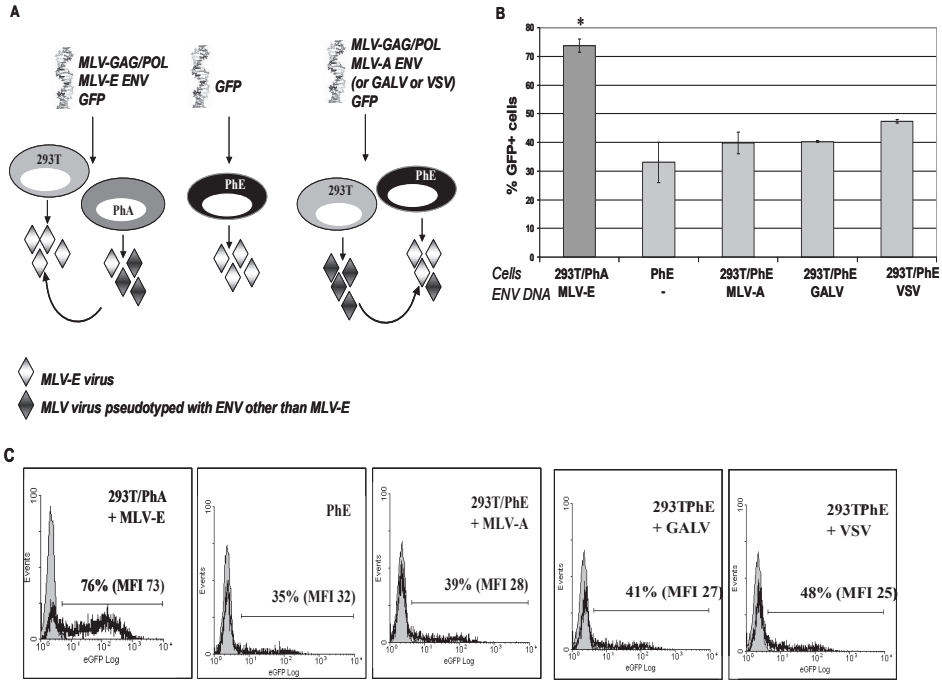

Figure 1. A co-culture of transfected 293T and Phoenix-A but not Ph-E cells, as a transient source of retrovirus, results in efficient transduction of primary murine T lymphocytes

A) Schematic illustration of packaging systems used for retroviral gene transfer of murine T cells. A monoculture of Ph-E cells or a co-culture of either 293T/Ph-A or 293T/Ph-E cells was transiently transfected with a GFP construct. In the case of 293T/Ph-A cells, MLV-GAG/POL and MLV-E DNA were added during transfections, whereas in the case of 293T/Ph-E cells MLV-GAG/POL and one of the following ENV genes were added: MLV-A, GALV or VSV. The different packaging systems result in supernatants that are composed of viruses pseudo-typed with indicated envelopes, and are used to transduce primary murine T lymphocytes. Production of MLV-E ENV-typed viruses, necessary to successfully transduce mouse T cells, is indicated by darkened virus particles. B) GFP expression of primary murine T cells transduced with different pseudo-typed retroviruses. Transduction efficiencies are shown as the mean percentage of mouse T cells expressing GFP of at least 4 experiments per condition +/- SEM. p values were obtained with a student's t-test (* packaging system: 293T/PhA + MLV-E DNA was compared to the other 4 packaging systems (PhE or 293T/PhE + MLV-A, VSV or GALV DNA, all with $p < 0.006$). C) Flow cytometry data representative for the results summarized in Figure 1B. Four days after transduction, mouse T cells were harvested, and 0.5×10^6 cells were used for analysis of GFP expression by flow cytometry.

transduction-mediating agent; and the number of infection cycles. All the optimisation experiments were done with retrovirus encoding GFP. The different parameters and outcomes of our optimization experiments are summarized in Table I.

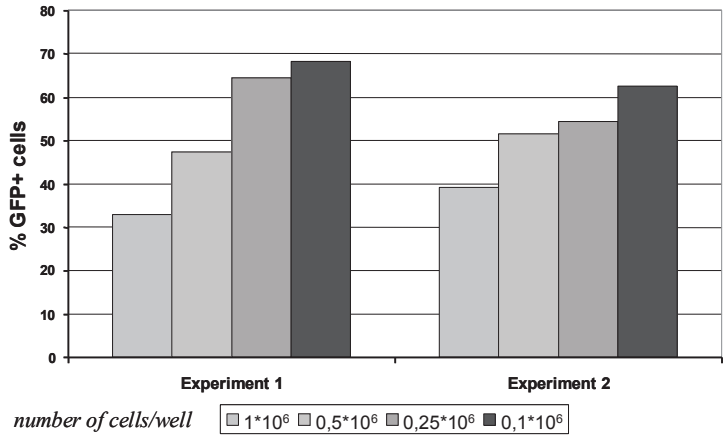

Figure 2. Low cell densities of primary murine T lymphocytes during gene transfer enhance retroviral transduction efficiencies

293T/Ph-E packaging cells transfected with GAG/POL, MLV-A and GFP DNA were used to produce retroviruses. Concanavalin A-activated primary mouse T cells were transduced at different cell densities, ranging from $1x10^6$ to $1x10^5$/well. Transduction efficiencies are indicated as the percentage of mouse T cells expressing GFP. Data shown are from two independent experiments.

A 293T/Ph-A packaging system most optimal for human TCR gene transfer into primary murine T cells

Following our optimized GFP transductions, we validated our transient virus production and T cell transduction protocol by TCR gene transfer. We believe that the use of a TCR directed against a human antigen, such as the melanoma antigen $gp100_{280-288}$, is critical to the translational value of *in vivo* models of tumours. Since TCRs of human origin are only minimally expressed by murine T cells, we murinized the gp100/A2 TCR by combining the variable region of the human TCR with the constant domain of a murine TCR (i.e. HDM2/A2 TCR), resulting in a reformatted gp100/A2

Table I: Summary of conditions to optimally activate, retrovirally transduce and expand primary murine T lymphocytes[ab]

	Murine primary T cells
Activation	2.5 µg/ml Conacavalin A *(tested: 1-10 µg/ml)* 100 U/ml IL-2 *(tested: 10-200 U/ml)* 24h *(tested: 24-48h)*
Transduction	6 µg/well Retronectin 1 hit: spin 1200g 1h 30ºC *(vs 2 hits, 1000g, 1h RT)* Incubate for 6h 37ºC/5% CO_2
Expansion	100 U/ml IL-2 *(tested: 10-200 U/ml)* 0.15-$1x10^6$ cells/ml

[a] GPF-positive retroviruses were derived from either 293T/Ph-A co-cultures transfected with MLV-E DNA or 293T/Ph-E co-cultures transfected with MLV-A DNA. Activation, transduction and expansion were performed in CMM in 24 well plates. See Materials and Methods for further technical details.

[b] A minimum of 2 experiments was performed for each parameter tested.

hu:mo TCR. Figure 3 demonstrates that 68% of murine T cells express the hu:mo TCR at their cell surface following transduction, whereas the expression of the human parental TCR (from which the murinized TCR was derived) is less than 5%, and demonstrates that murine TCR-C domains allow for successful surface expression of human TCRs by murine T cells. Murine T cells transduced with GFP and murine HDM2/A2 mouse TCR as control transgenes showed expression levels of >90% and 68%, respectively (Figure 3). When translating our results obtained with the various packaging systems with GFP as a reporter gene to TCRαβ transgenes, we confirmed the use of 293T/Ph-A cells as most optimal to transduce primary murine T cells (Figure 4). Please note that T cell transduction efficiencies mentioned in Figure 4 are somewhat lower than those in Figure 3, which is due to the fact that experiments were set up to compare packaging systems and were not yet optimized for every parameter as was the case for experiments described in Figure 3.

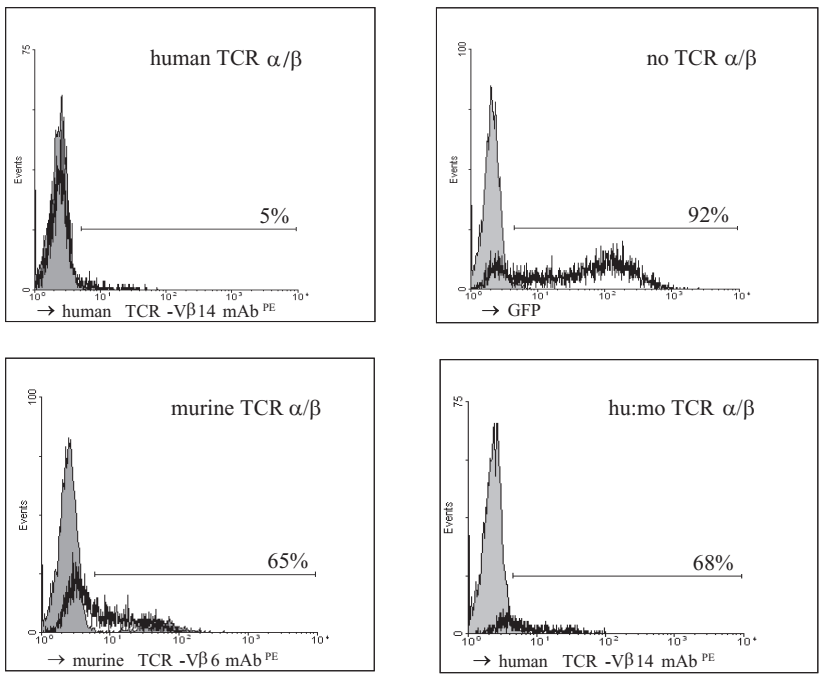

Figure 3. Primary murine T cells express high levels of introduced TCR at their surface following transduction with MLV-A and MLV-E pseudo-typed viruses positive for a gp100/A2 murinized TCR

Retrovirus supernatant was produced by a co-culture of 293T/Ph-A cells transfected with GAG/POL, MLV-E and transgene DNA. Hundred thousand ConA-activated primary murine T cells were transduced with GFP, or gp100/A2 hu:mo TCR; gp100/A2 human TCR, or HDM2/A2 murine TCR in one round of infection at 1200g and 30°C. T cells were analyzed by flow cytometry for expression of GFP or the TCRβ chain (PE labelled monoclonal antibody against human Vβ14 (both gp100/A2 TCRs) or murine Vβ6 (HDM2/A2 TCR)), following FSC/SSC gating on viable lymphocytes. Mock- and GFP- transduced lymphocytes were used as a negative and positive control, respectively. Transduction efficiencies are indicated as the percentage of mouse T cells expressing GFP, TCR-Vβ14 or TCR-Vβ6 at the X-axis. Data shown are from a representative experiment out of at least 4 for each construct.

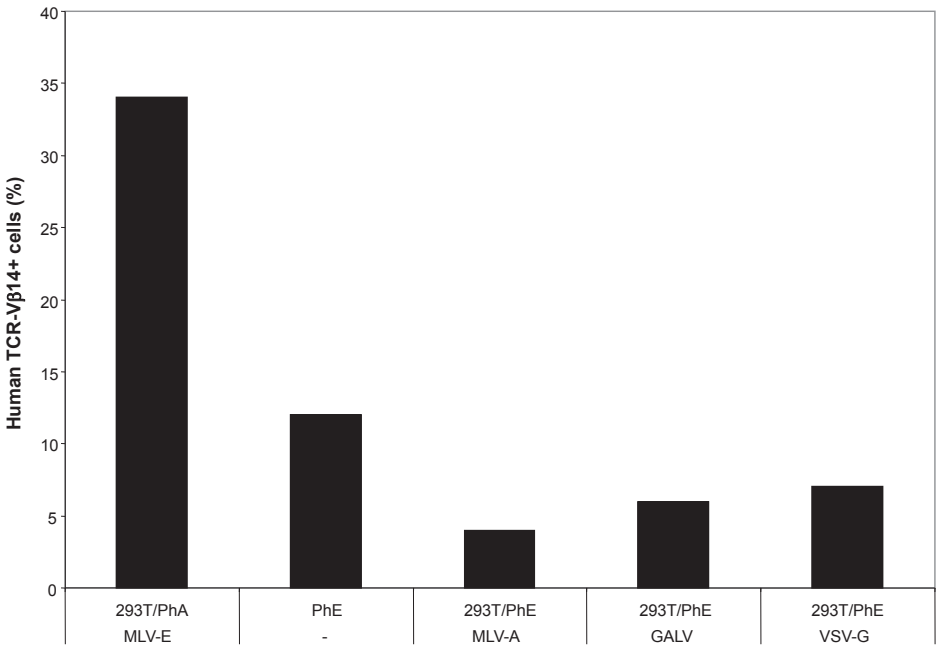

Figure 4. Successful retroviral transduction of primary murine T lymphocytes with a gp100-specific TCR depends on virus pseudo-typing

One million ConA-activated primary murine T lymphocytes were transduced with retroviral supernatant produced by 293T/Ph-A cells that were transfected with GAG/POL, MLV-E ENV and hu:mo gp100/A2 TCR DNA. Transduction was performed in two rounds of spin infection at room temperature. T cells were analyzed by flow cytometry for expression of the TCRβ chain (PE labelled monoclonal antibody against human Vβ14), following FSC/SSC gating on viable lymphocytes. Mock-transduced lymphocytes were used as negative control. Transduction efficiencies are indicated as the percentage of mouse T cells expressing Vβ14. Data shown are from a representative experiment out of 2.

Soluble CD3/CD28 mAb and ConA activation of primary murine T cells are equally efficient with respect to human TCR gene transfer

Activation of primary T cells through anti-CD3 and CD28 mAbs (CD3/CD28 mAbs) is commonly used to gene-transfer primary murine as well as human T cells and may be considered analogous to clinical gene transfer protocols (Rubinstein et al., 2003). To test whether CD3/CD28 mAb-based T cell activation could be extended to the presented transduction protocol, we used a mixture of soluble CD3/CD28 mAbs to activate T cells and tested its effect on the efficiency of TCR gene transfer (as described in the previous sections). Figure 5 demonstrates that CD3/CD28 mAb activation leads to transduction efficiencies that are similar to those following ConA T cell activation, both exceeding 50%.

Human TCR-transduced murine T cells proliferate and show antigen-specific function

Growth of gp100/A2 hu:mo TCR-transduced T cells was monitored during *ex vivo* culture for up to 2 weeks. We observed that cell numbers doubled at least 3 times during the first 5 days

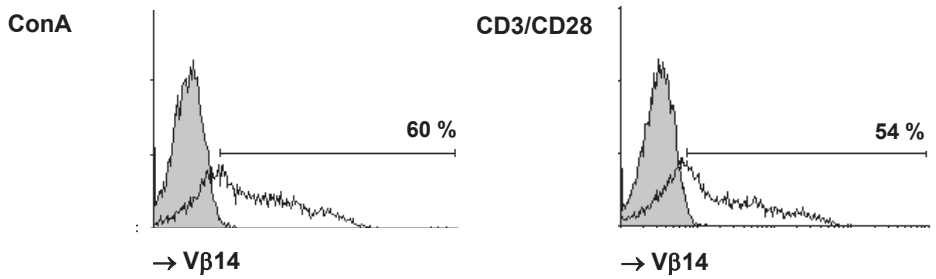

Figure 5. Soluble CD3/CD28 mAb and ConA activation of T cells are equally efficient with respect to human TCR gene transfer

Primary murine splenocytes were activated with either 2.5 µg/ml ConA or with a combination of soluble anti-CD3 and CD28 moAb (at 30 ng/ml ea.) in wells of a 24 well plate. Twenty-four hours post-activation, T cells were transduced with a gp100/A2 hu:mo TCR. Five days after start of culture, T cells were analyzed by flow cytometry for expression of the TCRβ chain as described in the legend to Figure 3. Mock-transduced lymphocytes were used as a negative control. Transduction efficiencies are indicated as the percentage of mouse T cells expressing Vβ14. Data shown are from a representative experiment out of 2.

of culture, after which cell yield could easily be maintained for the remainder of the culture period (Figure 6A). After retroviral transduction (day 2), T cells were harvested and seeded at cell densities of $0.15x10^6$ cells/ml, generally leading to a 10-fold increase in cell number within 5 days. In the second week, for practical reasons, T cells were cultured at $1x10^6$ cells/ml, which may have affected subsequent cellular expansion. Next to their proliferative capacity, TCR-transduced T cells were assayed for their antigen-specific function. A chromium release assay was performed at 2 weeks after the start of the culture. Importantly, we demonstrated that TCR-transduced T cells do not only show surface expression of the introduced TCR but also display gp100-specific cytotoxicity (Figure 6B).

DISCUSSION

In the present paper, we set up a quick and versatile method for TCR gene transfer into primary murine T cells based on transient production of retroviruses. Following the optimization of numerous parameters (virus pseudotype; T cell densities during transduction; amount of ConA to activate primary T cells; time of activation prior to gene transfer; amount of rIL-2 and source of serum used during T cell activation, transduction, and expansion; the use of fibronectin fragments CH-296 (Retronectin™) as a transduction-mediating agent; and the number of infection cycles), we observed that transduction efficiencies were mostly affected by pseudotype of retrovirus and T cell densities. The protocol that we defined as most optimal (see Table 1) allowed us to transduce > 90% of T cells with GFP and up to 68% of T cells with either a HDM2/A2 mouse TCR or a gp100/A2 human TCR containing murine TCR-C domains (i.e. murinized TCR).

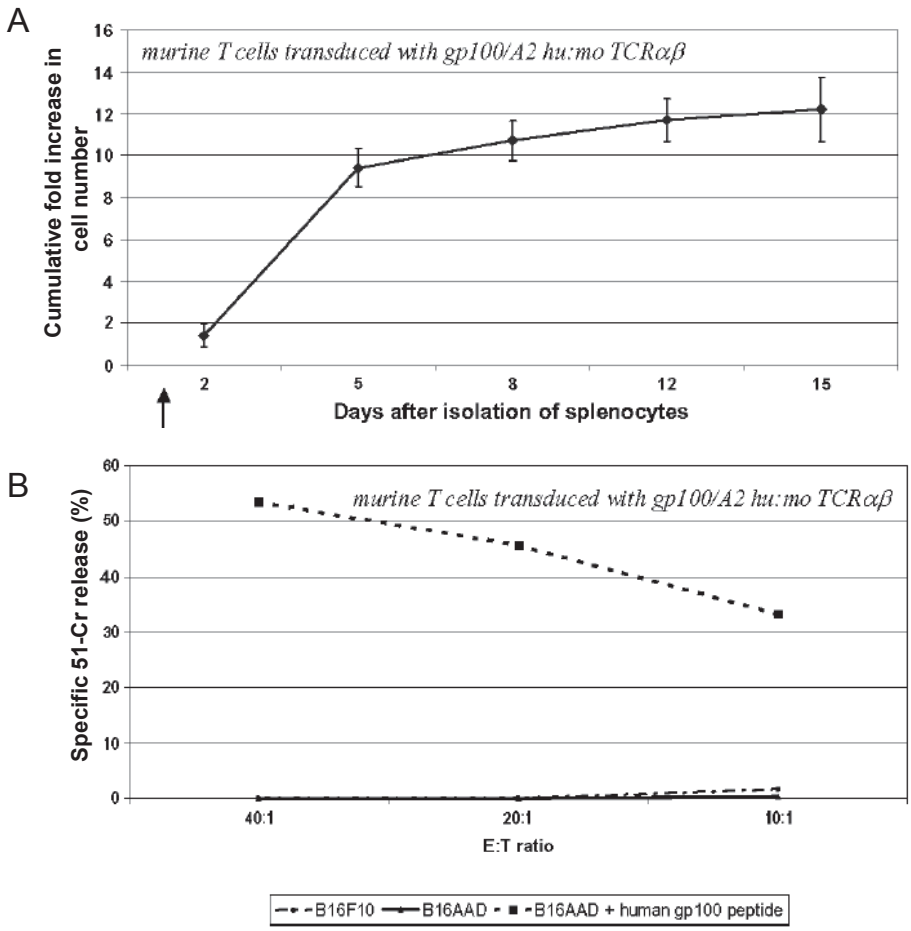

Figure 6. Human TCR-transduced murine T cells proliferate and show antigen-specific function

A) Gp100/A2 hu:mo TCR-transduced T cells proliferate in vitro. Gp100/A2 hu:mo TCR-transduced T cells were cultured at 0.15-1*10^6 cells/ml with 100 U/ml human IL-2 for up to 2 weeks. Medium was refreshed every 2 to 3 days, and number of viable cells was counted microscopically. Cell growth is depicted as cumulative fold increase in viable cell number (mean number ± SD, n=6). Cell number at day 1 (moment of TCR gene transfer, indicated by arrow) is set at 1.0. B) Gp100/A2 hu:mo TCR-transduced T cells show antigen-specific function. Gp100/A2 hu:mo TCR-transduced T cells were used in a standard 4h chromium release assay. B16AAD cells loaded with or without human gp100 peptide were used as target cells. B16F10 target cells served as a negative control. Effector to target ratios are indicated at the X-axis, and percentage of specific ^{51}-Cr release is indicated at the Y-axis. At the moment of cytotoxicity assay, 2 weeks after the start of the culture, TCRβ expression at the surface of transduced T cells was 14% (determined by flow cytometry).

Obtaining a high-titer, helper-free retroviral stock often necessitates generation of a receptor-positive clone of packaging cells, which is laborious as it involves the screening of tens to hundreds of clones, and is only successful in case permanent expression of the transgene does not adversely affect growth of the producer cell line. Here we present the first direct comparison of differently pseudotyped retroviruses, transiently packaged by different (combinations of) packaging cells which were transfected with different ENV DNAs, for their ability to transfer GFP and human TCR genes into primary murine T cells, and demonstrated that a 293T/PhA co-culture outperforms Ph-E-based cultures with respect to transduction efficiencies (Figures 1 and 4). Such a co-culture is an extension of the "ping-pong" system we described earlier to transduce human T cells (Eshhar et al., 2001). The virus supernatants from the various packaging systems did not differ in reverse transcriptase activity (C-type-RT Activity Assay, Cavidi Tech, Upsala, Sweden; data not shown), used as a measure of viral titres, suggesting that differences at the level of T cell transduction were due to differences in viral pseudo-typing. Moreover, mono-cultures of ecotropic virus-producing Ph-E or 293 T cells (the latter following transfection of ENV DNA) perform equally well with respect to gene transfer efficiencies, yet less than a 293T/Ph-A co-culture. It is of interest that even though both 293T/Ph-A cells transfected with MLV-E DNA and 293T/Ph-E cells transfected with MLV-A DNA produce a mixture of amphotropic and ecotropic viruses, the former packaging system results in two-fold higher T cell transduction efficiencies relative to the latter system. We believe that this may be because supernatants from these two systems might differ in their ratio between MLV-E and MLV-A-typed viruses (illustrated in Figure 1A). This ratio is expected to be higher and consequently facilitating mouse T cell transductions better in case of 293T/Ph-A cells, because these cells are transfected with MLV-E ENV DNA and both 293T and Ph-A cells will consequently produce MLV-E typed viruses, whereas in case of 293T/Ph-E cells only Ph-E cells will produce MLV-E types viruses. Of general note, we would like to mention that Ph-E cells showed a 1.5 to 2-fold lowered level of ENV expression and more readily lost this molecule from its surface in time when compared to Ph-A as demonstrated by flow cytometry of ENV expression at bi-monthly intervals (using 83A25 mAb which is able to detect both amphotropic and ecotropic envelopes). In fact, this observation was reproduced when using packaging cells from different laboratories (data not shown). Nevertheless, production of MLV-E pseudotyped viruses derived from Ph-E cells did not appear to be limited by a lowered level of MLV-E expression since transfection of Ph-E cells with additional MLV-E ENV DNA did not improve transduction efficiencies (data not shown).

When investigating which T cell densities were optimal for gene transfer, we experienced that for activation T cells need to be at a high concentration ($5x10^6$/ml, data not shown), whereas for transduction T cell densities should be low (i.e., $1x10^5$/ml, see Figure 2). Lower T cell densities result in higher virus particle per cell ratios and may thereby directly improve retroviral transduction. However, one cannot exclude that lower T cell densities facilitate cellular division and growth and thereby improve viral integration. It is of interest to note that

lowering T cell densities does not improve transduction efficiencies in the human system, in which 1 million cells per well is most optimal for retroviral transduction (Lamers et al., 2002). Murine T cells may express lower amounts of retrovirus receptors than human T cells, or retrovirus infection may simply be less efficient in murine T cells. Interestingly, we observed that transduction efficiencies were two- to three-fold higher when using fibronectin fragments when compared to transductions in which no fibronectin was used (data not shown). This is in contrast to the findings of Zhang and colleagues (Zhang et al., 2004), who observed no beneficial effect of fibronectin fragments on transduction efficiency.

With respect to TCR gene transfer, we believe that the use of a TCR directed against a human tumour antigen in preclinical models would facilitate further optimization and translation of TCR gene therapy into the clinic. To this end, we tested a human and hu:mo reformatted TCR both directed against human gp100/HLA-A2, the latter one constructed by linking human TCR-V domains to murine TCR-C domains (in analogy to Madsen and colleagues (Madsen et al., 1999)). The hu:mo TCR shows high surface expression on murine T cells (68%, Figure 3), which is in contrast to human but similar to mouse TCR (Figure 3). Since T cell activation upon incubation with CD3/CD28 mAbs rather than ConA may be considered analogous to clinical gene transfer protocols, we tested whether T cell activation through these antibodies could be implemented in our TCR gene transfer protocol. CD3/CD28 mAb-based activation of T cells leads to transduction efficiencies that are comparable to those following ConA-based T cell activation, and points to the robustness of the presented protocol.

The levels of TCR transgene surface expression observed in our experiments are expected to allow immediate monitoring of T cell function without the need to enrich receptor-transduced T cells. During *ex vivo* culture, gp100/A2 hu:mo TCR-transduced T cells doubled in cell number 3-4 times in the first week of culture, and resulting cell yield could easily be maintained for up to 2 weeks (Figure 6A). When tested 2 weeks post-transduction, gp100/A2 hu:mo TCR-transduced T cells show cytotoxicity in response to melanoma cells expressing human $gp100_{280-288}$ (Figure 6B). The observed expansion of antigen-specific TCR-transduced T cells is considered relevant since it allows *in vivo* adoptive T cell transfer experiments with high numbers of T cells. In extension to our observations, we are currently investigating how the expression of T cell differentiation markers (such as CD27, CD44, CD62L and CCR7) is related to function, and how T cell phenotype is affected by *ex vivo* administration of common-γ cytokines.

Taken together, the presented transduction protocol is fast (it takes only 5 days from transfection until flow cytometric analysis of transduced T cells), and is versatile with respect to the receptor of interest since it is based on transient production of transgene-positive retroviruses. In comparison, Zhang and colleagues (Zhang et al., 2003) demonstrated that 82% of T cells expressed a murine TCR following transduction, but they used a very laborious virus production system with multiple re-infections of different cell lines, and the need for selection of a producer cell line with G418. A novel contribution of the present paper to the

field is that gene transfer of a human TCR into primary murine T cells critically improved by the use of retroviruses pseudotyped with both MLV-A and MLV-E envelopes.

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CHAPTER 3

TCR gene-engineered T cells: limited T cell activation and combined use of IL-15 and IL-21 to ensure minimal differentiation and maximal antigen-specificity

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ABSTRACT

Clinical TCR gene therapy of melanoma represents a feasible and promising treatment rationale yet is currently challenged by objective response rates that stay behind those observed with conventional adoptive T cell therapy. Here, the phenotype and function of TCR-transduced T cells, considered to determine the efficacy of TCR gene therapy, were studied in relation to T cell activation and cytokine treatments. We observed that the lectin Concanavalin A (ConA), and to a lesser extent anti-CD3 and CD28 mAbs (soluble CD3/CD28), resulted in functional surface expression of the TCRαβ transgenes and enhanced fractions of $CD62L^{hi}/CD44^{lo}$ naive T cells. T cell functions and limited T cell differentiation were most significant when T cells were treated with a combination of IL-15 and IL-21 rather than IL-2. In comparison, anti-CD3 and CD28 mAbs coated to either latex or polystyrene beads (polystyrene or latex CD3/CD28) resulted in improved TCR expression levels and enhanced T cell differentiation irrespective of cytokine treatment, with effects most pronounced for polystyrene CD3/CD28. T cells demonstrated enhanced cytotoxic activity and IFNγ production when activated with CD3/CD28 beads and treated with IL-15 and IL-21, but at the same time displayed non-specific T cell responses. In contrast, ConA and soluble CD3/CD28 activations resulted in antigen-specific T cell responses. In short, we show that retroviral TCR engineering of primary T cells benefits from activation with ConA or soluble CD3/CD28 rather than immobilized anti-CD3 and CD28 mAbs with respect to T cell differentiation and antigen-specificity of T cell responses.

INTRODUCTION

T cell receptor (TCR) gene transfer represents a feasible and attractive therapeutic strategy to provide tumour-specific T cell immunity (Clay et al., 1999; Cooper et al., 2000; Orentas et al., 2001; Schaft et al., 2003; Willemsen et al., 2000), and at the same time circumvents the laborious nature and limited success of isolating and expanding tumour-reactive T cells from patients *ex vivo*. The clinical feasibility of this experimental therapy was recently demonstrated in trials with TCR-gene modified T cells in melanoma patients (Johnson et al., 2009; Morgan et al., 2006). Objective response rates with TCR-engineered T cells ranged from 12 to 30%, and were lower when compared to the objective response rates of over 50% generally observed with non-gene modified T cells (Dudley et al., 2002; Dudley et al., 2005; Dudley et al., 2008). In addition, recent clinical trials with T cells retrovirally modified with chimeric antibody-based receptors (CARs) showed little to no anti-tumour efficacies (Kershaw et al., 2006; Lamers et al., 2006b). Notably, preclinical studies showed that antigen-specific T cell effectiveness *in vivo* is inversely correlated to T cell differentiation (Gattinoni et al., 2005b; Wherry et al., 2003). Moreover, clinical anti-tumour efficacy of T cells appears to be directly related to T cell persistence (Ciceri et al., 2007; Kershaw et al., 2006; Robbins et al., 2004), which in turn is reported to be associated with differentiation state and replicative history of transferred T cells (Huang et al., 2005).

In clinical TCR and scFv gene therapy trials, T cells have generally been activated with soluble anti-CD3 mAb and cultured with IL-2, which induces significant cellular division necessary for efficient retroviral gene integration (Kershaw et al., 2006; Lamers et al., 2006b; Morgan et al., 2006). However, the method to activate and cytokine-treat T cells is not only important for its effect on cellular division but also for its effect on T cell differentiation and antigenic T cell function (Duarte et al., 2002; Sauce et al., 2002), which may provide a window for improvement of the therapeutic quality of gene-modified T cells. In preclinical gene transfer models, T cells are mostly activated with lectins such as Phytohaemagglutinin (PHA) or ConcanavalinA (ConA) (Fujio et al., 2000; Kessels et al., 2001; Kolen et al., 2002; Pouw et al., 2007; Zhang et al., 2004) or anti-CD3 mAb with or without anti-CD28 mAbs (Bondanza et al., 2006; Hollatz et al., 2008; Hori et al., 2003; Rubinstein et al., 2003; Sauce et al., 2002). A few studies directly compared TCR-dependent (anti-CD3/CD28 mAbs) versus TCR-independent (lectins) activation (Duarte et al., 2002; Hagani et al., 1999; Zhang et al., 2003), but data on phenotype and function of T cells in the setting of TCR gene transfer are lacking. IL-2 is most routinely used a T cell growth factor during T cell expansions. Recent reports suggest that other common-γ cytokines such as IL-15 and IL-21 represent better alternatives. For example, antigen priming of CD8 T cells in the presence of IL-15 or IL-21, but not IL-2, suppresses differentiation of naive T cells into effector T cells (Gattinoni et al., 2005b; Hinrichs et al., 2008). In addition, treatment of primary T cells derived from melanoma patients (Huarte et al., 2009) or

primary mouse T cells that are TCR engineered (Pouw et al.) with a combination of IL-15 and IL-21 results in improved T cell cytotoxicity and production of IL-2 and IFNγ.

In the present study, we tested T cell activation with ConA or anti-CD3/CD28 mAbs, either soluble or immobilized to polystyrene or latex beads, and T cell treatment with either IL-2 or a combination of IL-15 and IL-21 with respect to *in vitro* phenotype and function of TCR-engineered T cells. We showed that a single activation of primary T cells with bead-coated anti-CD3/CD28 mAbs, when compared to ConA or soluble anti-CD3/CD28 mAbs, resulted in increased TCR transgene surface expression and differentiation into $CD62L^{lo}$, $CD44^{hi}$ effector memory T cells that display non-specific T cell functions. In contrast, T cells activated with ConA or soluble CD3/CD28 antibodies demonstrated enhanced fractions of naive T cells and antigen-specific T cell responses, especially when T cells were treated with IL-15+IL-21.

MATERIALS AND METHODS

Cells and reagents

The packaging cells 293T and Phoenix-Amp (Ph-A), the B16 wildtype cells (B16F10) and B16 cells transfected either with HLA-A2 and hgp100 DNA's (B16gp100/A2) or AAD DNA (B16A2) were described previously (Pouw et al., 2007). Packaging and B16 cells were cultured in DMEM (Cambrex, Verviers, Belgium) supplemented with 200 nM L-Glutamine, 10% Fetal Bovine Serum (FBS, Greiner Bio-one, Alphen a/d Rijn, the Netherlands), 1% MEM non-essential amino acids, 100 µg/ml streptomycin and 100 U/ml penicillin (DMEM complete). B16gp100/A2 cells were grown under selection of 1 mg/ml G418 and 0.5 mg/ml Hygromycin B, and B16A2 cells were grown under selection of 1 mg/ml G418. Expression of HLA-A2 molecules was regularly verified by flow cytometry (using mAb BB7.2, BD Biosciences, San Jose, CA) following stimulation with rat IFNγ (Peprotech, Rocky Hill, NY). Monoclonal Abs used in this study were: APC-conjugated anti-mouse CD44 (IM7); PE-conjugated anti-mouse CD62L (MEL-14) (both from BD Biosciences); and PE-conjugated anti-human TCRVβ14 (CAS1.1.3, Beckman Coulter, Marseille, France). Other reagents used in this study were human rIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands), human rIL-15 (Peprotech); murine rIL-21 (R&D Systems, Minneapolis, MN); and the human $gp100_{280\text{-}288}$ wild type peptide (gp100 wt) YLEPGPVTA, (Schaft et al., 2003).

T cell activation

C57BL/6 mouse spleens (Erasmus Dierexperimenteel Centrum, Erasmus MC, Rotterdam, the Netherlands) were used according to protocols approved by the National Animal Experimenting Committee (Dierexperimentele Commissie), and dissected and homogenized into single-cell suspensions as described previously (Pouw et al., 2007). Cells were collected and cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 200 nM L-Glutamine, 10%

Table I: T cell activation conditions[a]

	Concentration	Bead : cell ratio	mAb (clone)	Provider
ConA	2.5 µg/ml	N/A[d]	N/A	Sigma, St. Louis, MS
Latex CD3/CD28	To coat 20x10^6 Latex beads:[b] anti-CD3: 0.05 µg/ml anti-CD28: 5 µg/ml	2.5:1	anti-CD3: 145-2C11 anti-CD28: 37.51	5-µm Latex beads: Interfacial Dynamics/Molecular Probes, Eugene, OR anti-CD3 and anti-CD28 mAbs: hybridoma
Polystyrene CD3/CD28	Polystyrene beads were pre-coated[c]	1:1	N/M[e]	4.5-µm Polystyrene beads: Dynal, Invitrogen, Breda, the Netherlands
Soluble CD3/CD28	anti-CD3: 30 ng/ml anti-CD28: 30 ng/ml	N/A	anti-CD3: 145-2C11 anti-CD28: 37.51	anti-CD3 and anti-CD28 mAbs: hybridoma

[a] T cells were activated by one of the indicated methods for 24 h, washed and expanded without stimulation in the presence of either 100 U/ml IL-2 or IL-15+IL-21 (50ng/ml each).

[b] Latex beads were resuspended with anti-CD3 and anti-CD28 mAbs, left to rotate for 20 min at 4°C, and washed twice with PBS with 2% FBS by pelleting at 1000 g for 10 min. Coated beads were resuspended in CMM (see Materials and Methods) and left to rotate for 30 min at RT prior to their use.

[c] Polystyrene beads were pre-coated and used according to the manufacturer's instructions.

[d] N/A = not applicable

[e] N/M = not mentioned

FBS, 1% MEM non-essential amino acids, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol and antibiotics (Complete Mouse Medium, CMM). T cells were activated by one of the following four methods: Concanavalin A (ConA); latex microbeads coated with anti-CD3 and anti-CD28 mAbs (latex CD3/CD28); polystyrene beads coated with anti-CD3 and anti-CD28 mAbs (polystyrene CD3/CD28); or soluble anti-CD3 and CD28 mAbs (soluble CD3/CD28). All T cell activations were performed for 24h and in the presence of 100 U/ml IL-2. Anti-CD3 and anti-CD28 mAbs were purified from hybridoma supernatants by affinity chromatography on sepharose-protein G (GE Healthcare, Milan, Italy). Details on source, preparation and use of T cell activation reagents are summarized in Table I.

TCR gene transfer and cytokine treatment

Following T cell activation by one of four methods (day 0), T cells were washed and retrovirally transduced with a murinized human TCRαβ specific for gp100$_{280-288}$/HLA-A2 (day 1) (for details on TCR gene transfer as well as TCR cloning see (Pouw et al., 2007)). T cells retrovirally transduced without transgenes (Mock T cells) were generated as controls for the TCR-transduced T cells (TCR T cells). TCR-transduced T cells were cultured at 0.15-1x10^6 cells/ml in 24 well plates in CMM supplemented with either 100 U/ml rIL-2 or IL-15 plus IL-21 (50ng/ml each). Cytokines were refreshed every 2-4 days.

Flow cytometry

T cells (at day 5 after the start of culture) were monitored for their TCRβ transgene expression and differentiation state by flow cytometry using a FACSCalibur (BD Biosciences) following staining with anti-TCR-Vβ14, CD44 and CD62L mAbs. For immunostaining, 0.1-0.5x10^6 T cells were washed with PBS and incubated with 10 µl of mAb for 30 min at 4 °C. T cells were washed again, fixed with 1 % paraformaldehyde and analyzed the same day with CellQuest software (BD Biosciences).

T cell expansion

Viability of T cells was determined by trypan-blue exclusion, and viable T cells were counted microscopically (Leitz Laburlux 12, Leica Geosystems BV, Rijswijk, the Netherlands) using Bürker counting chambers every 2nd or 3rd day for up to 1-2 weeks after T cell activation. T cell growth is expressed as cumulative T cell numbers.

Cytotoxicity and IFNγ secretion assays

Cytotoxic activities of TCR and Mock T cells (at day 6 after the start of culture) were measured in a standard 5h ^{51}Cr-release assay. B16F10, B16A2 or B16gp100/A2 target cells were labelled with 100 µCi $Na_2{}^{51}CrO_4$/10^6 cells for 2h at 37 °C and 5 % CO_2. In case of gp100 peptide-specific reactivity, B16A2 cells were pulsed with human gp100 peptide (final concentration of 10 µM) for 15 min at 37 °C and 5 % CO_2 prior to co-cultivation with effector T cells. Percentages of specific cytolysis, i.e. specific ^{51}Cr-release, were calculated as described previously (Weijtens et al., 1998). Cytokine secretion in response to the above-mentioned B16 target cells was measured in supernatants from 20h co-cultivations (6x10^4 T cells and 2x10^4 target cells per well in 96 well plates) by mouse IFNγ ELISA (U-CyTech, Utrecht, The Netherlands). During target cell stimulations no exogenous cytokines were added to the T cell cultures.

Statistical analyses

Statistical analyses of T cell phenotype, expansion, cytotoxicity and IFN γ production were performed with student's t-tests with statistical significance indicated by p values < 0.05. In all experiments the following symbols are used to indicate p<0.05: *: T cell activation methods compared to ConA (per cytokine treatment); †: IL-15+IL-21 treatment compared to IL-2 (per T cell activation method).

RESULTS

T cell activations with bead CD3/CD28 result in high TCR transgene surface expression

Murine T cells were activated by one of four methods (summarized in Table I), TCR gene-transduced, treated with different cytokines and analyzed for TCR surface expression by flow cytometry. T cell activation with ConA and treatment with IL-2 led to successful TCR gene transfer, with on average

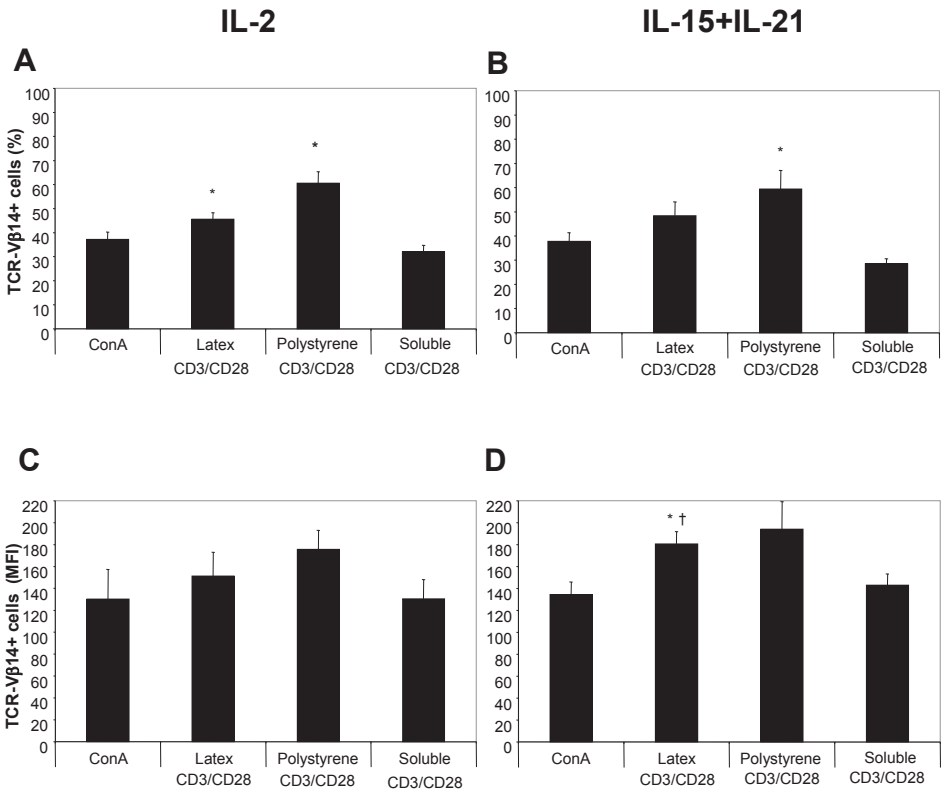

Figure 1. T cell activation with bead CD3/CD28 results in high TCR transgene surface expression

Murine splenocytes were activated by four methods for 24h, TCR-transduced and treated with either 100 IU/ml IL-2 (A and C) or a combination of IL-15 and IL-21 (50 ng/ml each) (B and D). See Table I for details on T cell activation conditions. Viable cells were monitored for the expression of the introduced TCRβ chain by flow cytometry (PE labelled mAb against human TCR-Vβ14) five days after start of the culture. TCR expression levels of T cells are indicated either as percentages of mouse T cells expressing TCR-Vβ14 (A and B) or mean fluorescence intensities (MFI) (C and D). Mean numbers ± SEM, n=11 for IL-2 T cells; n=5 for IL-15+IL-21 T cells. * = $p<0.05$ T cell activations vs ConA (per cytokine condition); † = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).

37 % of T cells expressing the introduced TCR-Vβ14 at day 5 after start of T cell activation (Figure 1A). Similar transduction efficiencies were obtained when T cells were activated with soluble CD3/CD28 (Figure 1A). Activation of primary murine T cells with bead CD3/CD28, especially polystyrene CD3/CD28, led to the highest percentages of T cells expressing the introduced TCR (Figure 1A, day 5: 46% and 65% for latex and polystyrene CD3/CD28, respectively, $p< 0.05$ when compared to ConA). Treatment of T cells with IL-15+IL-21 generally resulted in similar TCR transduction efficiencies, with activation with bead CD3/CD28 again providing the highest transgenic TCRβ surface expression levels (Figure 1B). Surface expression levels of TCR-Vβ14 on a per cell basis (mean fluorescence intensities, MFI) were in line with the observations made for percentages of TCRβ-positive T cells (Figures 1C and D). With respect to CD8 stainings, we observed that the level of surface expression of transgenic TCRβ in CD8 T cells was almost identical to that of total T cells (data not shown).

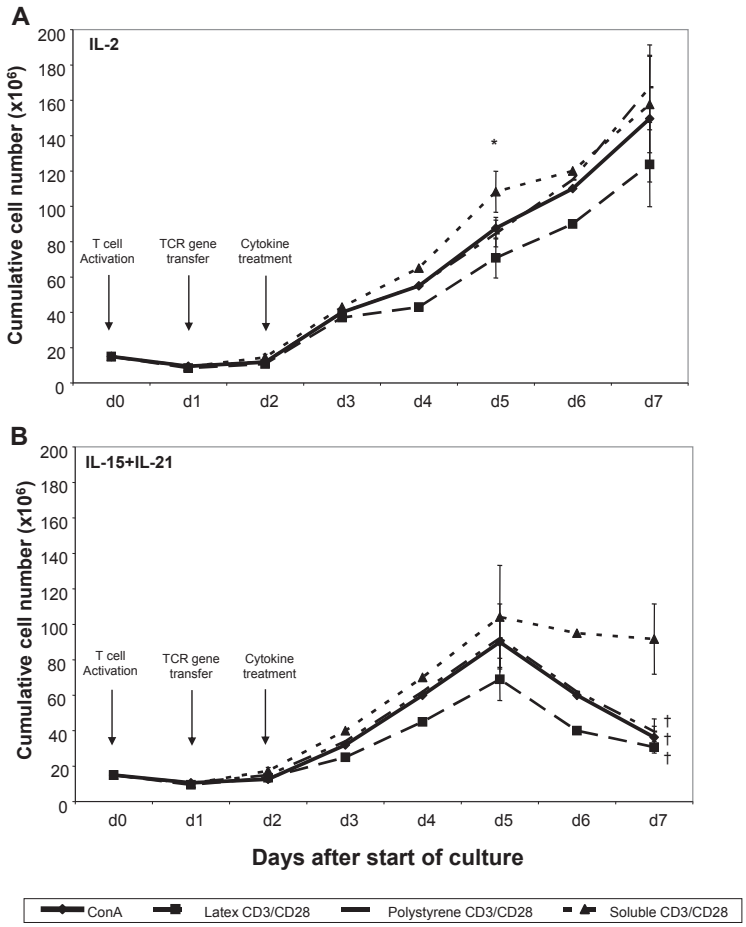

Figure 2. T cell expansion is decreased by IL-15+IL-21, but not affected by the different T cell activation stimuli

T cells were activated via four different methods for 24h (Table I), TCR transduced, seeded at 0.15-1x10^6 cells/ml and expanded in the presence of either 100 IU/ml human IL-2 or IL-15+IL-21 (50 ng/ml each) for up to one week. Medium including cytokines was refreshed every 2 to 3 days, and number of viable cells was counted microscopically and depicted as cumulative T cell numbers (mean number ± SEM, n=5). T cells were activated at day 0, TCR gene transfer was performed at day 1, and T cells received cytokine treatments from day 2 onwards. * = $p<0.05$ T cell activations vs ConA (per cytokine condition); † = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).

T cell expansion is decreased by IL-15+IL-21, but not affected by different T cell activation stimuli

In all T cell cultures, cell numbers doubled at least 5 times during the first 5 days following T cell activation (Figure 2). At day 5, T cells activated with soluble CD3/CD28 and treated with IL-2 resulted in the highest T cell numbers ($p<0.05$ when compared to ConA). After day 5, T cell numbers continued to increase in IL-2 cultures (Figure 2A), whereas in IL-15+IL-21 cultures T cell numbers declined (Figure 2B). For T cells treated with IL-15+IL-21, activation with soluble CD3/CD28 resulted in the

lowest reduction of T cell numbers at day 7. Despite the observed difference in T cell expansion, both IL-2 and IL-15+IL-21-treated T cells can be maintained for up to two weeks (data not shown).

T cell activations with ConA and soluble CD3/CD28, especially when treated with IL-15+IL-21, result in increased fractions of naive T cells

When studying CD8 T cell differentiation, we observed that flow cytometry percentages of $CD62L^{hi}$, $CD44^{lo}$ naive T cells were comparable for all IL-2 T cell cultures, except for T cells activated with polystyrene CD3/CD28, which contained less naive T cells (Figure 3A: polystyrene CD3/CD28 resulted in 4% of naive T cells, compared to 14-17% for the other T cell activations). Moreover, polystyrene CD3/CD28 T cells showed an increased fraction of fully differentiated $CD62L^{lo}$, $CD44^{hi}$ effector memory T cells (Figure 3A: 49% effector memory T cells for polystyrene CD3/CD28, versus 31-36% for the other T cell activations). For IL-15+IL-21 T cell cultures, we observed increased fractions of naive T cells and correspondingly decreased fractions of effector memory T cells for ConA and soluble CD3/CD28 T cell activations (Figure 3B vs 3A). T cells activated with polystyrene CD3/CD28 and treated with IL-15+IL-21 showed again the lowest and highest fractions of naive and effector memory T cells, respectively (Figure 3B). Table II summarizes the statistics with respect to CD8 T cell differentiation.

Table II. Anti-CD3 and CD28 mAbs coated to polystyrene significantly enhance the proportion of $CD62L^{lo}$/$CD44^{hi}$ T cells[a,b]

T cell activation	Cytokine	$CD62L^{lo}/CD44^{hi}$	$CD62L^{hi}/CD44^{hi}$	$CD62L^{lo}/CD44^{lo}$	$CD62L^{hi}/CD44^{lo}$
ConA	IL-2	35 (5)	51 (3)	2 (0)	12 (3)
	IL-15+IL-21	25 (5)	45 (4)	3 (1)	27 (2)†
Latex CD3/CD28	IL-2	45 (6)	44 (4)	2 (0)	8 (2)
	IL-15+IL-21	40 (6)	38 (5)	3 (1)	19 (3)†
Polystyrene CD3/CD28	IL-2	51 (2)	44 (2)	2 (0)	2 (0)*
	IL-15+IL-21	56 (2)*	31 (1)*†	5 (0)*†	9 (2)*†
Soluble CD3/CD28	IL-2	39 (6)	50 (4)	2 (0)	10 (2)
	IL-15+IL-21	22 (4)†	51 (5)	2 (1)	25 (2)†

[a] Splenocytes were activated as indicated in Table I, TCR gene-transduced, and expanded in the presence of either IL-2 or IL-15+IL-21. Subsequently, flow cytometric analysis was performed following staining with CD62L and CD44 mAbs at day 5 after the start of culture. Numbers represent mean percentages (SEM) of the various CD62L/CD44 T cell subsets. Number of experiments is 5 with each experiment being performed with TCR-transduced splenocytes derived from independent spleens.

[b] * = $p<0.05$ T cell activations vs ConA (per cytokine condition);
† = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).

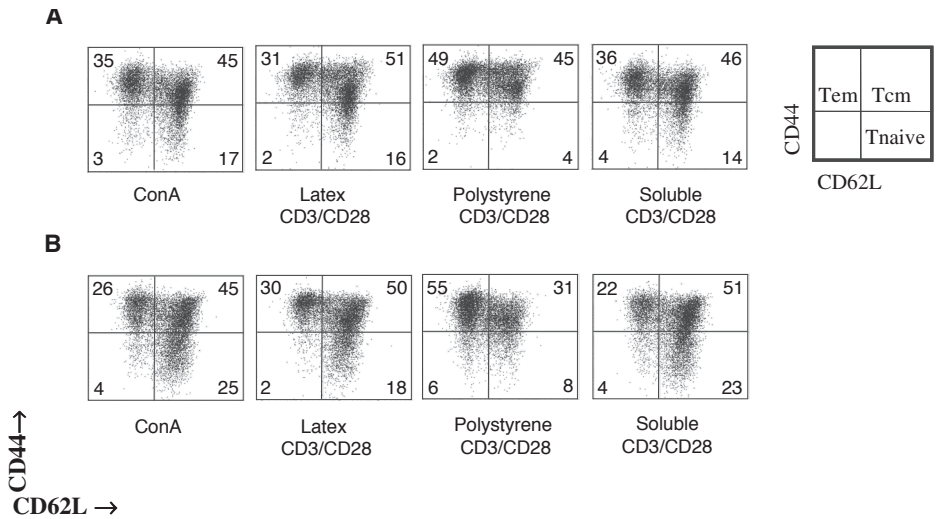

Figure 3. T cell activation with ConA and soluble CD3/CD28, especially when treated with IL-15+IL-21, results in enhanced fractions of naive T cells

Murine splenocytes were activated, TCR gene transduced and treated with either 100 U/ml IL-2 (A) or a combination of IL-15 and IL-21 (50 ng/ml each) (B). Five days after start of the culture, T cells were analyzed by flow cytometry following staining with anti-CD62L mAb^{FITC} and anti-CD44 mAb^{APC}. Percentages indicate the proportion of viable TCR-transduced T cells in the respective quadrant. Data of one out of 5 independent experiments is shown, all with similar results. Mean percentages ± SEM of the various CD62L/CD44 T cell subsets of all 5 experiments are shown in Table II.

T cells activated with ConA or soluble CD3/CD28 and treated with IL-15+IL-21 mediate enhanced and antigen-specific cytotoxicity

All T cell cultures demonstrated cytotoxicity according to chromium release assays towards human gp100 peptide-loaded B16A2 melanoma target cells. Latex and polystyrene CD3/CD28 T cell activations resulted in the highest levels of killing in both IL-2 and IL-15+IL-21-treated T cells (Figures 4A and B: $p<0.05$ compared to ConA T cells). When TCR T cells were tested for their capacity to recognize tumour cells that endogenously express human gp100 antigen (B16gp100/A2 cells), we observed that only latex CD3/CD28 T cells were able to kill these target cells to some extent when T cells were treated with IL-2 (Figure 4C). In contrast, all IL-15+IL-21-treated T cell cultures were able to respond towards B16gp100/A2 cells (Figure 4D), yet chromium releases were most pronounced for those T cell cultures that were activated with latex or polystyrene CD3/CD28 (Figure 4D: $p<0.05$ for polystyrene CD3/CD28 compared to ConA T cell cultures). Notably, IL-15+IL-21-treated T cell cultures activated with latex or polystyrene CD3/CD28, but not ConA or soluble CD3/CD28, clearly showed cytotoxicity (i.e., > 20% ^{51}Cr-release at E:T ratio of 40:1) towards non-peptide-loaded B16A2 cells (Figures 4F: $p<0.05$ for polystyrene CD3/CD28 compared to ConA T cell cultures). Moreover, Mock T cells activated with latex or polystyrene CD3/CD28 and treated with IL-2 showed enhanced cytotoxic activities versus B16gp100/A2 and B16A2 cells (*Supplementary Figures S1C and E*,

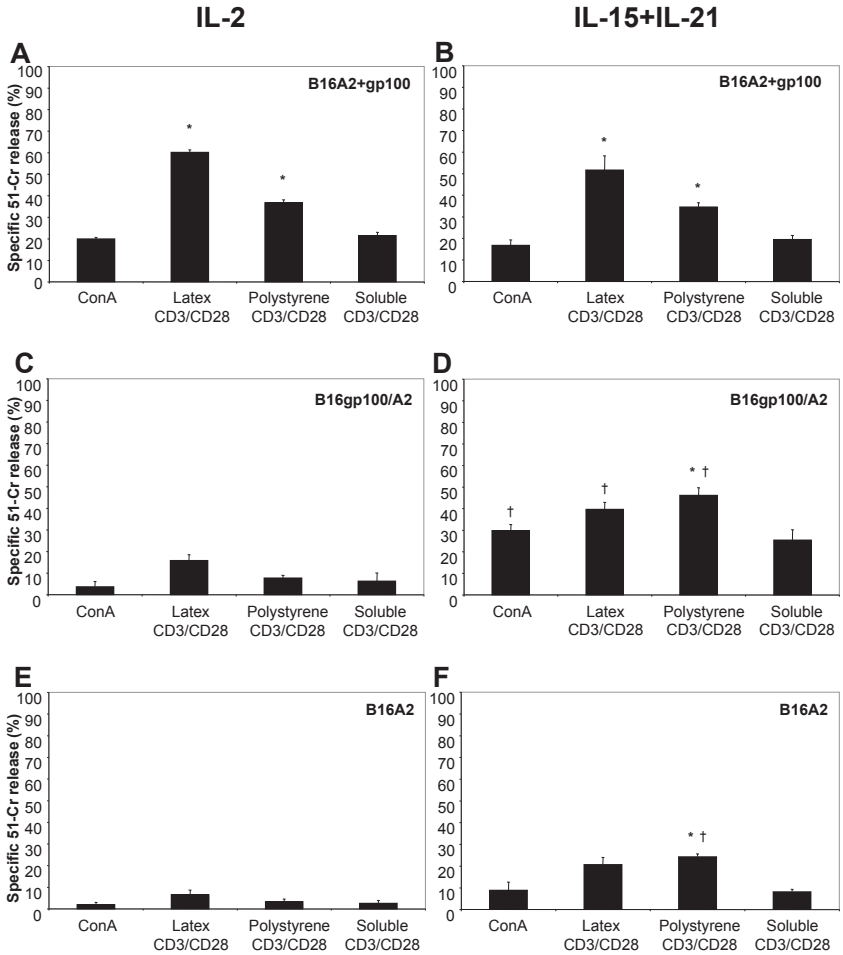

Figure 4. T cells activated with ConA or soluble CD3/CD28 mediate enhanced and antigen-specific cytotoxicity when treated with IL-15+IL-21

Six days after activation of T cells according to four different methods (Table I), TCR gene transfer and treatment with either 100 IU/ml IL-2 (A, C, E) or IL-15+IL-21 (50 ng/ml each) (B, D, F), T cells were used in a 5 h chromium release assay. B16A2 cells loaded with (A-B) or without (E-F) human gp100 peptide or B16gp100/A2 transfectants (C-D) were used as target cells. T cell activation conditions are indicated at the X-axes, and percentages of specific ^{51}Cr release at E:T ratios of 40:1 are indicated at the Y-axes (mean ± SEM; n=3; * = $p<0.05$ T cell activations vs ConA (per cytokine condition); † = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).

respectively). IL-15+IL-21 Mock T cells showed detectable cytotoxicity towards B16gp100A2 and B16A2 cells for all T cell activation conditions tested, although most significant for the bead-stimulated T cell cultures (*Supplementary Figure S1*).

T cells activated with ConA or soluble CD3/CD28 and treated with IL-15+IL-21 mediate enhanced and antigen-specific IFNγ production

Next, TCR T cells were tested for their ability to produce IFNγ by ELISA. All T cell cultures demonstrated IFNγ release after stimulation with human gp100 peptide-loaded B16A2 cells, with no differences neither among T cell activation conditions nor cytokine treatments (Figures 5A and B). Stimulation of IL-2-treated T cells with B16gp100/A2 cells resulted in low levels of IFNγ secretion (Figure 5C), whereas stimulation of IL-15+IL-21-treated T cells with these target cells resulted in significantly increased IFNγ secretion for all T cell activation conditions (Figure 5D). In line with the cytotoxicity data, IL-15+IL-21-treated T cell cultures that were activated with latex or polystyrene CD3/CD28, but not ConA or soluble CD3/CD28, showed detectable IFNγ secretion (i.e., > 200 pg/ml IFNγ) towards non-peptide-loaded B16A2 cells (Figure 5F) (note that IL-2-treated T cell cultures presented with overall low IFNγ levels irrespective of T cell activation conditions, see Figure 5E). Furthermore, IL-15+IL-21-treated Mock T cells activated with either latex or polystyrene CD3/CD28 showed significant IFNγ releases versus B16A2 and B16gp100/A2 cells (*Supplementary Figure S2*).

DISCUSSION

In the present study, we tested T cell activation with ConA or anti-CD3/CD28 mAbs, either soluble or immobilized to polystyrene or latex beads, and T cell expansion with either IL-2 or a combination of IL-15 and IL-21 with respect to *in vitro* phenotype and function of TCR-engineered T cells. In particular, we tested these T cell activation methods (summarized in Table I) for their effects on TCR transgene expression, T cell expansion, T cell differentiation state, and anti-tumour responses.

T cell activation with bead CD3/CD28, especially polystyrene CD3/CD28, led to the highest TCR-Vβ14 expression levels, which appeared independent of cytokine treatment (Figure 1). Bead CD3/CD28 may result in efficient retroviral transduction as a consequence of up-regulated expression (or conformational activation) of very late activation antigen-4 (VLA-4) and VLA-5 molecules on T cells, which has been reported to occur following effective cross-linking of CD3 and CD28 molecules. Up-regulated expression levels of VLA molecules mediate adhesion of T cells to fibronectin (as is present in the transduction-mediating reagent Retronectin™) and take part in T cell co-stimulation (Kotani et al., 1994; Mittelbrunn et al., 2004; Sato et al., 1995; Shimizu et al., 1990). T cells activated with ConA or soluble CD3/CD28 showed comparable TCR surface expression levels, which is in agreement with previous reports (Pouw et al., 2007; Zhang et al., 2003). With respect to T cell expansion, soluble CD3/CD28 was slightly superior when compared to ConA or bead-coated anti-CD3/CD28 mAbs (Figure 2), which is in line with findings on improved expansion of primary human T cells stimulated with soluble CD3/CD28 when compared to bead-coated CD3/CD28 mAbs (Hollatz

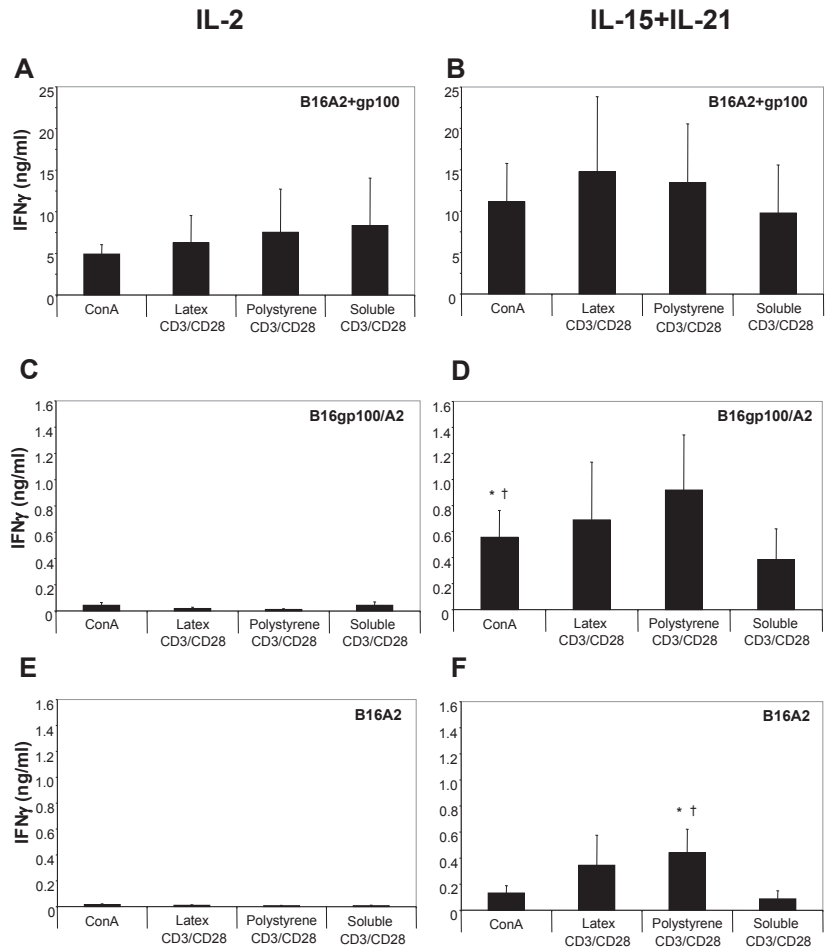

Figure 5. T cells activated with ConA or soluble CD3/CD28 and treated with IL-15+IL-21 mediate enhanced and antigen-specific IFNγ production

Six days after activation of T cells according to four different methods (Table I), TCR gene transfer and treatment with either 100 IU/ml IL-2 (A, C, E) or IL-15+IL-21 (50 ng/ml each) (B, D, F), T cells were used in a 20h tumour cell stimulation assay. B16A2 cells loaded with (A-B) or without (E-F) human gp100 peptide or B16gp100/A2 transfectants (C-D) were used as target cells. T cell activation conditions are indicated at the X-axes, and absolute levels of IFNγ present in supernatants (determined by commercial ELISA) are indicated at the Y-axes (mean ± SEM; n=5; * = $p<0.05$ T cell activations vs ConA (per cytokine condition); † = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).

et al., 2008). Treatment of TCR-transduced T cells with IL-15+IL-21 resulted in decreased cell numbers between day 5 and 7, yet maintained cell numbers thereafter up to week 2 (data not shown). This limited T cell expansion in the presence of IL-15+IL-21 versus IL-2 may in part be explained by decreased surface expression of IL-2Rα chain by T cells upon treatment with IL-15+IL-21 (Pouw et al, manuscript submitted).

Our experiments with respect to T cell differentiation demonstrated that T cells activated with polystyrene CD3/CD28 contained predominantly effector memory T cells, especially when treated with IL-15+IL-21 (Figure 3 and Table II). Expectedly, a significantly decreased fraction of naive T cells was observed, suggesting that polystyrene CD3/CD28 activation recruits a wider repertoire of CD8 T cell subsets into cell cycle, when compared to the other T cell activation conditions. These findings are in extension to those of Duarte and colleagues who observed that primary human T cells, irrespective of expression of a Nerve Growth Factor receptor transgene, showed enhanced T cell differentiation towards $CD45^{hi}$, $CCR7^{lo}$ effector T cells following stimulation with plastic-bound CD3/CD28 when compared to the lectin PHA (Duarte et al., 2002). However, T cells activated with latex CD3/CD28 (Figure 3) or cell-sized beads coated with CD3/CD28 mAbs (Bondanza et al., 2006) contain predominantly central memory T cells, implying that latex and potentially other beads, but not polystyrene beads, induce T cell differentiation that is halted at the stage of central memory T cells. The observed T cell differentiation induced by polystyrene CD3/CD28 is not a direct consequence of the immobilization of anti-CD3/CD28 mAbs per se since identical mAbs used both in soluble form and coated to latex beads yielded less T cell differentiation (Figure 3 and Table II). Differences between polystyrene and latex beads and their use in T cell activation may affect the simultaneous and optimal presentation of the CD3 and CD28-mediated signals to T cells, and constitute a more plausible explanation for their distinct effects on T cell activation and differentiation. First, polystyrene beads used in this study were 4.5 µm polymer beads, whereas latex beads were 5 µm beads. Second, both types of beads were coated with possibly different clonotypes and concentrations of anti-CD3 and/or anti-CD28 antibodies that have their particular epitope specificities and/or ligand-binding affinities. Finally, polystyrene and latex beads were used in different bead to T cell ratios to activate T cell cultures (i.e., polystyrene beads were used according to the manufacturer's instructions, whereas latex beads were used according to optimizations performed by authors). See Table I for further details on beads used for T cell activations. Generally, naive and central memory T cells have been implicated to be superior to effector memory T cells in adoptive T cell therapy (Berger et al., 2008; Hinrichs et al., 2009), which is anticipated to be related to the ability of $CD62L^{hi}$ T cells to migrate to peripheral lymph nodes and to generate new waves of T cells following local antigen encounter (Klebanoff et al., 2005a). Hence, polystyrene CD3/CD28 T cell activation may not be favoured for adoptive T cell therapeutic applications.

Next, we studied the effects of T cell activation and cytokine treatment on T cell functions. T cell cytotoxicity and IFNγ secretion towards human gp100-peptide-loaded as well as hgp100-transfected B16 cells were most prominent for T cells activated with CD3/CD28 beads (Figures 4 and 5). T cell functions were enhanced following treatment with IL-15+IL-21 compared to IL-2, and preserved the superior responsiveness of T cell cultures that were stimulated with CD3/CD28 beads. However, part of the observed cytotoxicity and IFNγ secretion of bead-stimulated T cells appeared to be non-specific. On the one hand, TCR T cells showed responses

above background levels (that is > 20% ^{51}Cr-release at E:T ratio of 40:1, and/or > 200 pg/ml IFNγ release at E:T ratio of 3:1) towards human gp100-negative B16A2 cells (Figures 4E, 4F, 5E and 5F) and on the other hand Mock T cells showed responses above background levels towards both B16gp100/A2 and B16A2 cells (*Supplementary Figures S1 and S2*). The observed non-specificity of bead-stimulated T cells was most pronounced for polystyrene CD3/CD28-activated and IL-15+IL-21-treated T cells (Figures 4, 5 and *Supplementary Figures S1 and S2*), and appeared more prominent at day 14 after the start of the T cell cultures (compared to the presented day 6 data, not shown). Importantly, ConA and soluble CD3/CD28 T cells did neither show functional responses of TCR T cells versus antigen-negative target cells (Figures 4 and 5) nor Mock T cell responses versus antigen-positive target cells (*Supplementary Figures S1 and S2*). The only exception to this was a detectable yet small non-specific cytotoxicity versus B16gp100/A2 cells observed for ConA and soluble CD3/CD28 T cells that were treated with IL-15+IL-21 (24% and 29% specific lysis at E:T 40:1 for ConA and soluble CD3/CD28 T cells, respectively, and < 20% at E:T ratios of 20:1 or less: *Supplementary Figure S1D* and data not shown). Intriguingly, B16gp100/A2 cells elicited higher cytotoxic activities in Mock T cells when compared to B16A2 cells.

We conclude that T cell activations with CD3/CD28 beads may represent strong and more prolonged TCR-mediated stimuli providing T cells enhanced effector T cell activities that do not entirely depend on TCR:cognate pMHC interactions. These preclinical findings may have clinical value. Firstly, the inclusion of an immobilized T cell-stimulating anti-CD28 mAb may contribute to the observed T cell non-specificity due to its ability to lower the threshold for triggering T cell activation (Viola and Lanzavecchia, 1996). In fact, T cells stimulated with immobilized anti-CD3/CD28 mAbs have been reported to express increased levels of perforin and show enhanced allo-reactivity (Duarte et al., 2002). Also, NK cell cytotoxic activity of lymphocyte cultures activated with anti-CD3/CD28 mAbs has been observed previously (Anderson et al., 1989; Leeuwenberg et al., 1985; Massaro et al., 1990; Rubinstein et al., 2003; Stankova et al., 1989). It is noteworthy, that Lamers and colleagues (Lamers et al., 2002) did not observe non-specific cytotoxicity of human T cells activated with an anti-CD3 mAb (without CD28 mAb) when compared to PHA. Secondly, treatment of TCR-engineered T cells with IL-15+IL-21 results in enrichment of naive T cells (Pouw et al, manuscript submitted) and enhanced T cell cytotoxicity and IFNγ production of effector memory T cells present in the same T cell pool (Pouw et al., 2010). The observed effects on T cell differentiation are in line with reports on pmel-1 T cells (transgenic for gp100 TCR, not TCR-transduced) which show that IL-15 *or* IL-21 treatment enriches for less differentiated T cells that display decreased *in vitro* T cell responsiveness (Gattinoni et al., 2005b; Hinrichs et al., 2008). We observed that IL-21 rather than IL-15 is responsible for enrichment of naive T cells (Pouw et al., manuscript submitted). Both cytokines, when used separately, result to some extent in enhanced cytotoxicity and production of IL-2, but not IFNγ (Pouw et al., 2010). Notably, the combination of IL-15 and IL-21 significantly enhanced both T cell cytotoxicity and T cell cytokine production,

potentially as a consequence of enhanced gene transcription of perforin 1, granzymes A and B, and IFNγ (Pouw et al., 2010). Based on data in the current paper, we argue that the enhanced T cell response profile generated by IL-15+IL-21 treatment may further contribute to non-specific T cell responses of CD3/CD28 bead stimulated T cells. A summary of our findings with respect to the four T cell activation methods and two cytokine treatments tested is provided in Table III.

Taken together, our data demonstrate that strong T cell activation with anti-CD3 and CD28 mAbs coated to beads, most prominently polystyrene beads, in contrast to the lectin ConA or soluble anti-CD3 and CD28 mAbs, results in efficient TCR gene transfer but is limited by enhanced differentiation into effector T cells that are endowed with non-specific T cell responsiveness. Conversely, weaker T cell activation conditions such as ConA and soluble anti-CD3/CD28 mAbs, show a somewhat lower TCR gene transfer efficiency yet preservation of naive T cells and T cells capable of antigen-restricted T cell activity. The use of IL-15+IL-21 instead of IL-2 to expand T cells results in less differentiated T cells and increased T cell functions, which are *not* accompanied by non-specific T cell functions in case of ConA and soluble CD3/CD28 T cell stimulations. Presented data provide a rationale to test suboptimal T cell stimulations and optimize intensity and durations of CD3/CD28-based T cell stimulations and treatment with IL-15 and IL-21 for clinical use of TCR-engineered T cells.

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Table III. Effects of T cell activation and expansion protocols on T cell parameters[a]

T cell activation	Cytokine	TCR expression[b]	T cell expansion[c]	Preservation of Tn[d]	T cell Response (CTX/IFNγ)[e]	Antigen Specificity (CTX/IFNγ)[f]
ConA	IL-2	++	+++	++	+/+	+++/+++
	IL-15+IL-21	++	+	+++	++/+++	++/+++
Latex CD3/CD28	IL-2	++	+++	+	+/+	++/+++
	IL-15+IL-21	++	+	++	+++/+++	+/+
Polystyrene CD3/CD28	IL-2	+++	+++	+	+/+	++/+++
	IL-15+IL-21	+++	+	+	+++/+++	-/+
Soluble CD3/CD28	IL-2	++	+++	++	+/+	+++/+++
	IL-15+IL-21	++	++	+++	++/++	++/+++

[a] Splenocytes were activated as indicated in Table I, transduced with gp100/A2 TCRab transgenes, and expanded in the presence of either IL-2 or IL-15+IL-21. Subsequently, T cells were monitored for the expression of the introduced TCR (by flow cytometry for TCR-Vb14), T cell expansion (by counting cell numbers), T cell differentiation (by flow cytometry for CD62L and CD44), and antigen-specific and non-specific cytotoxicity and IFNg production (by chromium release and ELISA, respectively, using antigen-positive and negative target cells).

[b] Expression of TCRb transgene (day 5): + <20%; ++ 20-50%; +++ > 50% TCR-Vβ14+ T cells.

[c] Expansion of TCR T cells (day 7): + < 60x106; ++ 60-100x106; +++ > 100x106 T cells.

[d] Preservation of naive TCR T cells (Tn) (day 5): + <10%; ++ 10-20%; +++ >20% CD62Lhi/CD44lo T cells.

[e] Responsiveness of TCR T cells (day 6): - no response towards gp100 peptide-loaded B16A2 cells (< 20 % 51Cr-release / < 200 pg/ml IFNg); + response towards gp100 peptide-loaded B16A2 cells (> 20 % 51Cr-release / > 200 pg/ml IFNg); ++ response towards B16gp100/A2 cells (> 20 % 51Cr-release / > 200 pg/ml IFNg); +++ enhanced response towards B16gp100/A2 cells (> 40 % 51Cr-release / > 400 pg/ml IFNg).

[f] Antigen-specificity of TCR T cells (day 6): - response towards either gp100 peptide-loaded B16A2 cells or B16gp100/A2 cells and response towards B16A2 cells (> 20 % 51Cr-release / > 200 pg/ml IFNg); + response towards either gp100 peptide-loaded B16A2 cells or B16gp100/A2 cells > response towards B16A2 cells (at least 2-fold difference); ++ response towards either gp100 peptide-loaded B16A2 cells or B16gp100/A2 cells and no response towards B16A2 cells; +++ response towards either gp100 peptide-loaded B16A2 or B16gp100/A2 cells and neither response of TCR T cells towards B16A2 cells nor Mock T cells towards gp100 peptide-loaded B16A2 and B16gp100/A2 cells.

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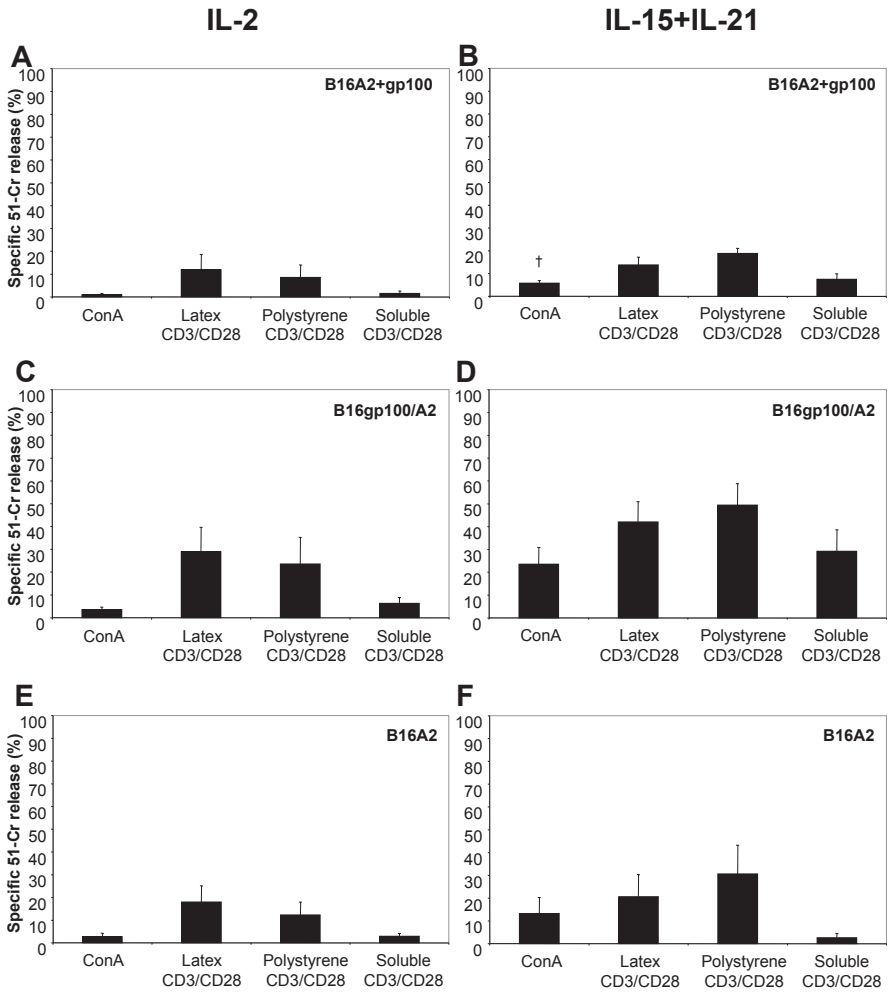
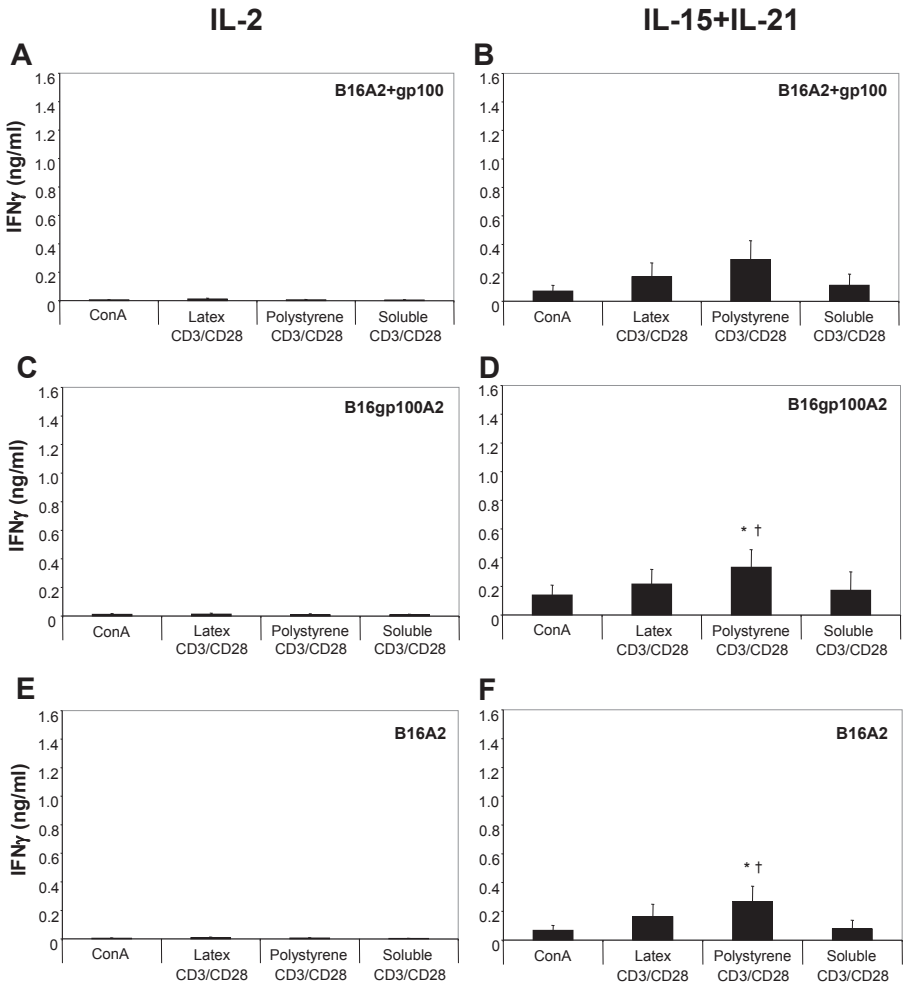
SUPPLEMENTARY FIGURES



Supplementary Figure S1. Mock T cells activated with bead-coated CD3/CD28 mAbs show non-specific cytotoxicity

Six days after activation of T cells according to four different methods (Table I), Mock transduction, and expansion with either IL-2 (A, C, E) or IL-15+IL-21 (B, D, F), T cells were used in a 5h chromium release assay. B16A2 cells loaded with (A-B) or without (E-F) human gp100 peptide or B16gp100/A2 cells (C-D) were used as target cells. T cell activation conditions are indicated at the X-axes, and percentages of specific 51Cr release at E:T ratios of 40:1 are indicated at the Y-axes (mean±SEM; n=3). * = $p<0.05$ T cell activations vs ConA (per cytokine condition); † = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).



Supplementary Figure S2. Mock T cells activated with bead-coated CD3/CD28 mAbs and expanded in IL-15+IL-21 show non-specific IFNγ production

Six days after activation of T cells according to four different methods (Table I), Mock transduction, and expansion with either 100 U/ml IL-2 (A, C, E) or IL-15+IL-21 (50ng/ml each) (B, D, F), T cells were used in a 20 h tumour cell stimulation assay. B16A2 cells loaded with (A-B) or without (E-F) human gp100 peptide or B16gp100/A2 cells (C-D) were used as target cells. T cell activation conditions are indicated at the X-axes, and absolute levels of IFNγ present in supernatants (determined by commercial ELISA) are indicated at the Y-axes (mean±SEM; n=5). * = $p<0.05$ T cell activations vs ConA (per cytokine condition); † = $p<0.05$ IL-15+IL-21 vs IL-2 (per T cell activation condition).

CHAPTER 4

IL-21 results in de-differentiation of primary murine T lymphocytes

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ABSTRACT

IL-21 has recently been reported to suppress differentiation of naive T cells into effector T cells, and consequently aid the *in vivo* persistence and antigen-specific T cell responses. However, mechanisms behind IL-21-driven effects on T cell differentiation and activation remain elusive. Here, we demonstrate that short-term exposure of primary T cells to IL-21, and to a lesser extent IL-21+IL-15, resulted in a significant enrichment of $CD62L^{hi}/CD44^{lo}$, $CD27^{int}/CCR7^{hi}$ T cells, which was paralleled by differential expression of genes involved in T cell differentiation. IL-21 reduced both the proliferation and apoptosis of naive T cells. IL-21 down-regulated the expression of IL-2 receptor components, which was reversed by IL-15, up-regulated the expression of IL-21R, and prevented T cell expansion. Notably, IL-21 induced a phenotypic reversal of highly purified $CD44^{+}$ effector memory T cells into $CD44^{-}$ T cells. Interestingly, IL-21+IL-15 significantly up-regulated the expression of genes related to T cell effector functions such as perforine, granzymes and IFNγ. Taken together, our data show that IL-21 de-differentiates $CD44^{hi}$ T cells into naive T cells, and in combination with IL-15, enhances T cell activation, properties that may have implications for T cell therapy.

INTRODUCTION

Members of the common-γ chain cytokine family (i.e., γc cytokines) include interleukin(IL-)2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-2 is bound by a heterotrimeric receptor composed of the IL-2Rα (CD25), the common-β chain (i.e., IL-2Rβ or CD122) and the γc (CD132), whereas IL-15 is bound by a heterotrimeric receptor composed of the IL-15Rα, CD122 and CD132 (Leonard and Spolski, 2005). IL-21, in analogy to IL-4, IL-7 and IL-9, is bound by a heterodimeric receptor consisting of a specific receptor subunit, in case of IL-21 the IL-21R, and CD132. In addition to the γc receptor chain, IL-2 and IL-21 share Janus kinases (Jaks) and Signal Transducers and Activators of Transcription (STATs), representing a rapid cytosol-to-nuclear signalling pathway, as well as biological effects on T cells (Kasaian et al., 2002; Parrish-Novak et al., 2000). Interestingly, these two cytokines have specific but unique effects on T cell homeostasis, T cell differentiation and T cell effector functions. In example, IL-2 is reported to result in activation-induced cell death (AICD) (Lenardo, 1991), inhibition of memory CD8 T cell proliferation and survival (Ku et al., 2000), and proliferation of regulatory T cells that suppress or eliminate activated T cells (Shevach, 2000). IL-2 provided during antigen priming of CD8 T cells results in secondary expansion of memory CD8 T cells (Williams et al., 2006). Moreover, IL-2 accelerates differentiation of naive T cells into memory effector CD8 T cells, which may adversely affect T cell function *in vivo* and as such may compromise clinical responses of IL-2 treated adoptively transferred T cells (Evans et al., 1995; Gattinoni et al., 2005b; Hinrichs et al., 2008). Notably, in our current clinical phase I trial to treat metastasized renal cell carcinoma with scFv gene-modified T cells that have been expanded with IL-2, we observed that transduced T cells of patients, who *all* showed progressive disease, have a late effector CD8 T cell phenotype [Lamers, C. *et al.*, manucript in preparation]. In analogy to IL-2, IL-21 acts as a co-mitogen for antigen-activated effector CD8 T cells (Hinrichs et al., 2008; Moroz et al., 2004; Parrish-Novak et al., 2000). However, in contrast to IL-2, IL-21 does not induce proliferation of regulatory T cells (Peluso et al., 2007), but rather results in long-term sustainment of CD8 T cell numbers (Moroz et al., 2004) and accumulation of memory CD8 T cells (Allard et al., 2007). In addition, antigen priming of CD8 T cells in the presence of IL-21 suppresses differentiation of naive T cells into effector T cells (Hinrichs et al., 2008). Next to IL-21's ability to promote homeostasis of memory CD8 T cells, it is of interest that IL-21 was shown to enhance the tumour-infiltrating ability and anti-tumour activity of CD8 T cells (Di Carlo et al., 2004; Sondergaard et al., 2007). In fact, IL-21 turns out to be superior to IL-2 and IL-15 in inducing anti-tumour responses of endogenous as well as adoptively transferred CD8 T cells, and achieving long-term tumour control (Moroz et al., 2004). Clinical application of rIL-21 shows good tolerance and anti-tumour activity in patients with metastatic renal cell carcinoma and melanoma (Thompson et al., 2008), which is most likely associated with the observed *in vivo* activation of CD8 T cells and NK cells (Frederiksen et al., 2008).

Despite the clinical potential of IL-21 in anti-tumour therapy and its appreciated role in inducing anti-tumour T cell responses, the mechanisms behind IL-21-driven effects on T cell differentiation and activation remain elusive. Here, we analyzed how IL-2, IL-15 and/or IL-21 affected differentiation, proliferation and apoptosis of primary murine T cells by extensive phenotyping and gene expression profiling. We report that short-term exposure to IL-21, and to a lesser extent IL-21+IL-15, resulted in a significant enrichment of naive T cells. IL-21 reduced both the proliferation and apoptosis of naive T cells, and induced a phenotypic reversal of highly purified $CD44^+$ effector memory T cells into $CD44^-$ T cells. IL-21 down-regulated the expression of IL-2 receptor components, which was reversed by the addition of IL-15, and up-regulated the expression of its own receptor, and consequently did not support the expansion of T cells. IL-21, but not IL-15, induced differential expression of genes involved in T cell differentiation, and in combination with IL-15 up-regulated the expression of genes related to T cell effector functions.

MATERIAL AND METHODS

Ethics statement

Mice were housed according to guidelines of the Erasmus Medical Center, and procedures were carried out in compliance with standards for use of laboratory animals. Animal experiments performed in this manuscript have been approved by the animal experimental committee of the Erasmus Medical Center (DEC-consult).

Cells and reagents

The packaging cell lines 293T and Phoenix-amp were cultured as described elsewhere (Pouw et al., 2007). Antibodies (all anti-mouse mAbs unless stated otherwise) used in this study were: PE- or biotin-conjugated CD27 (LG.3A10); APC-conjugated CD44 (IM7); FITC- or PE-conjugated CD62L (MEL-14); APC-conjugated CD25 (PC61); FITC-conjugated CD122 (TM-b1); biotin-conjugated CD132 (TUGm2); PE-conjugated Bcl-2 (3F11); PE-conjugated IgG isotype control (A19-3); PE-conjugated active Caspase-3 (C92-605) (all from BD Biosciences, Breda, the Netherlands); PE- or APC-conjugated CCR7 (4B12, eBiosciences, San Diego, CA); PE-conjugated anti-human TCRVβ14 (CAS1.1.3, Beckman Coulter, Marseille, France); unconjugated Bcl-3 (C-14); unconjugated Bcl-6 (N3) (both from Santa Cruz Biotechnology, Santa Cruz, CA); PE-conjugated anti-human Bcl-x_L (7B2.5; Southern Biotech, Birmingham, AL); unconjugated BIM (14A8; Chemicon International, Temecula, CA); PE-conjugated anti-rat IgG (polyclonal $F(ab')_2$, Caltag Laboratories, Invitrogen, Breda, the Netherlands); biotin-conjugated IL-15Rα (FDN03; R&D Systems, Minneapolis, MN); unconjugated IL-21R (Abcam; Cambridge, UK) and FITC-conjugated anti-rabbit IgG (polyclonal IgG, Santa Cruz Biotechnology). Other reagents used in this study were Streptavidin (SaV)-FITC conjugate (BD Biosciences); Concanavalin A

(ConA, Sigma, St. Louis, MS); human rIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands); human rIL-15 (Peprotech, Rocky Hill, NY); murine rIL-21 (R&D Systems); and carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Paisley, UK).

Cytokine stimulation of T cells

Single cell suspensions of C57BL/6 mouse spleens (Erasmus MC animal housing facility, Rotterdam, The Netherlands), obtained in compliance with a national animal license, were activated with 2.5 µg/ml Concananvalin A (ConA) and 100 U/ml IL-2 for 24h as described previously (Pouw et al., 2007). Following T cell activation, T cells were cultured at 0.15-1x10^6 cells/ml in 24 well plates in complete mouse medium (Pouw et al., 2007) supplemented with 100 U/ml IL-2, 50 ng/ml IL-15, 50 ng/ml IL-21 or combinations of these cytokines (as indicated in the Results section).

Flow cytometry and FACSort

T cells were monitored for T cell differentiation markers, cellular division and apoptosis by flow cytometry using a FACSCalibur (BD Biosciences). To analyze expression of CD27, CD44, CD62L and CCR7, 0.1-0.5x10^6 T cells were washed with PBS, incubated at 4ºC (or 37ºC for CCR7 detection) with 10 µl of mAb added to cell pellet for 30 min, washed again, and fixed with 1% paraformaldehyde prior to flow cytometry analysis. CFSE labelling of T cells (0.42µM) was performed prior to cytokine stimulations and analyzed as CFSE dilution over time. For intracellular detection of active Caspase-3, Bcl-x_L, Bcl-2, Bcl-3, Bcl-6 and BIM, T cells were stimulated in the presence of brefeldin A (1 mg/ml) for 20h and subsequently stained using the Cytofix/Cytoperm kit (BD Biosciences) in 96 well plates according to the manufacturer's protocol. In some experiments, T cells were incubated with CD62L and CD44 mAbs for 30 min at 4ºC prior to intracellular stainings. Conjugated and matched isotype control mAbs were used to determine non-specific intracellular staining. Flow cytometry analysis was performed using CellQuest Pro software 5.2.1, and dot plots were used to present staining results of viable T cells (gated on FSC and SSC properties) and in some cases total T cells. Quadrants were set to separate distinct T cell subpopulations in IL-2 cultures, mostly based on the markers CD62L and CD44, and used as a reference for other T cell cultures. For cell sorting purposes, $CD44^{hi}$ or $CD44^{hi}/CD62L^-$ T cells were sorted using a FACSAria system (BD Biosciences).

Microarray analysis

RNA was extracted from T cells by Rneasy Mini kit following RNase-Free DNase treatment (Qiagen, Valencia, CA). Concentration of RNA was measured on a nanodrop ND-100 (NanoDrop Technologies Inc., Wilmington, DE) and its quality was checked (RNA integrity number > 7) on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). 1 µg RNA was labelled with cyanine 3 (test samples) and cyanine 5 (common reference) by the Low NA input Linear Amplification kit (Agilent). A common reference was generated by labelling 1 µg portions of

a pool comprising 1 µg of each test RNA, and mixing the resulting material afterwards. Mouse Genome 4x44K microarrays were hybridized overnight at 65°C, scanned with a DNA Microarray Scanner, and analyzed with Feature Extraction 9.5.1 (Agilent). To process data, outlier removed median signals were used in the R 2.5.0 and Bioconductor 2.0 MAANOVA packages. All slides were subjected to a set of quality control checks and, after log2 transformations, data were normalized by a global lowess smoothing procedure and analyzed using a two-stage mixed linear model (Kerr et al., 2000; Wolfinger et al., 2001). A permutation based Fs test (Cui and Churchill, 2003) was used to infer effects due to transgene or cytokine stimulation. p values were adjusted to reduce the false discovery rate (FDR) to 5%.

Statistical analyses

Statistical analysis of T cell phenotype was performed with the student's t-test with statistical significance indicated by p values < 0.05.

RESULTS

IL-21 results in T cells that predominantly have a naive T cell phenotype

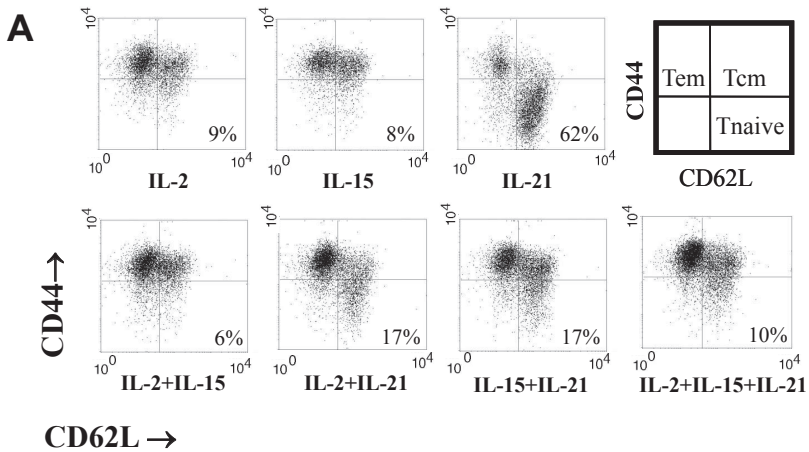
Primary murine T cells were activated with ConA and cultured in the presence of IL-2, IL-15, IL-21 or combinations thereof for up to 12 days and analyzed for their differentiation state. Only in the IL-21 culture, a clear naive T cell population was present based on CD62L and CD44 co-staining results (Figure 1A and B). Naive T cells were also significantly enriched, albeit to a lesser extent, when T cells were cultured in presence of IL-21+IL-15 or IL-21+IL-2, but not IL-21+IL-15+IL-2. The effects of the different cytokine treatments on all CD62L/CD44 T cell subsets are summarized in Table I. Percentages of $CD62L^+/CD44^-$ T cells were stable over time from day 3 onwards until the end of cytokine cultures (day 12), and depended on the

Table I. IL-21 significantly affects CD62L, CD44 T cell phenotype[a]

	$CD62L^-, CD44^+$	$CD62L^+, CD44^+$	$CD62L^-, CD44^-$	$CD62L^+, CD44^-$
IL-2	**48±7**	**31±5**	**9±3**	**12±6**
IL-15	**33±8** **	**44±11** **	**7±3**	**16±7**
IL-21	**14±6** **	**9±7** **	**13±4** **	**65±11** **
2+15	**46±11**	**34±6**	**8±1**	**13±6**
2+21	**44±13**	**21±4** **	**10±6**	**25±14** *
21+15	**31±10**	**30±6**	**10±4**	**29±9** **
2+21+15	**39±16**	**36±7** *	**6±2**	**19±8**

[a] Primary murine T cells were ConA activated and cultured with IL-2 (100 U/ml), IL-15 (50 ng/ml), IL-21 (50 ng/ml) or combinations of these cytokines and analyzed by flow cytometry for the expression of CD62L and CD44. Staining results with anti-CD62L mAb^{FITC} and anti-CD44 mAb^{APC} are shown for viable T cells at day 3 following culture with different cytokines. Percentages correspond to the fractions of viable T cells with a given CD62L, CD44 phenotype, and are depicted as mean±SEM, n=15. Statistically significant differences when compared to IL-2 are indicated by asterisks (* $p < 0.05$ and ** $p < 0.005$).

presence of cytokines rather than the absence of a T cell growth factor such as IL-2. T cells cultured in the presence of IL-21 are also significantly enriched for CCR7$^+$ T cells, when compared to T cells cultured in other cytokines (*Supplementary Figure S1*). In addition to CCR7$^+$ T cells, IL-21 cultures showed an increased percentage of CD27^{low} cells, whereas CCR7$^-$/CD27$^+$ T cells predominate in all other cytokine cultures.



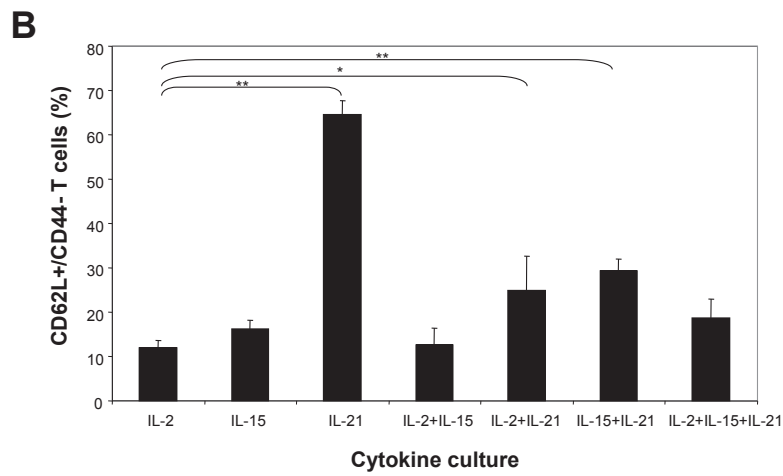


Figure 1. IL-21 results in T cells that predominantly have a CD62L$^+$/CD44$^-$ T cell phenotype

Primary murine T cells were activated with ConA and subsequently cultured with IL-2 (100 U/ml), IL-15 (50 ng/ml), IL-21 (50 ng/ml) or combinations of these cytokines and analyzed by flow cytometry for the expression of CD62L and CD44. Staining results are shown for viable T cells at day 3 following culture with different cytokines. Quadrants separate distinct CD62L and CD44 T cell subpopulations in IL-2 cultures, and are used as a reference for other T cell cultures. Percentages correspond to the fractions of viable T cells with a CD62L$^+$, CD44$^-$ phenotype. (A) A representative experiment, n=15 and (B) mean±SEM for all experiments, n=15 (* $p<0.05$ and ** $p<0.005$ when compared to IL-2). Figure (A) shows an insert with a schematic overview of the CD62L/CD44 phenotype of naive, central and effector memory T cells (Tnaive, Tcm and Tem, respectively).

IL-21 inhibits proliferation of naive T cells

Our initial analysis of T cell growth demonstrated that T cells expand to significant numbers in the presence of IL-15 (day 9: 60-fold expansion) but not IL-21 (no expansion) (*Supplementary Figure S2*). In fact, IL-21 cultured cells were lost and could not be monitored past the 3rd to 5th day following cytokine culture. T cells of IL-21 cultures survived and could be maintained in culture until the end of the experiment by the co-presence of IL-15 and, to a lesser extent, IL-2. Simultaneous use of IL-21, IL-15 and IL-2 did not further enhance T cell expansion and persistence compared to IL-21+IL-15 or IL-21+IL-2 (*Supplementary Figure S2*). Figure 2A demonstrates that whereas only half of IL-21 cultured T cells (at day 4 after CFSE labelling) have divided multiple times, this was true for >75% of IL-2, IL-15 or IL-21+IL-15 cultured T cells. The decreased proliferation rate of IL-21 cultured T cells appeared to be restricted to the CD62L$^+$/CD44$^-$ T cell subset constituting the majority of IL-21 cultured T cells (Figure 2B: >90% of CD44$^-$ ((R2+3+4)/(R1+2+3+4)x100%) and CD62L$^+$ T cells ((R6+7+8)/(R5+6+7+8)x100%) proliferated at most 2 times). To better understand IL-21's effects on T cell proliferation, we investigated protein and gene expression of different γc cytokine receptor components in cytokine cultured T cells. Figure 3A and 3B demonstrate that IL-21 significantly down-regulated both mRNA and cell surface expression of CD25 and CD122. And although IL-15 down-regulated the surface expression of CD25 to some extent (1.2 vs 3.9 fold when compared to IL-21), it restored the expression levels of CD25 and CD122 when co-administered together with IL-21. Furthermore, IL-21 up-regulated mRNA levels of CD132 and IL-21R to a small but significant extent, although not apparent at protein levels, yet did not affect the expression level of IL-15Rα. IL-15 was observed to down-regulate the expression of its own receptor at the protein level, most likely as a consequence of ligand-driven receptor internalization (Dubois et al., 2002).

IL-21 decreases Caspase-3 levels most predominantly in naive T cells

IL-21 significantly down-regulated expression of active Caspase-3, Bcl-x_L and Bcl-2 in viable T cells (Figure 4A and *Supplementary Figure S3*: $p<0.05$ when compared to IL-2). IL-15, when added together with IL-21 to T cells, restored the lowered expression levels of these apoptosis molecules. Microarray experiments confirmed down-regulated expression of *casp3*, *bcl2* and *bcl2l1* (bclxl) genes in IL-21 T cells and normalized expression of these molecules in IL-21+IL-15 T cells (Figure 4B). The flow cytometric expressions of Bcl-3, Bcl-6 and BIM, although showing a trend towards a decrease upon culture with IL-21, were not statistically different among the various T cell cultures (Figure 4A and *Supplementary Figure S3*). Interestingly, IL-21 up-regulated expression of *bcl3* and *bcl6* genes in IL-21 T cells (Figure 4B). Looking into more detail, down-regulated expression of Caspase-3, Bcl-x_L and Bcl-2 occurred predominantly in naive T cells (Figure 5: 10 vs 54% CD62L$^+$/CD44$^-$ T cells were positive for Caspase-3; 54 vs 87% for Bcl-x_L; and 5 vs 43% for Bcl-2 in IL-21 and IL-2 cultures, respectively).

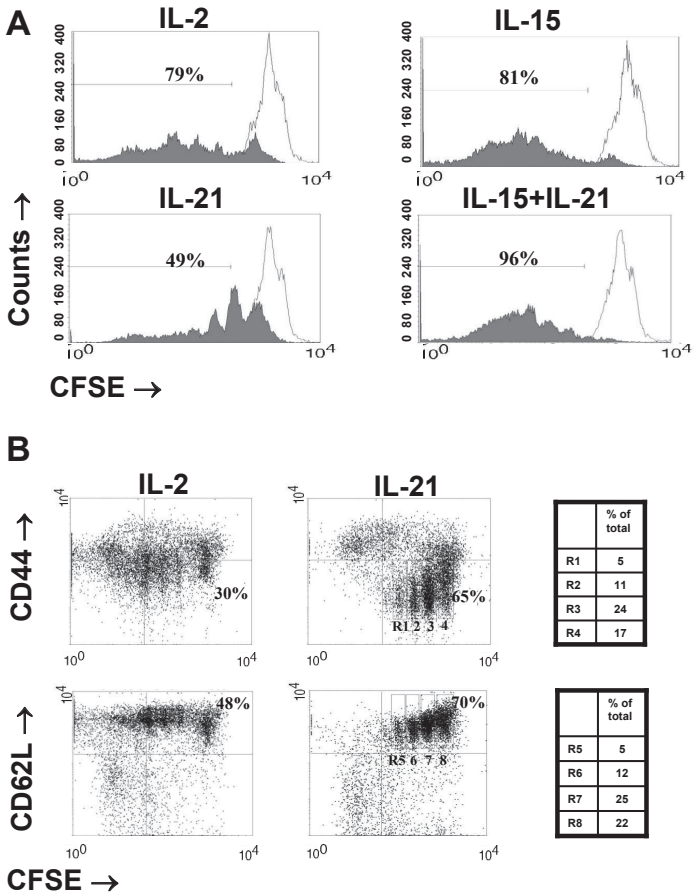


Figure 2. IL-21 inhibits proliferation of primary murine splenocytes when compared to IL-2

Freshly isolated primary murine splenocytes were labeled with CFSE (0.42 µM) and activated with ConA for 24 h in the presence of either IL-2, IL-15, IL-21 or IL-21+IL-15 with cytokines being refreshed every 2-3 days. T cells were analyzed by flow cytometry for CFSE dilution and the CD62L and CD44 markers at days 1, 4 and 8 after the start of culture. (A) CFSE dilution is shown for viable T cells at day 1 (open histograms) and day 4 (filled histograms). Marker M1 is set on CFSE negative cells at day 1 and percentages correspond to fractions of T cells within M1 (i.e., cells that underwent division). (B) CFSE dilutions of viable T cells cultured for 4 days in either IL-2 or IL-21 are plotted against CD62L or CD44. Tables next to Figure (B) show percentages of T cells present in gates R1-4 (CD44) and R5-8 (CD62L) (R1-4 and R5-8 are both based on CFSE signals at day 4 that correspond to 3, 2, 1 and 0 cellular divisions, respectively). Results are from a representative experiment out of 3 independent experiments with similar results.

IL-21 reverts CD44+ T cells into CD44- T cells

To follow up on T cell proliferation and apoptosis experiments in relation to the observed enrichment of naive T cells, we next studied the effect of IL-21 on differentiated T cells. IL-2 cultured T cells were sorted either for $CD44^{hi}$ or $CD44^{hi}/CD62L^{-}$ and subsequently cultured in IL-2 or IL-21 (Figure 6A and B). T cells cultured in IL-2 predominantly remained $CD44^{hi}$, whereas T cells cultured in IL-21 displayed a significant shift towards the $CD44^{-}$ phenotype.

Also, CD62L re-appeared on T cells, but this marker did not discriminate between IL-2 and IL-21 cultured T cells. These results imply that the IL-21-induced enrichment of $CD44^-$ T cells can be completely recapitulated when starting from differentiated T cells.

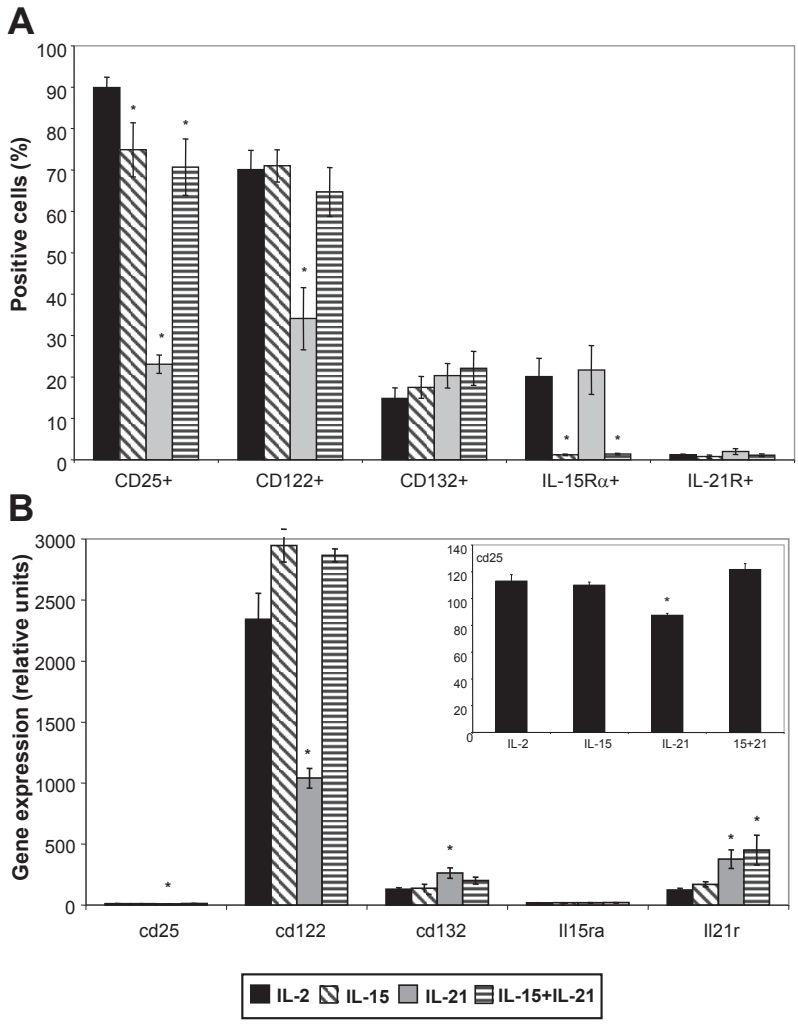


Figure 3. IL-21 down-regulates expression of CD25 and CD122

Murine splenocytes were activated and cultured as described in legend to Figure 1. At day 3 T cells were analyzed for the expression of γc cytokine receptors. (A) Cytokine receptors for which T cells were stained with antibodies and analyzed by flow cytometry are indicated at the X-axis. Percentages of viable T cells that were positive for a cytokine receptor are indicated at the Y-axis (mean±SEM, n=5). (** = $p<0.005$ when compared to IL-2). (B) Pellets of T cells were used for RNA isolation, after which RNA was labeled and used to hybridize mouse genome 4x44K micro array chips (Agilent). See Materials and Methods for details. Y-axis shows relative expression levels of the receptor compared to the common reference. Two independent experiments (starting from T cell cultures) gave similar results (* = $p<0.05$ when compared to IL-2). For statistical analysis data from both experiments were pooled (* = $p<0.05$ when compared to IL-2).

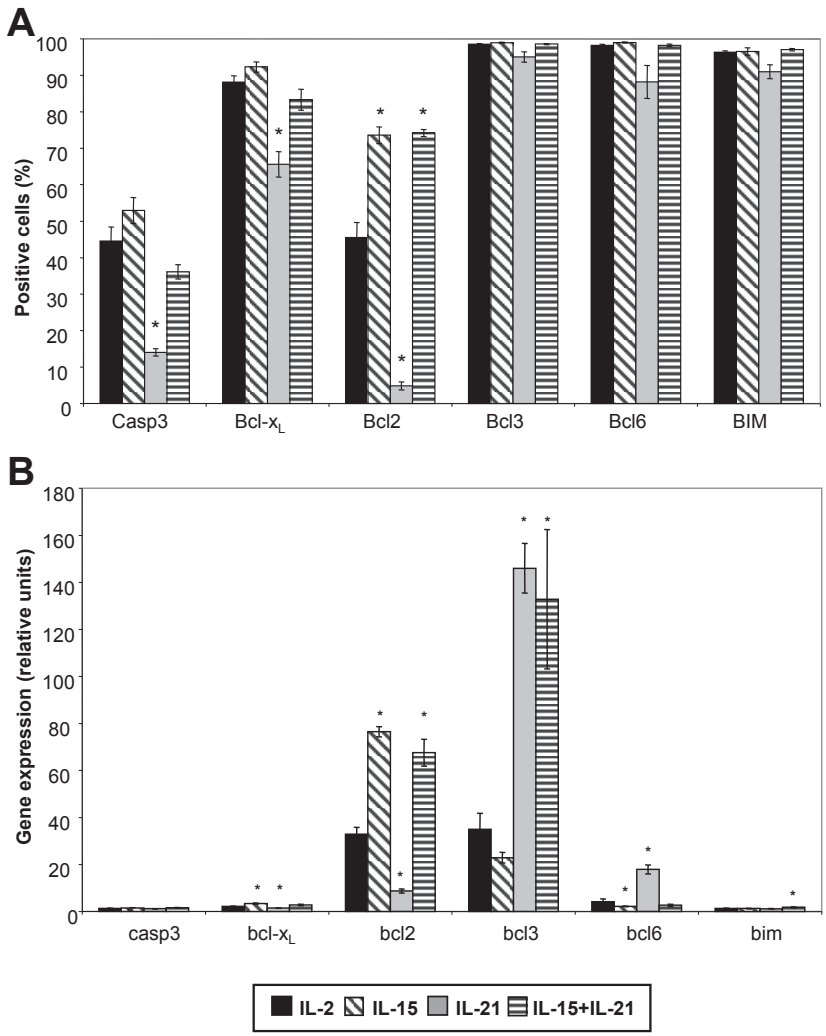


Figure 4. IL-21 down-regulates the expression of Caspase-3, Bcl-2 and Bcl-x_L in viable T cells

Primary murine T cells were activated and cultured as described in legend to Figure 1 and analyzed for protein and gene expression of Caspase-3, Bcl-x_L, Bcl-2, Bcl-3, Bcl-6 and BIM. (A) Flow cytometry results are shown for viable T cells at day 3 following culture with different cytokines. Shown are percentages of positive cells for an apoptosis marker (mean±SEM, n=5; * = $p<0.05$ when compared to IL-2). A representative experiment, n=5, is shown in Supplementary Figure S4. (B) T cells were analyzed for RNA expression levels as indicated in the legend to Figure 3B. Y-axis shows relative expression levels of the apoptosis markers compared to the common reference. Two independent experiments (starting from separate T cell cultures) gave similar results. For statistical analysis data from both experiments were pooled (* = $p<0.05$ when compared to IL-2).

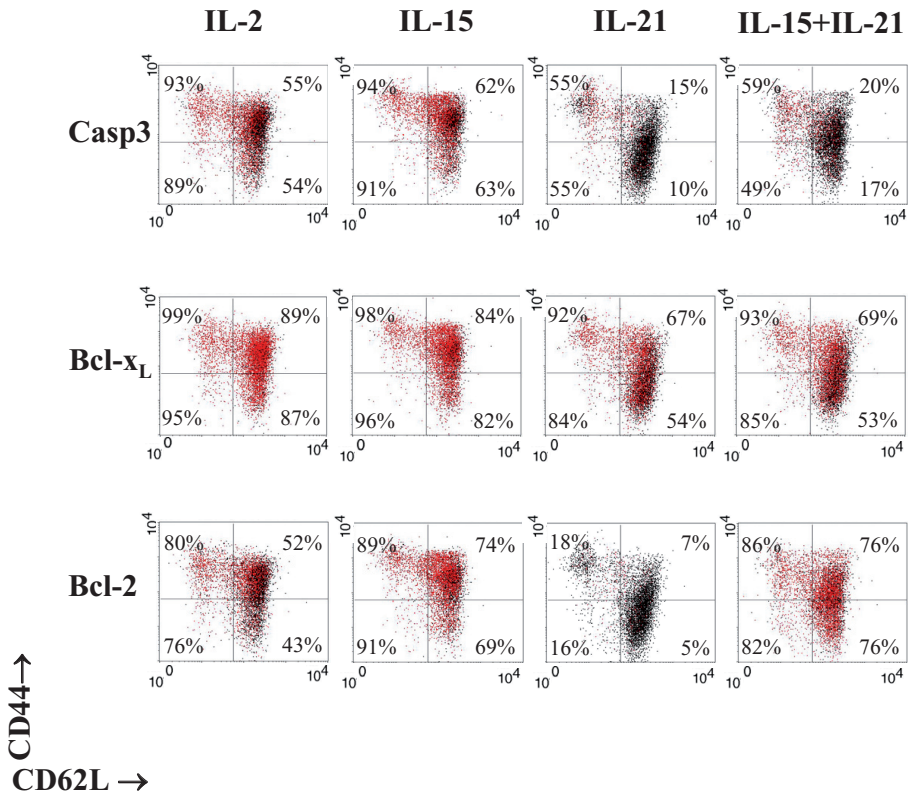


Figure 5. IL-21 prevents apoptosis of naive T cells

Primary murine T cells were stained for surface expression of apoptosis markers as described in legend to Figure 4. Expressions of active Caspase-3, Bcl-x_L and Bcl-2 (depicted as red dots) in viable T cells (gated on FSC and SSC properties) cultured in IL-2, IL-15, IL-21 or IL-21+IL-15 for 3 days are plotted against CD62L or CD44. Percentages correspond to T cells present in the indicated quadrant that are positive for the apoptosis marker. Results are from a representative experiment, n=3. Expressions of Caspase-3, Bcl-x_L and Bcl-2 within CD62L/CD44 subsets of total T cells (non-gated) are presented in *Supplementary Figure S5*.

IL-21, especially when combined with IL-15, up-regulates genes involved in T cell effector functions

Short-term cultures of primary T cells differentially expressed a total of 2712, 2212 or 6375 genes ($p<0.05$) when comparing IL-21, IL-15 or IL-21+IL-15 versus IL-2 cultures, respectively (the top 20 of which are shown in *Supplementary Figures S5A to C*). Our analysis showed that IL-21, IL-15 or the combination of these cytokines predominantly influence the expression of cytokine genes or genes involved in cytokine signalling (see *Supplementary Figure S5* for functional grouping based on ontology analysis). When IL-21 or IL-15 was used individually, they both up-regulated the gene expression of IFNγ (*ifng*). However, IL-21 uniquely up-regulated the expression of chemokine (C-C motif) ligands 3 and 4 (*ccl3* and *4*), lymphotoxin A (l*ta*), suppressor of cytokine signalling 2 and 3 (*socs2* and *3*), cytokine inducible SH2-containing

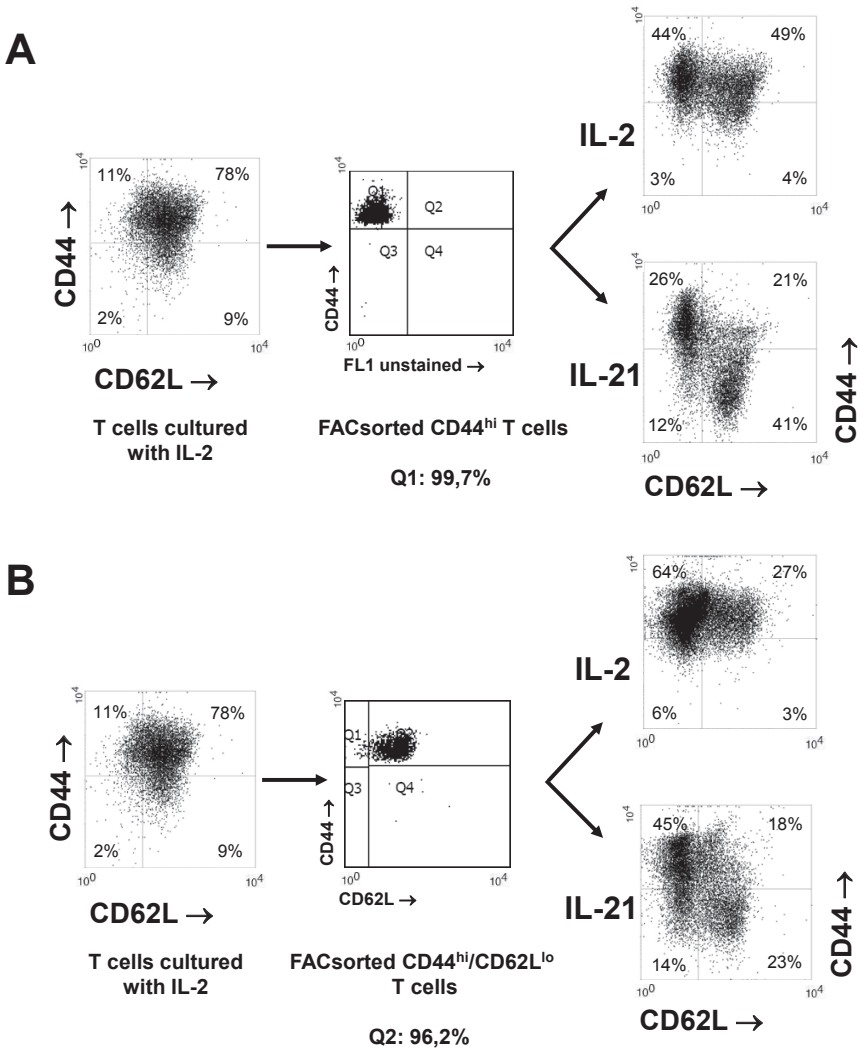


Figure 6. IL-21 reverts CD44^{hi} T cells into CD44^{-} T cells

Murine splenocytes were activated with ConA for 48 h in the presence of IL-2, after which (A) CD44^{hi} T cells and (B) CD44^{hi}/CD62L^{-} T cells were sorted on a FACSAria Flow Sorter and subsequently cultured in either IL-2 or IL-21 for 3 subsequent days. T cells were analyzed for expression of CD62L and CD44, and percentages in dot plots correspond to the fractions of viable T cells present in the corresponding quadrant. Results are from a representative experiment, n=3.

protein (*cish*) and cyclin-dependent kinase and Abl enzyme substrate 1 (*cables1*), whereas IL-15 up-regulated the expression of Epstein-Barr virus induced gene (*ebi3, Il27 component*) and the IL-7 receptor (*il7r, cd127)* (*Supplementary Figures S5A and B*). In addition, IL-21 down-regulated the expression of genes involved in cellular motility, and up-regulated the expression of some B cell related genes, possibly as a result of remaining B cells in IL-21 cultures of splenocytes (*Supplementary Figure S5A*). IL-15 up-regulated expression of genes involved

in T cell as well as NK cell activation, such as tumour necrosis factor receptor super family, member 9 (*tnfrsf9*, *cd137*) and killer cell lectin-like receptor, subfamily A, members 7, 16 and 23 (*klra7, 16 and 23*) (*Supplementary Figure S5B*). When IL-21 and IL-15 were used simultaneously, micro-array analyses again revealed differential expression of cytokine genes, such as *ifng, ccl3, ccl4*, *il10* and *ebi3* (*Supplementary Figure S5C*). Most prominently, the gene encoding IFNγ showed a 25-fold up-regulated expression level, pointing to a synergistic effect of IL-21 and IL-15 on IFNγ mRNA levels (when used individually, IL-21 and IL-15 resulted in a 7 and 3 fold up-regulated expression, respectively).

Looking at molecules used to define differentiated T cells, data show that IL-21 up-regulated the expression of *sell* (CD62L) and *ccr7*, and at the same time down-regulated the expression of c*d44*. Furthermore, IL-21 down-regulated the expression of eomesodermin (*eomes*), whereas IL-15 down-regulated the expression of T-cell-specific transcription factor 7 (*tcf7*) and lymphoid enhancer-binding factor (*lef1*), both involved in Wnt signalling (Figure 7B). Looking at T cell effector molecules, such as perforin 1 (p*rf1*), granzymes A and B (*gzma and gzmb*), and the above-mentioned *ifng*, we observed enhanced expression in IL-21, and especially in IL-21+IL-15 T cells (Figures 7A and C).

DISCUSSION

We studied the effects of IL-21, IL-15 and IL-2 on the differentiation, proliferation and apoptosis of primary T cells. We observed that non-stimulated T cells cultured with IL-21, and to a lesser extent IL-21+IL-15, are enriched for T cells expressing the markers CD62L, CD27 and CCR7 but not CD44, whereas T cells cultured with IL-2 or IL-15 are enriched for T cells expressing CD44 (Figure 1 and *Supplementary Figure S1*). High expression of CD62L and CCR7, both required for cell re-entry into lymph nodes (Arbones et al., 1994; Galkina et al., 2003), and of the co-stimulatory CD27, together with the absence of the activation marker CD44, point to a naive T cell phenotype upon exposure to IL-21. These data are in line with earlier reports on an increased CD62L expression by antigen-stimulated pmel-1 TCR transgenic T cells in the presence of IL-21, and to a lesser extent IL-15, in comparison to IL-2 (Gattinoni et al., 2005b; Hinrichs et al., 2008). IL-21's induced expression of CD62L was previously reported not to diminish either by the co-presence of IL-2 or secondary stimulation with antigen and IL-2 (Hinrichs et al., 2008). In our studies, the lack of antigen and use of primary T cells during T cell expansion, conditions chosen because of their relevance to clinical trials (Lamers et al., 2006a), may explain the absence of up-regulated CD62L expression upon exposure to IL-15 or a combination of IL-21 and IL-2. With respect to T cell expansion and proliferation, we found that upon administration of IL-21, T cells do not expand during a 2-week period (*Supplementary Figure S2*) and show a decreased proliferative capacity that was restricted to the $CD62L^{+}/CD44^{-}$ T cell subset (Figure 2). Decreased proliferation of IL-21 cultured T cells may be

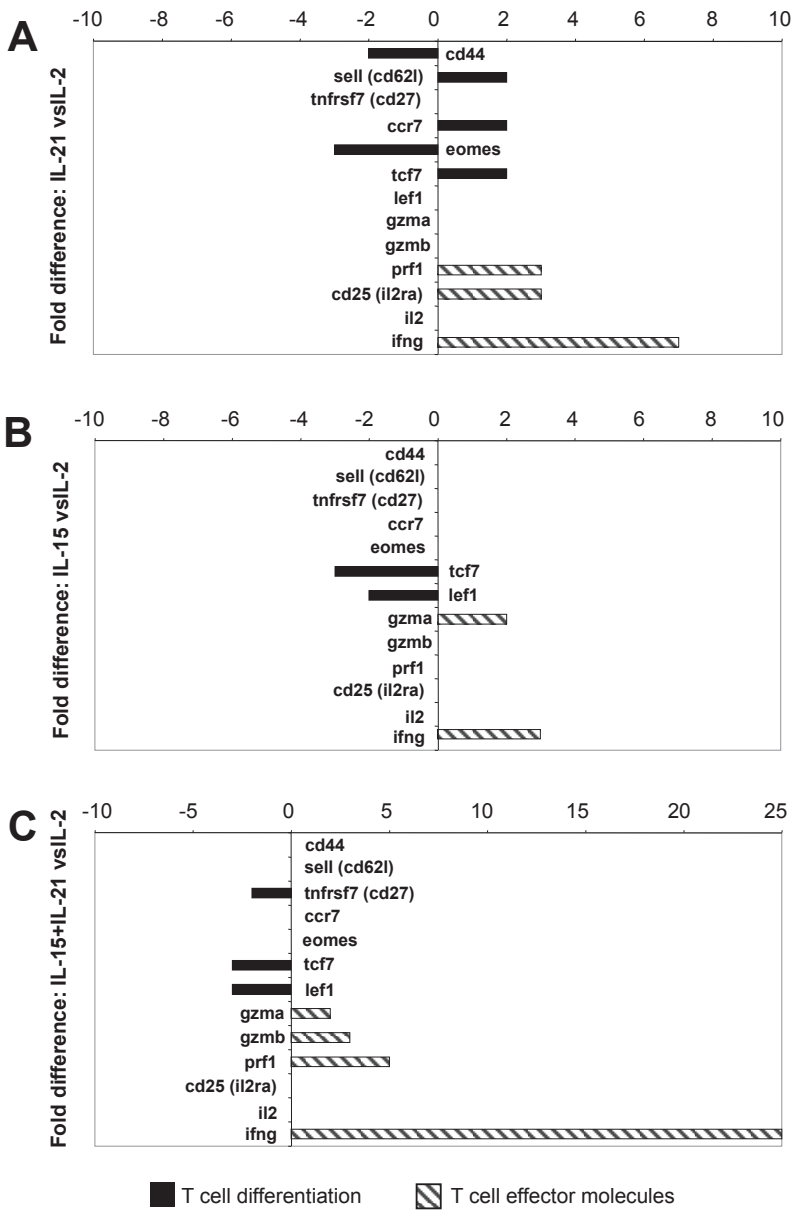


Figure 7. A combination of IL-21 and IL-15 up-regulates expression of T cell effector molecules

T cells were cultured and analyzed by micro arrays as described in the legend to Figure 3B. Differential expression of the following genes was determined when comparing (A) IL-21 versus IL-2, (B) IL-15 versus IL-2 and (C) IL-21+IL-15 versus IL-2: cd44, sell (CD62L), tnfrsf7 (CD27), ccr7, eomes, tcf7, lef1, gzma, gzmb, prf1, cd25 (IL-2Rα), il2 and ifng (all genes having $p<0.05$ when compared to IL-2). X- and Y-axes show fold differences and gene notations, respectively. Two independent experiments (starting from separate T cell cultures) gave similar results. For statistical analysis data from both experiments were pooled. Mock-transduced T cells did not differ from TCR-transduced T cells with respect to gene expression profiles (data not shown).

explained by decreased gene and cell surface expression of the IL-2 receptor subunits CD25 and CD122 when compared to IL-2 cultured T cells (Figure 3). Although IL-21R protein levels were generally low, IL-21 up-regulated the expression of its own receptor at the mRNA level (Figure 3) and as such may maintain T cells in a non-responsive state towards endogenous IL-2. The lack of T cell expansion in IL-21 cultures is in contrast to reports on anti-CD3 mAb or antigen-stimulated mouse T cells in which IL-21 promoted T cell expansion (Hinrichs et al., 2008; Parrish-Novak et al., 2000), but in agreement with a report on antigen-independent proliferation of spleen-derived as well as peripheral blood-derived human CD8 T cells (Alves et al., 2005). In the latter report, proliferation of human T cells was absent in the presence of IL-21, but strongly induced in the presence of IL-15. When IL-21 and IL-15 were combined in our T cell cultures, IL-15 was able to 'normalize' the lowered surface expressions of CD25 and CD122 and to restore the decreased proliferation of T cells (Figure 2A). When investigating T cell apoptosis, we found that IL-21 significantly down-regulated gene and protein expressions of Caspase-3, Bcl-x_L and Bcl-2 in viable T cells (Figure 4 and *Supplementary Figure S3*). Please note that our results do not imply that IL-21 has anti-apoptotic activities towards primary T cells. On the contrary, when analyzing total T cells, IL-21 resulted in an over-expression of Caspase-3 with protective Bcl factors being virtually absent (see *Supplementary Figure S5*). These pro-apoptotic activities of IL-21 are in agreement with results reported for antigen-specific CD8 T cells (Barker et al., 2007) and contribute to the lack of T cell expansion observed in our IL-21 T cell cultures (*Supplementary Figure S3*). Importantly, IL-21 induced Caspase-3^{hi} T cells that predominantly belong to the central and effector memory T cells (evident from total T cells: *Supplementary Figure S4*), which suggests that naive T cells are more resistant to IL-21 induced apoptosis (evident from viable, and thus surviving T cells: Figure 5). IL-15 protected T cells from apoptosis, which was most evident from the enhanced Bcl-2 expression (Figure 4). When combined with IL-21, IL-15 significantly restored Caspase-3 and Bcl expression levels, although levels remained below those obtained with IL-15 only. This may explain the apparent discrepancy between IL-21+IL-15's promoting effect on T cell proliferation (Figure 2) and lack of effect on T cell expansion (*Supplementary Figure S3*). The decreased levels of Caspase-3 in viable IL-21 T cell cultures, and restored Caspase-3 levels in viable IL-21+IL-15 T cell cultures nicely paralleled the proliferative capacity of these T cell cultures (Figure 2) and extended previous observations that pointed to an essential role of Caspase-3 in proliferating T cells (Algeciras-Schimnich et al., 2002).

To learn whether limited apoptosis of naive T cells drives IL-21's ability to enrich $CD62L^+$/$CD44^-$ T cells, we started from T cells purified for a differentiated phenotype (i.e., $CD44^{hi}$ or $CD44^{hi}$/$CD62L^-$ T cells) and found that IL-21, but not IL-2, reverted $CD44^+$ T cells into $CD44^-$ T cells (Figures 6A and B). Prior to exposure to IL-21, sorted T cells contained < 1% of viable $CD44^{lo}$ T cells, which accounted at most for 10% of the total number of viable $CD44^{lo}$ T cells that were obtained following an IL-21 culture. A likely explanation of these data is that next to limited apoptosis, a phenotypical switch contributes to IL-21-induced enrichment of naive

T cells. Additional CFSE labelling experiments showed that a significant proportion of the newly evolved $CD44^-$ T cells have not diluted their CFSE label (data not shown), indicating that the enrichment of $CD44^-$ T cells does not require cell division. Interestingly, CD44hi T cells proliferate and undergo apoptosis in a setting of allogeneic bone marrow transplantation (Alpdogan et al., 2008), and CD44 signalling results in enhanced Caspase and decreased Bcl levels in leukemic T cells (Cordo Russo et al., 2008; Guy et al., 2002; Maquarre et al., 2005). From these findings, we hypothesize that IL-21 treated T cells may down-regulate CD44 expression as a feedback mechanism to prevent full-blown apoptosis. The potential of CD8 T cells to revert into a less-differentiated phenotype upon antigenic stimulation, accompanied by re-expression of CCR7 and CD62L, has been shown previously (Perret and Ronchese, 2008; van Leeuwen et al., 2005). Moreover, in melanoma patients treated with adoptive T cell therapy, transferred T cells undergo a transition from late effector to effector memory T cells and show an up-regulated expression of CD27 and CD28 following a clinical anti-tumour response (Powell et al., 2005). Our current report, however, shows for the first time that T cell treatment with IL-21 results in de-differentiation of primary $CD44^{hi}$ (CD8) T cells.

Finally, we observed that most of the genes of which expression was influenced by IL-21 were related to cytokines and cytokine-mediated signalling, such as *ccl3*, *ccl4* and *ifng* (*Supplementary Figure S5A*). Interestingly, in various mouse tumour models it was observed that IL-21 resulted in tumour infiltration of CD8 T cells, which was accompanied by expression of IFNγ and IFNγ-induced CXC chemokines such as IFNγ-inducible protein 10 (IP-10), monokine induced by IFNγ (MIG) and IFNγ-inducible T cell alpha chemo attractant (I-TAC) (Di Carlo et al., 2004; Sondergaard et al., 2007). These chemokines as well as the CC chemokines *ccl3* and *ccl4* may further enhance leukocyte trafficking towards the tumour. Looking at T cell differentiation, IL-21 but not IL-15 up-regulated the expression of *sell* (CD62L) and *ccr7*, and down-regulated the expression of *cd44* (Figure 7), which extends the observed IL-21-induced enrichment of $CD62L^+$, $CCR7^+$, $CD44^-$ T cells (Figure 1 and *Supplementary Figure S1*). Targets of Wnt signalling such as *lef1*, reported to have an up-regulated expression upon exposure to antigen and IL-21 (Hinrichs et al., 2008), were not found to have a differentially regulated expression upon exposure to IL-21 in our study (*Supplementary Figure S5A*) and showed a down-regulated expression in IL-15 T cell cultures (*Supplementary Figure S5B*). IL-21+IL-15 cultures most significantly regulated *ifng*, the expression of which was 25-fold increased when compared to IL-2 cultures (*Supplementary Figure S5C*, $p<0.006$). The effect of IL-21+IL-15 on *Ifng* gene expression is of a synergistic nature and may be the consequence of activation of distinct downstream mediators. In example, IL-21 signals predominantly through Jak3, STAT1 and STAT3, whereas IL-15 signals predominantly through STAT5 and ERK1/2 (de Totero et al., 2008), with regulatory sites in the IFNγ promoter being responsive to both STATs and ERK-dependent transcription factors (Egerton et al., 1998; Strengell et al., 2003). In addition, IL-15 induced genes involved in NK cell proliferation and activation (*Supplementary Figure S5B*), and may preferentially expand NK cells that contain high levels of IFNγ (Terabe et al., 2008).

The enhanced IFNγ mRNA levels are most likely responsible for a sharp increase in T cell IFNγ protein production in response to gp100/A2-positive melanoma cells. Indeed, we have observed that T cells pre-treated with IL-21+IL-15 show enhanced antigen-specific production of IFNγ protein, which is almost exclusively confined to $CD62L^-/CD44^+$ effector memory T cells (Pouw et al., 2010). In addition to *Ifng*, gene expression of other T cell effector molecules, including P*rf1*, G*zma*, and G*zmb*, is also specifically up-regulated in IL-21+IL-15 T cell cultures (Figure 7C). This suggests that the combination of these two cytokines results in an increased potential of T cells to respond to antigen-positive target cells. In fact, the enhanced effector CD8 T cell phenotype by IL-21+IL-15 may provide a molecular basis to explain the findings by Zeng and colleagues (Zeng et al., 2005), who reported enhanced anti-melanoma responses of CD8 T cells upon *in vivo* administration of both IL-21 and IL-15 relative to either cytokine separately.

In summary, short-term exposure to IL-21 enriches T cells for a naive T cell phenotype by reversal of $CD44^+$ effector memory T cells into $CD44^-$ T cells and limits proliferation and apoptosis of these T cells. IL-21+IL-15-treated cultures demonstrated decreased apoptosis of memory T cells and are characterized by up-regulated expression of T cell effector molecules. We believe that T cell or TCR gene therapy of tumours may benefit from pre-treatment of T cells with a combination of IL-21 and IL-15.

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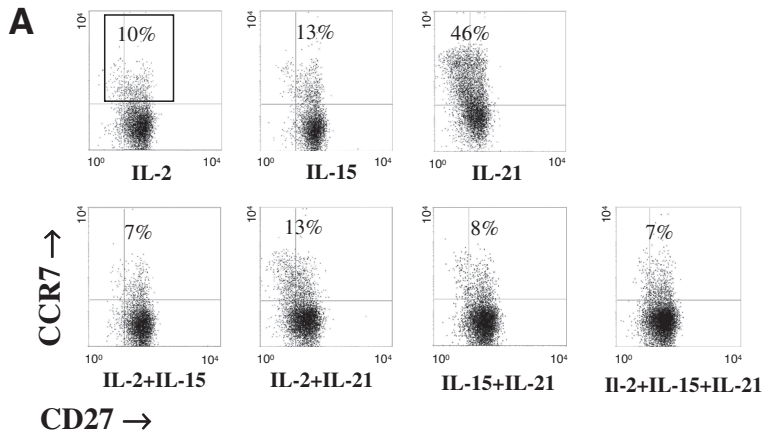
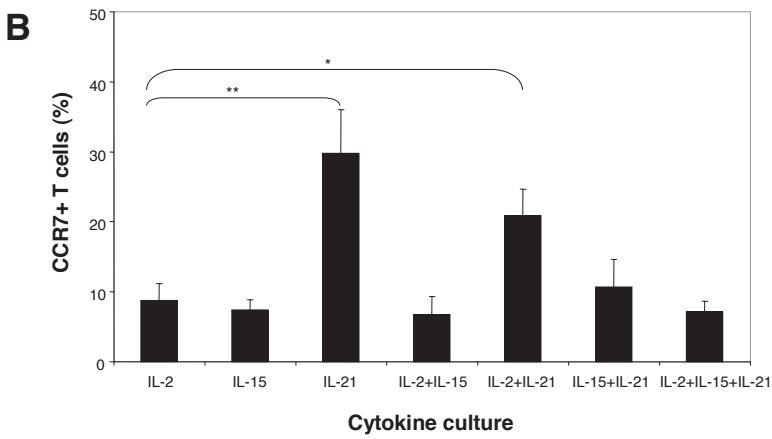
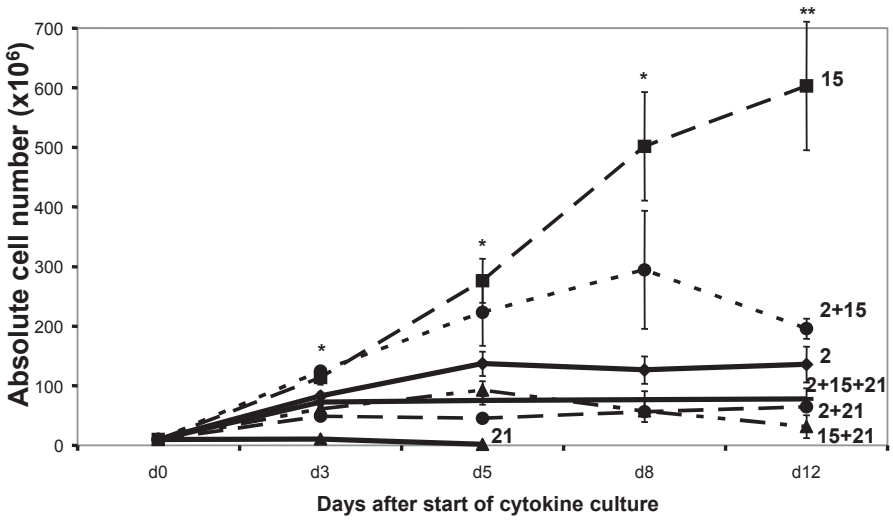
SUPPLEMENTARY FIGURES



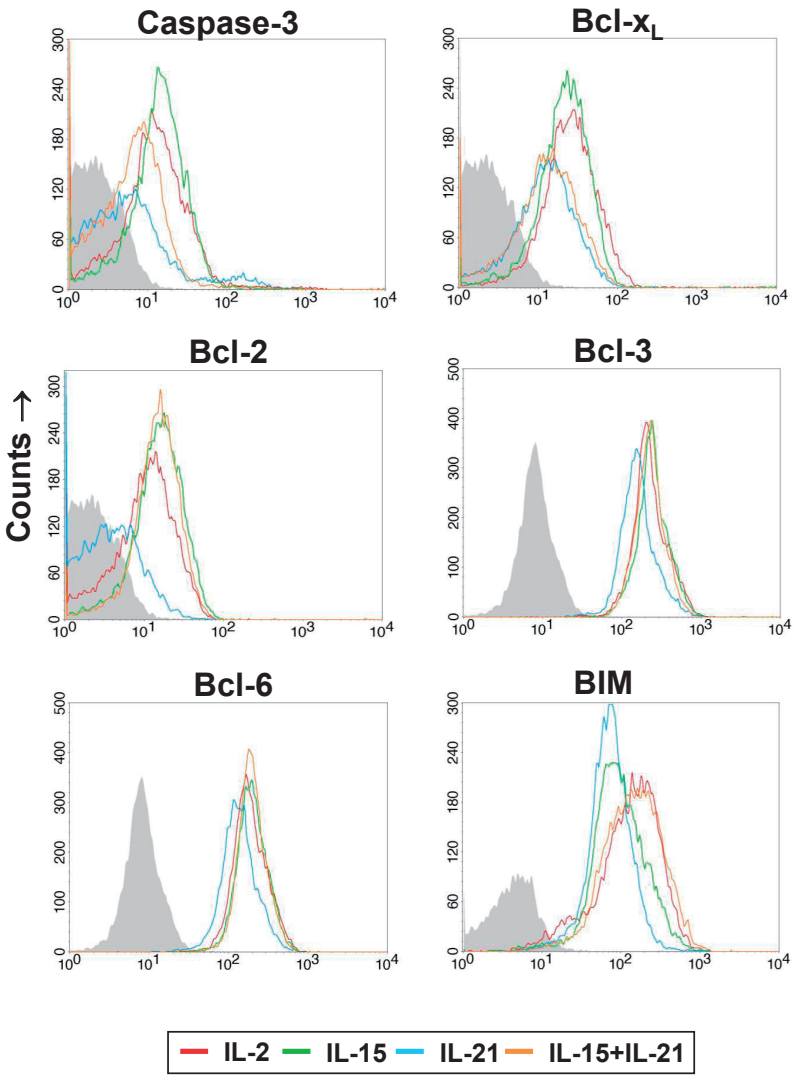


Supplementary figure S1. IL-21 results in T cells that predominantly have a CCR7+ T cell phenotype
T cells were activated with ConA and subsequently cultured with cytokines as described in the legend to Figure 1, and analyzed by flow cytometry for the expression of CD27 and CCR7. Staining results are shown for viable T cells at day 3 following culture with different cytokines. Percentages correspond to the fractions of viable T cells showing a CCR7+ phenotype. (A) A representative experiment, n=15 and (B) mean±SEM for all experiments, n=15 (* $p<0.05$ and ** $p<0.005$ when compared to IL-2).

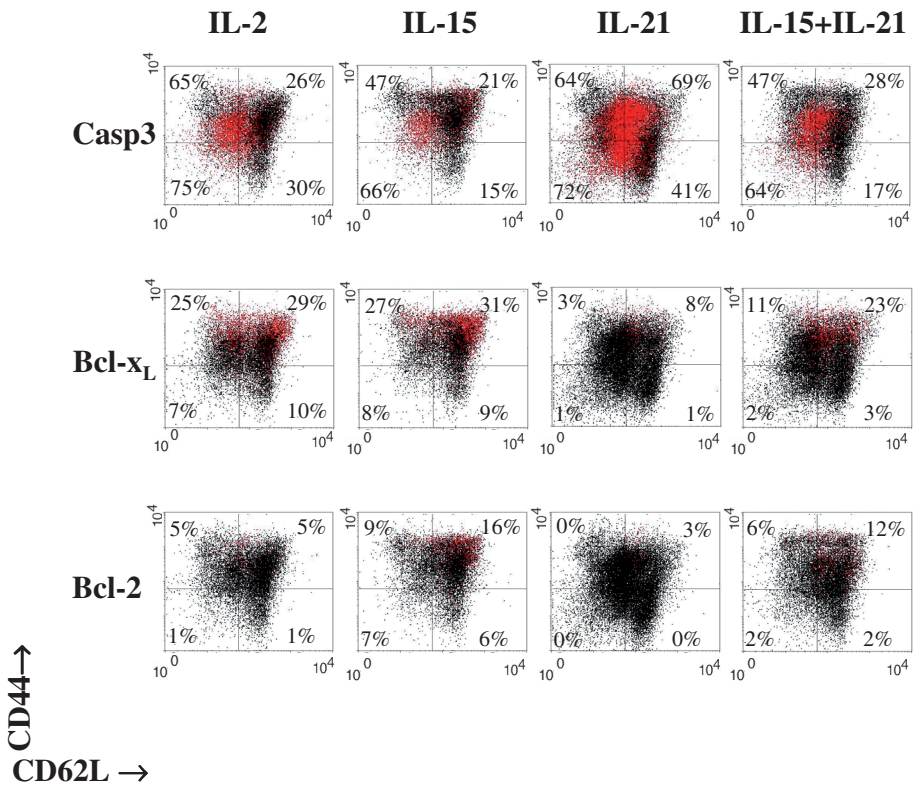


Supplementary figure S2. IL-21 results in loss of T cells cultured without antigen, and T cells survive by the co-presence of IL-15

T cells were expanded at 0.15-1x106/ml in the presence of either IL-2, IL-15, IL-21 or combinations of these cytokines. T cells were harvested, counted, and received fresh medium including cytokine(s) every 2 to 3 days. Viable T cells (based on trypan-blue exclusion) were counted microscopically (Leitz Laborlux 12, Leica Geosystems BV, Rijswijk, The Netherlands) using Bürker counting chambers. Cell growth is monitored up to two weeks from the start of culture and depicted as mean cell numbers ± SEM (x106), n=15. T cells cultured in IL-15 differed significantly ($p<0.05$) from either all other cultures (**) or all other cultures except IL-2+IL-15 (*).

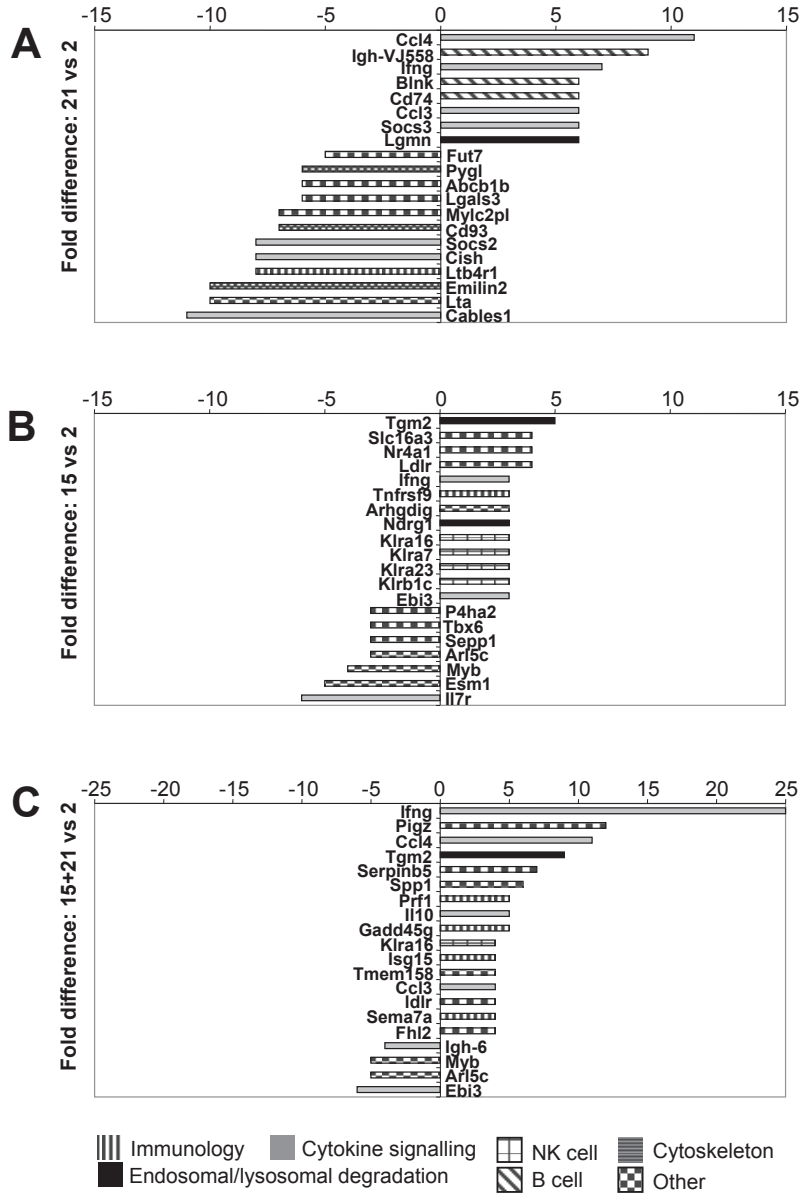


Supplementary figure S3. IL-21 down-regulates the surface expression of Caspase-3, Bcl-x$_L$ and Bcl-2
Primary murine T cells were stained as described in legend to Figure 4A. Expression of apoptosis markers is shown for viable T cells at day 3 following culture with different cytokines. Grey histograms represent staining with isotype control antibodies. Results are from a representative experiment, n=5. Statistics on all 5 experiments are shown in Figure 4A.



Supplementary figure S4. IL-21 up-regulates surface expression of Caspase-3 predominantly in memory T cells

Primary murine T cells were stained for expression of active Caspase-3, Bcl-x_L and Bcl-2 as described in legend to Figure 5. Expression of active Caspase-3, Bcl-x_L and Bcl-2 (depicted as red dots) in total T cells (non-gated) cultured in IL-2, IL-15, IL-21 or IL-21+IL-15 for 3 days is plotted against CD62L or CD44. Percentages correspond to T cells present in the indicated quadrant that are positive for the apoptosis marker. Results are from a representative experiment, n=3. Expressions of active Caspase-3, Bcl-x_L and Bcl-2 within CD62L/CD44 subsets of viable T cells (gated on FSC and SSC properties) are presented in Figure 5.



Supplementary figure S5. IL-21 differentially regulates expression of cytokine genes and those involved in cytokine signaling

T cells were cultured with different cytokines for 3 days, and analyzed for RNA expression as indicated in the legend to Figure 3B. The 20 most differentially expressed genes for (A) IL-21 versus IL-2, (B) IL-15 versus IL-2 and (C) IL-21+IL-15 versus IL-2 T cell cultures are graphically presented (all genes having $p<0.05$ when compared to IL-2). X- and Y-axes show fold differences and gene notations, respectively. Functionally similar gene products (based on gene ontogeny analysis) are grouped by different patterns as indicated below the Figure. Two independent experiments (starting from separate T cell cultures) gave similar results. For statistical analysis data from both experiments were pooled.

CHAPTER 5

Combination of IL-21 and IL-15 enhances tumour-specific cytotoxicity and cytokine production of TCR-transduced primary T cells

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ABSTRACT

IL-21, and to a lesser extent IL-15, inhibits differentiation of antigen-primed CD8 T cells and promotes their homeostasis and anti-tumour activity. Here, we investigated molecular mechanisms behind tumour-specific responses of primary murine T lymphocytes engineered to express a TCR directed against human gp100/HLA-A2 following short-term exposure to IL-15 and/or IL-21. We demonstrated that IL-15+IL-21, and to a lesser extent IL-21, enhanced antigen-specific T cell cytotoxicity, which was related to enhanced expression of granzymes A and B, and perforin 1. Furthermore, IL-15+IL-21 synergistically enhanced release levels and kinetics of T cell IFNγ and IL-2, but not IL-10. Enhanced secretion of IFNγ was accompanied by increased gene expression and cytosolic protein content, and was restricted to effector memory T cells. To summarize, we show that IL-15+IL-21 improves antigen-specific responses of TCR-transduced effector T cells at multiple levels, which provides a rationale to treat T cells with a combination of these cytokines prior to their use in adoptive TCR gene therapy.

INTRODUCTION

TCR gene transfer represents an attractive therapeutic strategy to provide patients with tumour-specific T cell immunity (Clay et al., 1999; Cooper et al., 2000; Orentas et al., 2001; Schaft et al., 2003; Willemsen et al., 2000). The clinical feasibility of this experimental therapy has recently been demonstrated in trials with TCR-gene modified T cells to treat melanoma patients who were pre-conditioned with lympho-ablative chemotherapy (Johnson et al., 2009; Morgan et al., 2006). Objective response rates in these trials ranged from 12% to 30%, and although impressive, still lagged behind the 50% objective response rates observed in earlier trials with non-gene modified T cells (Dudley et al., 2002; Dudley et al., 2005).

One way to enhance the efficacy of TCR gene therapy, other than choosing high-affinity or genetically improved TCR transgenes (Johnson et al., 2009; Varela-Rohena et al., 2008), is to expose T cells to common-γ cytokines other than interleukin-2 (IL-2) prior to their adoptive transfer. Notably, antigen priming of CD8+ T cells in the presence of IL-21, and to a lesser extent IL-15, suppresses differentiation of naive T cells into effector T cells, which in turn enhances *in vivo* persistence of T cells and antigen-specific effectiveness (Gattinoni et al., 2005b; Hinrichs et al., 2008). In fact, clinical anti-tumour efficacy of T cells irrespective of gene-modification appears to be directly related to T cell persistence (Ciceri et al., 2007; Kershaw et al., 2006; Robbins et al., 2004), which in turn is reported to be associated with differentiation state and replicative history of transferred T cells (Huang et al., 2005).

IL-2, IL-15 and IL-21 all act as co-mitogens for antigen-activated effector CD8 T cells (Moroz et al., 2004; Waldmann, 2006). In contrast to IL-2, however, IL-15 enhances survival of CD8 effector T cells (Hsu et al., 2005) and IL-21 does not induce proliferation of regulatory T cells (Peluso et al., 2007). Next to their effects on CD8 T cell homeostasis, IL-15 and IL-21 are able to enhance the *in vivo* anti-tumour effects of CD8 T cells (Brentjens et al., 2003; Di Carlo et al., 2004; Hinrichs et al., 2008; Klebanoff et al., 2004; Sondergaard et al., 2007; Teague et al., 2006). In example, adoptively transferred T cells resulted in potentiated tumour regression when pre-treated with IL-15 or IL-21 (Brentjens et al., 2003; Hinrichs et al., 2008; Klebanoff et al., 2004). It is of interest that IL-21 turns out to be superior to IL-15 in inducing anti-tumour responses and achieving long-term tumour control by endogenous as well as adoptively transferred CD8 T cells (Hinrichs et al., 2008; Moroz et al., 2004). Moreover, combined *in vivo* treatment with IL-15 and IL-21 enhanced CD8 T cell expansion and anti-melanoma responses relative to the administration of either cytokine separately (Zeng et al., 2005).

Here, we investigated molecular mechanisms behind functional responses of primary murine T lymphocytes engineered to express a TCR directed against human gp100/HLA-A2 and treated with IL-15 and/or IL-21. Unexpectedly, we observed that a combination of IL-15 and IL-21 promotes antigen-specific T cell responses at multiple levels. First, IL-15+IL-21 enhanced T cell cytotoxicity in response to hgp100-positive tumour cells, which paralleled up-regulated expression of granzymes A, B and perforin-1. Second, these cytokines showed

a synergistic effect in enhancing levels and kinetics of secreted IFNγ and IL-2, but not IL-10, in an antigen-specific manner. The enhanced IFNγ protein production, the most significant response noted upon co-treatment with IL-15 and IL-21, was due to enhanced IFNγ mRNA levels and was restricted to the subset of CD62L-/CD44+ effector memory CD8 T cells.

MATERIAL AND METHODS

Cells and reagents

The packaging cell lines 293T and Phoenix-amp, and the melanoma cell lines B16 wildtype (B16), B16 transfected with AAD DNA (human HLA-A2 containing the α3 loop of murine H2-Kd, termed B16A2) and B16 transfected with HLA-A2 and hgp100 DNA's (B16gp100/A2) (kindly provided by dr. G. Adema, Nijmegen, The Netherlands) were cultured as described elsewhere (Pouw et al., 2007). B16A2 was grown under selection of 1 mg/ml G418, and B16gp100/A2 was grown under selection of 1 mg/ml G418 and 0.5 mg/ml Hygromycin B. Monoclonal Abs used in this study were: PerCP-conjugated anti-CD3ε (145-2C11), FITC-conjugated anti-CD4 (RM4-5), Allophycocyanin(APC)-conjugated anti-CD8α (53-6.7), APC-conjugated anti-CD11c (HL3), PE-conjugated anti-CD19 (1D3), PE-conjugated anti-NK1.1 (PK136), PE- or biotin-conjugated anti-CD27 (LG.3A10), APC-conjugated anti-CD44 (IM7), FITC- or PE-conjugated anti-CD62L (MEL-14), PE-conjugated anti-IFNγ (XMG1.2), APC-conjugated anti-IL-2 (JES6-5H4), APC-conjugated anti-IL-10 (JES5-16E3) (all from BD Biosciences, Breda, the Netherlands), PE- or APC-conjugated anti-CCR7 (4B12, eBiosciences, San Diego, CA), and PE-conjugated anti-human TCR-Vβ14 (CAS1.1.3, Beckman Coulter, Marseille, France). Other reagents used in this study were human gp100 wildtype peptide (YLEPGPVTA) (Schaft et al., 2003), Streptavidin (SaV)-FITC conjugate (BD Biosciences), Concanavalin A (ConA, Sigma, St. Louis, MS), human rIL-2 (Proleukin, Chiron, Amsterdam, The Netherlands), human rIL-15 (Peprotech, Rocky Hill, NY) and murine rIL-21 (R&D Systems).

TCR gene transfer and cytokine stimulation

Single cell suspensions of C57BL/6 mouse spleens (Erasmus MC animal housing facility, Rotterdam, The Netherlands) were obtained in compliance with a national animal license. T cells were genetically modified with TCRαβ genes prior to cytokine exposure as described elsewhere (Pouw et al., 2007). In short, T cells were stimulated for 24h with 2.5 μg/ml Con A in the presence of 100 U/ml IL-2 (which is considered the start of culture, t= day 0). Subsequently, T cells were retrovirally transduced via retronectin-mediated virus supernatant centrifugations with transgenes encoding a human TCRαβ specific for $gp100_{280-288}$/HLA-A2 (gp100/A2) that had previously been murinized for TCR-Cα and Cβ. Mock-transduced T cells were exposed to the same procedure except for the use of virus-free instead of virus-containing supernatant, and were used as experimental controls for TCR-transduced T cells. Following T cell activation

and gene transfer (at t=day 2), T cells were cultured at 0.15-1x10^6 cells/ml in 24 well plates in medium (Pouw et al., 2007) supplemented with 100 U/ml rIL-2, 50 nanogram (ng)/ml IL-15, 50 ng/ml IL-21 or 50 ng/ml IL-21 + 50 ng/ml IL-15. Concentrations of the different cytokines were pre-determined by titration experiments (ranging from 10-100 ng/ml, data not shown). T cell exposure times to cytokines are indicated in the various Figures. Cytokines were refreshed every 2-3 days.

Cytotoxicity and cytokine secretion

Cytotoxic activity of TCR-transduced T cells was measured in a standard 4-6h ^{51}Cr-release assay, principally as described (Pouw et al., 2007; Weijtens et al., 1998). B16F10, B16gp100/A2 and B16A2 cells were used as target cells, and in some experiments B16A2 cells were pulsed with human gp100 peptide (final concentration: 10 µM) for 15 min at 37ºC and 5% CO_2 prior to co-cultivation with effector T cells. Cytokine secretion in response to the above-mentioned B16 target cells, and ConA and medium as controls, was measured in supernatants from 20h co-cultivations (6x10^4 T cells and 2x10^4 target cells per well in 96 well plates) by mouse IFNγ ELISA (U-CyTech, Utrecht, The Netherlands), mouse IL-2 ELISA (Endogen, Pierce Biotechnology, Rockford, IL) and mouse IL-10 ELISA (U-CyTech). During target cell stimulations no exogenous cytokines were added to the T cell cultures.

Flow cytometry and FACSort

Immune monitoring comprised detection of leukocyte markers, T cell differentiation markers, TCR transgene expression and intracellular cytokines by flow cytometry using a FACSCalibur (BD Biosciences). To analyze surface expression of leucocyte or T cell markers as well as the introduced human gp100/A2 TCRαβ, 0.1-0.5x10^6 T cells were washed with PBS, incubated for 30 min at 4ºC (or 37ºC for CCR7 detection) with 10 µl of mAb (or 5 µl of the TCR-Vβ14 mAb) added to a cell pellet, washed again, and fixed with 1% paraformaldehyde prior to flow cytometry. For intracellular detection of IFNγ, IL-2 and IL-10, T cells were stimulated with target cells in the presence of brefeldin A (1 mg/ml) for 20h and subsequently stained using the Cytofix/Cytoperm kit (BD Biosciences) in 96 well plates according to the manufacturer's protocol. Medium and ConA (10 µg/ml) were used as negative and positive stimulation controls, respectively. Conjugated and matched isotype control mAbs were used to determine non-specific intracellular stainings. In some experiments, T cells were incubated with CD62L and CD44 mAbs for 30 min at 4ºC prior to intracellular IFNγ staining. Flow cytometry analysis was performed using CellQuest Pro software 5.2.1. Data are presented either as absolute numbers of cells present in a fixed volume that were positive for a certain cytokine (see *Supplementary Figure S1*) or as dot plots of IFNγ-stained cells with quadrants set at IL-2 T cells that were stimulated with medium (Figure 7).

Microarray analysis

TCR-transduced T cells were cultured in the presence of IL-2, IL-15, IL-21 or IL-15+IL-21 for 3 days, washed and co-cultured with B16 or B16gp100/A2 cells ($2x10^6$ T cells and $0.5x10^6$ target cells in 2 ml medium) for another 18h without exogenous cytokines. Cellular RNA was extracted by the Rneasy Mini kit following RNase-Free DNase treatment (Qiagen, Valencia, CA). Concentration was measured on a nanodrop ND-100 (NanoDrop Technologies Inc., Wilmington, DE), and quality was checked (RNA integrity number > 7) on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). One µg of RNA was labelled with cyanine 3 (test samples) and cyanine 5 (common reference) by the Low NA input Linear Amplification kit (Agilent). A common reference was generated by labelling 1 µg portions of a pool comprising 1 µg of each test RNA, and mixing the labelled portions afterwards. Mouse Genome 4x44K microarrays were hybridized overnight at 65°C, scanned with a DNA Microarray Scanner, and analyzed with Feature Extraction software 9.5.1 (Agilent). To process data, outlier removed median signals were used in the R 2.5.0 and Bioconductor 2.0 MAANOVA packages. All slides were subjected to a set of quality control checks and, after log2 transformations, data were normalized by a spacial lowess smoothing procedure and analyzed using a two-stage mixed linear model (Kerr et al., 2000; Wolfinger et al., 2001). Analyses were performed using full factorial design with a group-means-parameterized linear model quantifying Array (random), Slide (random) and Batch (random) effects in addition to the reference and the experimental effects. For each analysis the mixed model was re-parameterized with three different contrast matrices to test the hypotheses of interest. For hypothesis testing an Empirical Bayes Fs test was used and its null distribution was estimated from a mixed distribution based on pooling the statistics from a limited number (40) of permutations (Storey and Tibshirani, 2003; Yang and Churchill, 2007).

Statistical analyses

Statistical analyses of T cell phenotype and function were performed with a two-tailored student's t-test for paired samples with statistical significance indicated by $p < 0.05$.

RESULTS

IL-15 decreases the number of TCR transgene-positive T cells, whereas IL-21 increases the expression levels of TCR transgenes per cell

Primary murine T cells were retrovirally transduced with TCRαβ directed against human $gp100_{280-288}$/HLA-A2 (gp100/A2) and subsequently treated either with IL-2, IL-15, IL-21 or the combination of IL-15 and IL-21 (i.e., IL-15+IL-21). Analysis of TCR-Vβ14 transgene expression revealed that differences among T cell cultures were small, yet significant when analyzing large series of TCR transductants (n=18). Percentages of TCR-Vβ14+ T cells following exposure to IL-15 were decreased compared to T cell cultures exposed to IL-2 (Figure. 1A: 46, 35, 44 and 48% TCR-Vβ14+ cells for

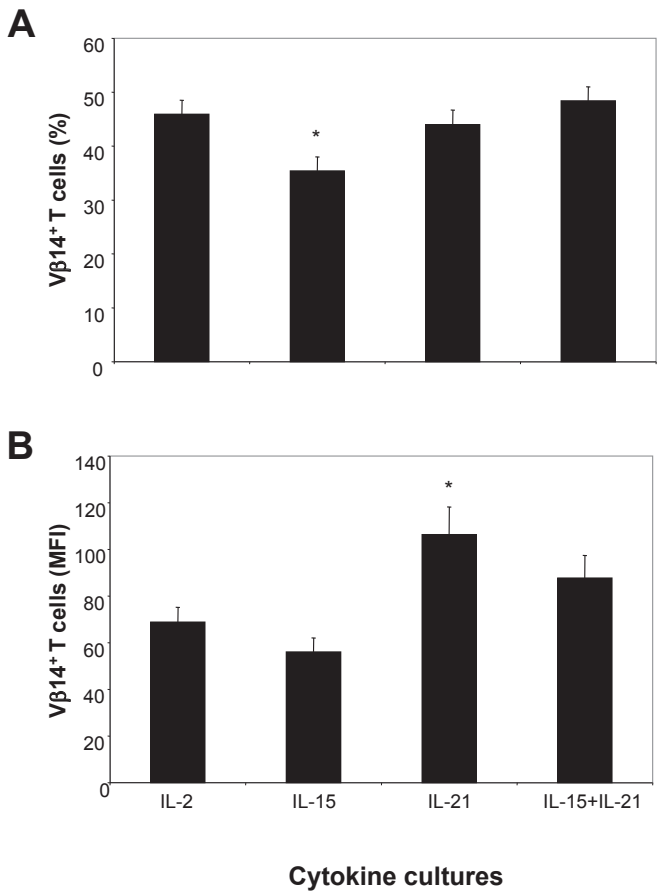

Figure 1. IL-15 decreases the number of TCR transgene-positive T cells, whereas IL-21 increases the expression levels of TCR transgenes per cell

Primary murine T cells were retrovirally transduced with TCRαβ directed against human $gp100_{280-288}$/HLA-A2 (gp100/A2) and subsequently treated either with IL-2 (100 U/ml), IL-15 (50 ng/ml), IL-21 (50 ng/ml) or IL-15+IL-21 (50 ng/ml each). T cells were analyzed by flow cytometry for expression of TCR-Vβ14 within the FSC/SSC lymphocyte gate. Mock-transduced T cells were used as negative controls and showed <5% TCR-Vβ14 staining (data not shown). Transduction efficiencies at day 5 after start of culture (i.e., 3 days after addition of various cytokines) are indicated as percentages (A) or mean fluorescence intensities (MFI) (B) of T cells expressing TCR-Vβ14 (mean±SEM, n=18, * = $p<0.01$ compared to IL-2).

IL-2, IL-15, IL-21 and IL-15+IL-21 T cell cultures, respectively, IL-15 versus IL-2 $p<0.05$). Although percentages of TCR-Vβ14+ T cells in IL-21 T cell cultures did not differ from those in IL-2 T cell cultures, the Mean Fluorescence Intensitiy (MFI) of TCR-Vβ14+ T cells was increased in IL-21 T cell cultures relative to that of IL-2 T cell cultures (Figure 1B: MFI of 69, 56, 106 and 88 for IL-2, IL-15, IL-21 and IL15+IL-21 T cell cultures, respectively, IL-21 versus IL-2 $p<0.05$). Percentages as well as MFI of TCR-Vβ14+ T cells following exposure to IL-15+IL-21 were not significantly different from IL-2 T cell cultures. Mock-transduced T cells consisted of less than 5% TCR-Vβ14+ T cells (data not shown).

T cells treated with a combination of IL-15 and IL-21 show enhanced antigen-specific cytotoxicity

Chromium release assays demonstrated that T cells were able to recognize human $gp100_{280-288}$ peptide-loaded B16 melanoma target cells expressing human HLA-A2 (B16A2), irrespective of the cytokine added to the T cell cultures (Figure 2A). B16gp100/A2 cells, which present endogenously processed human gp100 in the context of HLA-A2 were not, or only to a low extent, killed by T cells that had been cultured in the presence of IL-2 or IL-15 (Figure 2B). However, B16gp100/A2 cells were clearly lysed by IL-21 cultured T cells, an effect that was even more pronounced when T cells were cultured in the presence of IL-15+IL-21 (Figure 2B). Cytotoxic responses towards peptide loaded B16A2 cells and B16gp100/A2 cells were qualitatively similar in that IL-15+IL-21 cultured T cells showed the highest cytotoxicity, IL-2 cultured T cells the lowest cytotoxicity, and IL-15 or IL-21 cultured T cells an intermediate cytotoxicity. Mock-transduced T cells showed no background activity, except for some background activity (up to 20% at E:T> 40:1) of IL-15+IL-21 T cells towards B16gp100/A2 (not towards B16A2 with or without gp100 peptide).

The abilities of T cells to kill tumour cells paralleled gene expression levels of effector molecules. Figure 3 shows that gene expression of granzyme A (*Gzma*, Figure 3A), granzyme B (*Gzmb*, Figure 3B) and perforin 1 (*Prf1*, Figure 3C) were most profoundly increased in IL-15+IL-21 treated T cells following antigen stimulation (4- to 5-fold increase for *Gzma*, *Gzmb* and *Prf1*, $p<0.05$ when compared to IL-2 T cells). Interestingly, IL-15 reduced gene expression of *Gzma*, but not of the other lytic effector molecules (Figure 3A; $p<0.05$ when compared to IL-2 T cells). Gene expression levels of *Gzmb*, but not *Gzma* or *Prf1*, were slightly but significantly up-regulated in T cells cultured with IL-15+IL-21 following stimulation with antigen-negative B16 cells (Figure 3B).

Antigen-specific secretion of IFNγ is enhanced and shows accelerated kinetics upon combined treatment with IL-15 and IL-21

In cytokine release assays, IL-2 and IL-15 T cell cultures showed low levels of IFNγ (Figure 4A), IL-2 (Figure 4B) or IL-10 (Figure 4C) secretion in response to B16gp100/A2 target cells. IL-21 cultured T cells secreted higher levels of IL-2 and IL-10, but not IFNγ, whereas the highest levels of secretion for all three cytokines were observed for IL-15+IL-21 cultured T cells (Figure 4). Secreted levels of IFNγ and IL-2 by IL-15+IL-21 T cells reached statistical significance when compared to IL-2 T cells. IL-10 levels were generally low and not statistically different among the different cytokine cultures. Notably, IL-15+IL-21 cultured T cells secreted 27-fold more IFNγ than IL-2 cultured T cells (Figure 4A: 2400 and 90 pg/ml, respectively), whereas IL-15 or IL-21 alone hardly induced secretion of IFNγ (Figure 4A: 22pg/ml and 143pg/ml, respectively). This observed synergy between IL-15 and IL-21 for IFNγ secretion was also evident, although to a lesser extent, for IL-2 and IL-10 secretion, with a 13-fold increase in both IL-2 and IL-10 secretion for IL-15+IL-21 T cells compared to IL-2 T cells (Figures 4B and C, with IL-15 and IL-21 alone resulting in only a 2- to 5-fold increase in IL-2 or IL-10 secretion). Stimulation with antigen-negative B16 cells resulted in low levels of secreted IFNγ and negligible levels

of secreted IL-2 or IL-10 for IL-15+IL-21 T cells (Figure 4). Mock-transduced T cells showed no cytokine secretion in any condition tested (data not shown).

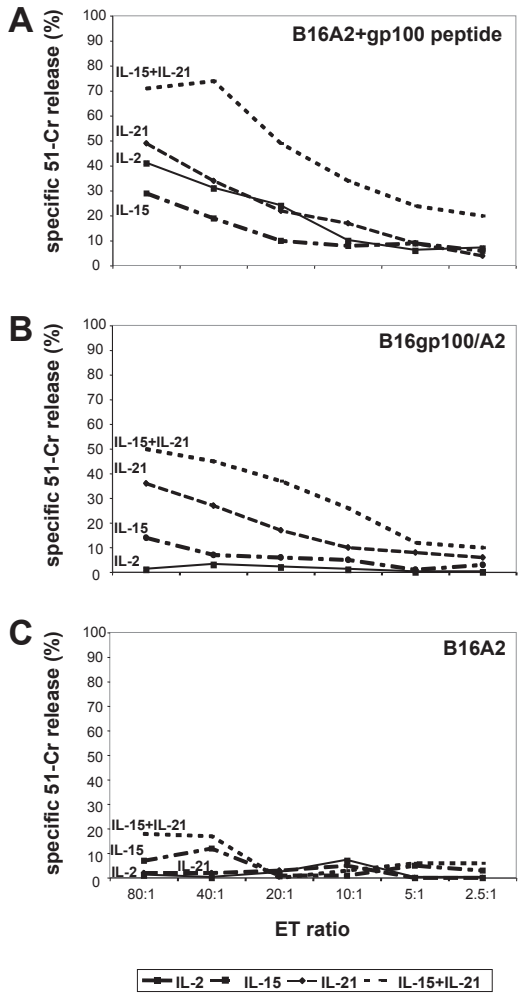

Figure 2. T cells treated with a combination of IL-15 and IL-21 show enhanced antigen-specific cytotoxicity
Murine splenocytes were gp100/A2 TCR-transduced and cultured with IL-2, IL-15, IL-21 or IL-15+IL-21 as described in legend to Figure 1. Cytokine-stimulated T cells were co-cultured for 4h with ^{51}Cr-labeled target cells. Target cells included B16A2 cells loaded with human gp100 peptide (A), B16gp100/A2 cells (B) or B16A2 cells (C). Effector to target cell ratios and percentages of specific ^{51}Cr-release are indicated in the Figures. Mock-transduced T cells served as negative controls. Only in case of mock-transduced T cells cultured with IL-15+IL-21 there was some background activity towards B16gp100/A2 (not towards B16A2 with or without gp100 peptide) up to 20% of ^{51}Cr-release at E:T ratio's of >40:1. At the moment of cytotoxicity assay, 2 weeks after start of culture, TCR-Vβ14 surface expression was around 40% (data not shown). Results are from a representative experiment out of 6 experiments with similar results.

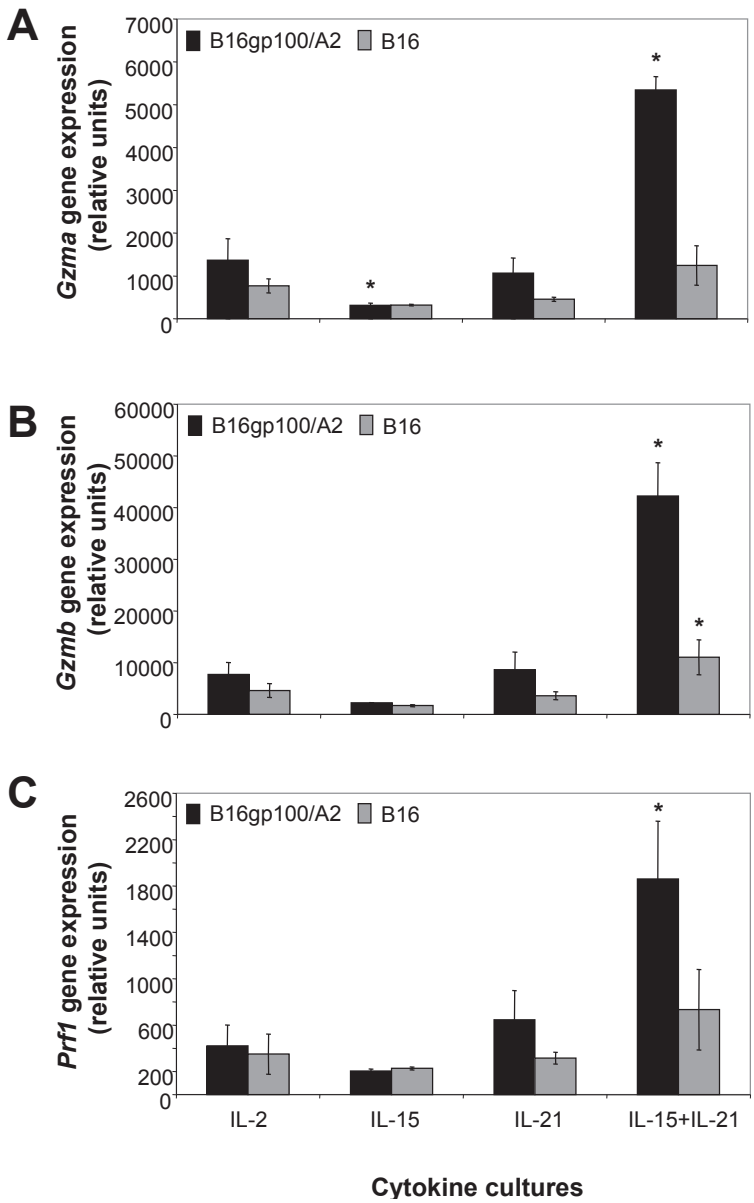

Figure 3. A combination of IL-15 and IL-21 enhances antigen-specific *Gzma*, *Gzmb* and *Prf1* gene expression in T cells

Murine splenocytes were gp100/A2 TCR-transduced, cultured with cytokines as described in legend to Figure 1. Five days after start of culture, T cells were stimulated with B16gp100/A2 or B16 cells for 20 hours. Stimulated T cells were analyzed by microarray for gene expression of Granzyme A (*Gzma*, A), Granzyme B (*Gzmb*, B) or Perforin 1 (*Prf1*, C). See Materials and Methods for details about microarray procedures. Y-axes show relative gene expression for cytokine cultures compared to a common reference. Two independent experiments (starting from separate T cell cultures) gave similar results (mean±SD, * = $p<0.05$ compared to IL-2).

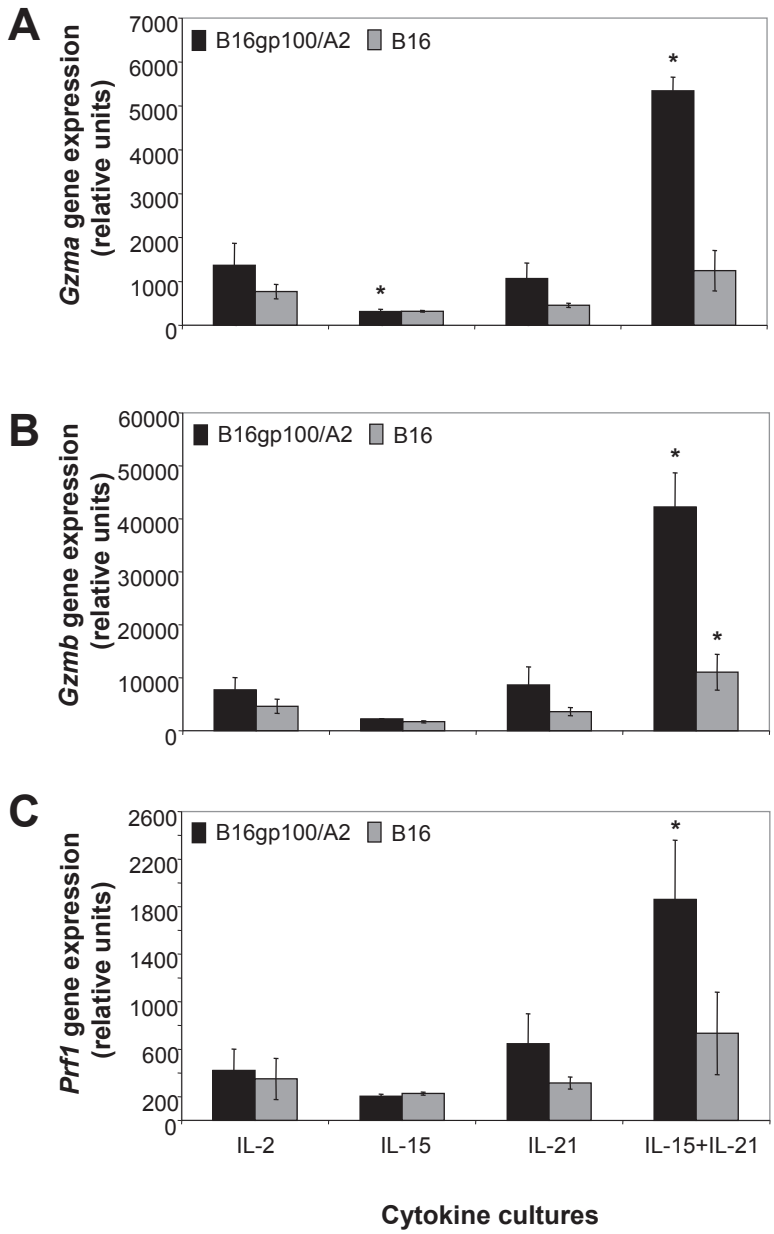

Figure 4. Antigen-specific secretion of IFNγ is enhanced upon combined treatment with IL-15 and IL-21
Murine splenocytes were gp100/A2 TCR-transduced, cultured with cytokines and stimulated with B16 target cells as described in legend to Figure 3. Stimulated T cells were analyzed by commercial ELISA for the secretion of IFNγ (A), IL-2 (B) and IL-10 (C). During the stimulation assay, no exogenous cytokines were added to the T cell:target cell co-cultures. T cells cultured with medium or ConA served as negative and positive controls, respectively. Mock-transduced T cells showed no cytokine secretion in any condition tested (data not shown). The different T cell cultures and the levels of cytokines that were present in supernatants are indicated on the X- and Y-axis, respectively (mean±SEM, n=4 (IL-2 and IL-10) or n=10 (IFNγ), * = $p<0.05$ and ** = $p<0.005$ compared to IL-2).

Next, we investigated the kinetics of antigen-specific cytokine secretion, and observed that IL-15+IL-21 cultured T cells secreted IFNγ at enhanced levels already at 4h after stimulation (Figure 5A, $p<0.05$ when compared to IL-2 T cells). Kinetics of IL-2 secretion by IL-15+IL-21 T cells were similar but somewhat delayed when compared to IFNγ secretion (Figures 5A and B: 4% of maximum IL-2 level as measured at t=20h was secreted after 4h compared to 23% for IFNγ), whereas kinetics of IL-10 secretion were accelerated when compared to IFNγ or IL-2 (Figure 5C: 78% of maximum IL-10 level was secreted after 4h of stimulation).

The combination of IL-15 and IL-21 enhances antigen-specific *Ifng* gene expression in T cells

Studying mRNA expression, we observed that IL-2 and IL-15 cultured T cells show low levels of *Ifng* gene expression in response to B16gp100/A2 target cell stimulation (Figure 6A). IL-21 cultured T cells expressed significantly higher levels of *Ifng* (Figure 6A: 2031 versus 650 relative gene expression units for IL-21 and IL-2 cultured T cells, respectively, $p<0.05$). In line with IFNγ protein levels, highest *Ifng* gene expression levels were observed for IL-15+IL-21 T cells (Figure 6A: 6461 relative gene expression units, $p<0.05$ when compared to IL-2). No differences were found in *Il2* and *Il10* gene expression levels for T cells cultured in the presence of different common-γ cytokines (Figures 6B and C).

Effector memory T cells are the major source of enhanced antigen-specific IFNγ protein production upon combined treatment with IL-15 and IL-21

To determine the cell type responsible for IFNγ production, especially relevant in the context of IL-15 and IL-21's reported inhibitory actions on T cell differentiation, we performed intracellular cytokine stainings in combination with stainings for T cell differentiation markers. The number of IFNγ+ T cells after stimulation with B16gp100/A2 target cells was enhanced in IL-21 T cell cultures, although not to a statistically significant extent, and highest in IL-15+IL-21 T cell cultures, which corroborated the secretion data (*Supplementary Figure S1A*: $p<0.005$ when compared to IL-2 T cell cultures). See Figure 7A for a representative dotplot example. No significant differences could be found with respect to IL-2 or IL-10+ T cells after stimulation with B16gp100/A2 target cells (*Supplementary Figures S1B and C*). CD62L/CD44/IFNγ triple stainings revealed that CD62L-/CD44+ effector memory T cells were the major source of antigen-induced IFNγ (Figure 7B). Antigen-negative B16 stimulation resulted in low numbers of intracellular cytokine-positive T cells (*Supplementary Figure S1*), and mock-transduced T cells showed no cytokine production in any condition tested (data not shown).

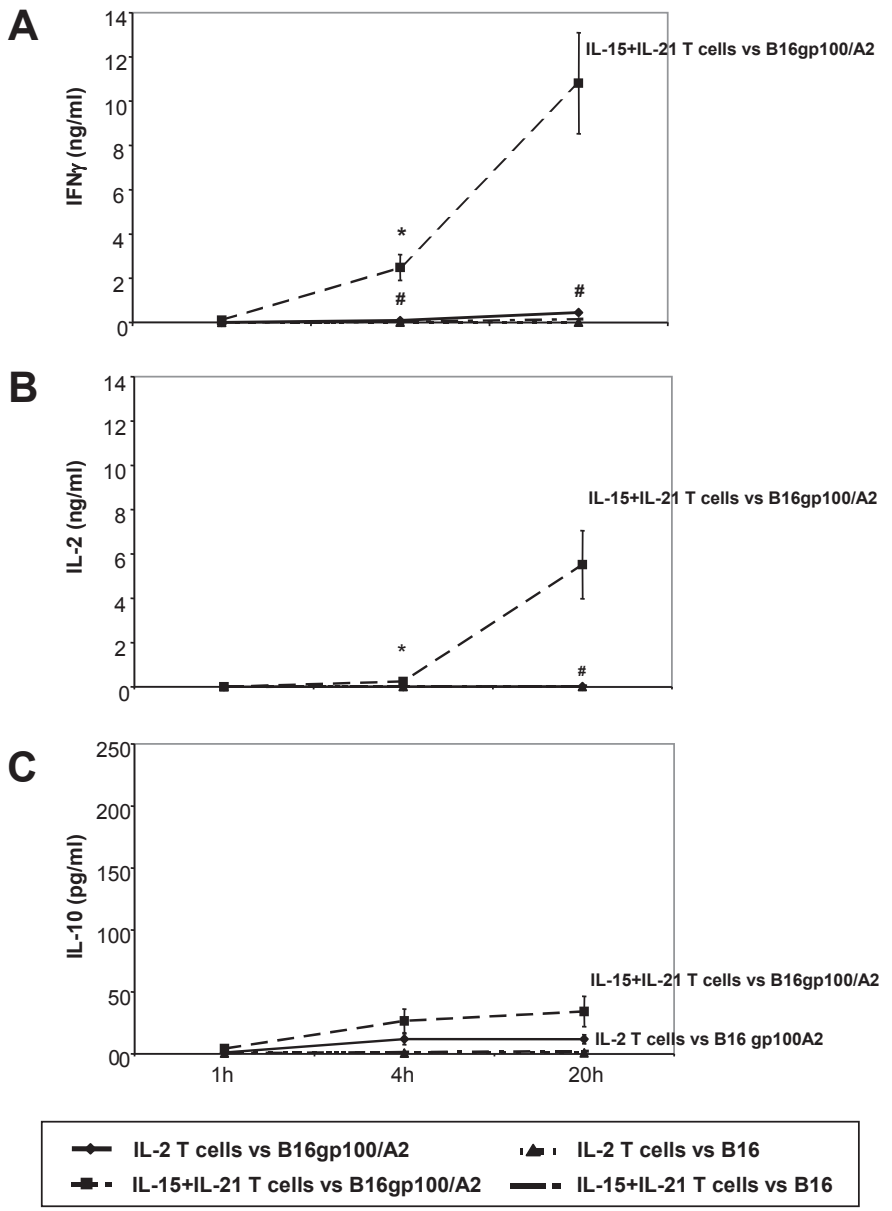

Figure 5. Antigen-specific secretion of IFNγ and IL-2, but not IL-10, shows accelerated kinetics upon combined treatment with IL-15 and IL-21

Murine splenocytes were gp100/A2 TCR-transduced and cultured with cytokines as described in legend to Figure 1. Cytokine cultured T cells were stimulated with B16gp100/A2 and B16 cells for 1, 4 or 20h, and analyzed by commercial ELISA for the secretion of IFNγ (A), IL-2 (B) or IL-10 (C). Mock-transduced T cells showed no cytokine secretion at any time-point tested (data not shown). Time points following target cell stimulations are indicated at the X-axes. Absolute levels of cytokines present in supernatants are indicated at the Y-axes (mean±SEM, n=3, * and # = $p<0.05$ compared to IL-2 for B16gp100/A2 and B16, respectively).

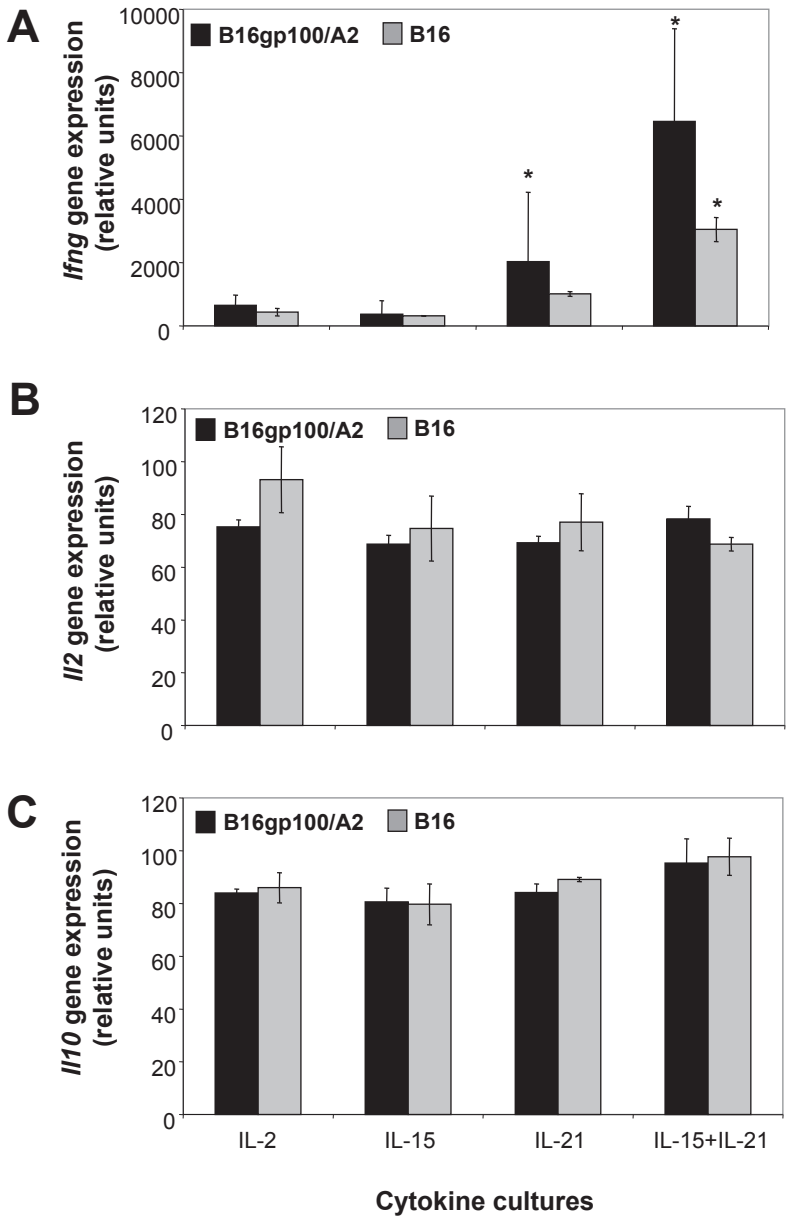

Figure 6. The combination of IL-15 and IL-21 enhances antigen-specific *Ifng* gene expression in T cells
Murine splenocytes were gp100/A2 TCR-transduced, cultured with cytokines as described in legend to Figure 1. Five days after start of culture, T cells were stimulated with B16gp100/A2 or B16 cells for 20 hours. Stimulated T cells were analyzed by microarray for gene expression of *Ifng* (A), *Il2* (B) or *Il10* (C). See Materials and Methods for details about microarray procedures. Y-axes show relative gene expression for cytokine cultures compared to a common reference. Two independent experiments (starting from separate T cell cultures) gave similar results (mean±SD, * = $p<0.05$ compared to IL-2).

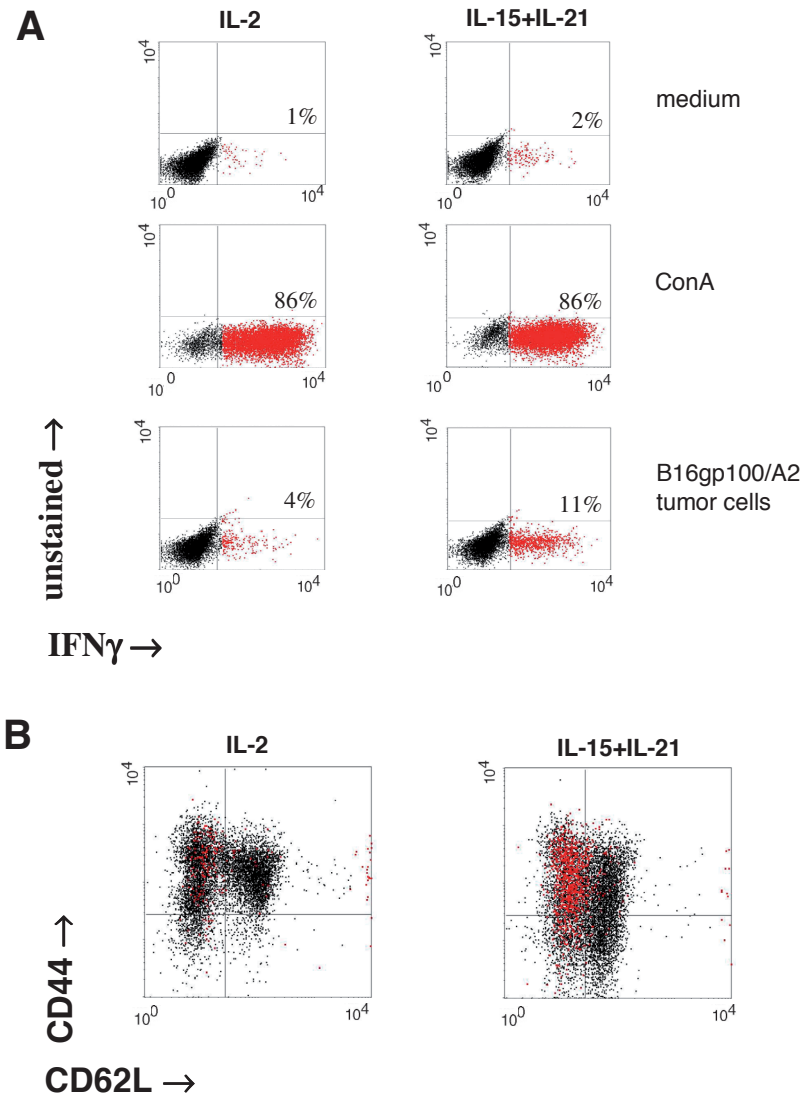

Figure 7. IL-15+IL-21-induced IFNγ production is restricted to CD62L+/CD44- effector memory T cells
Murine splenocytes were gp100/A2 TCR-transduced and cultured with cytokines as described in legend to Figure 1. Five days after start of culture, T cells were stimulated with medium, ConA (10 ng/ml) or B16gp100/A2 cells, and analyzed by flow cytometry for the expression of IFNγ (A). In (B) IFNγ staining was combined with the T cell differentiation markers CD62L and CD44. In the latter case, T cells were first stained with anti-CD62L mAb^{FITC} and anti-CD44 mAb^{APC}, followed by intracellular staining with anti-IFNγ mAb^{PE}. Cells were FSC/SSC gated on viable lymphocytes and percentages of IFNγ^{+} T cells (in red) were determined. Shown are the results for IL-2 and IL-15+IL-21 cultures. Results are from a representative experiment out of 3 independent experiments with similar results.

DISCUSSION

In the present paper we studied molecular mechanisms behind tumour-specific responses of primary murine T cells treated with IL-2, IL-15, IL-21 or a combination of IL-15 and IL-21 in the setting of TCR gene transfer. Combined treatment with IL-15 and IL-21 of primary TCR-engineered T cells resulted in enhanced tumour-specific responsiveness at the following levels. First, cytotoxicity and the expression of lytic effector molecules were increased. Second, levels of secreted IL-2 and IFNγ were enhanced, likely regulated at the post-transcriptional and transcriptional level, respectively. Intriguingly, IL-15 and IL-21 inhibit T cell differentiation, yet they acted most profoundly on IFNγ production by effector memory T cells.

Analysis of TCR transgene expression in TCR-engineered T cells revealed that differences upon short-term culture with various cytokines were small, yet IL-15 decreased percentages and IL-21 increased MFIs of TCR transgene-positive T cells (Figure 1). Currently, we cannot rule out whether IL-15 and IL-21 as single cytokines act directly on TCR surface expression or indirectly via their effects on T cell growth (IL-15 enhances T cell growth, whereas IL-21 does not support T cell growth when compared to IL-2, data not shown). Importantly, IL-15+IL-21, a combination of cytokines with significant consequences for TCR-mediated functions (see below), did not alter transduction efficiencies. This indicates that the effects of IL-15+IL-21 on T cell function must be related to T cell properties rather than TCR surface expression levels. Moreover, IL-15+IL-21 results in similar T cell numbers at the day of phenotypical and functional analyses when compared to IL-2 (data not shown). It is of interest to note that the potentially beneficial effect of IL-21 on T cell differentiation (Hinrichs et al., 2008), in particular the enrichment of T cells with a CD62L+/CD44- naive T cell phenotype, is independent of prior exposure to IL-2 (inherent to our transduction protocol) and genetic introduction of TCR transgenes (*Supplementary Figure S2*). The same holds true for IL-15+IL-21 treatment of T cells, which results in an enrichment of CD62L+/CD44- T cells, although to a lesser extent when compared to IL-21, that is not compromised following TCR gene transfer (Figure 7B, and described in more detail in Pouw et al., manuscript submitted).

T cells cultured in the presence of IL-21, but not IL-2 or IL-15, clearly lysed hgp100/HLA-A2-expressing B16 melanoma cells (Figure 2). Antigen-specific cytotoxicity observed for IL-21 T cell cultures is in line with findings by Casey and colleagues showing that IL-21, in contrast to IL-2, enhances T cell cytotoxicity (Casey and Mescher, 2007). In addition, we noted that IL-21 up-regulated perforin 1 gene expression (Figure 3C: $p<0.05$ B16gp100/A2 when compared to B16), which is in agreement with a report by Ebert and colleagues demonstrating up-regulated perforin-mediated cytotoxic activity of human intra-epithelial lymphocytes after exposure to IL-21 (Ebert, 2009). The enhanced cytotoxicity was even more pronounced when T cells were cultured in the presence of IL-15+IL-21 (Figure 2). Enhanced cytotoxic killing of IL-15+IL-21 T cells coincided with significantly increased gene expression of the effector molecules granzyme A, granzyme B and perforin 1 (Figure 3). Both IL-15 and IL-21 have

been shown to up-regulate intracellular granzyme B and perforin expression (Alves et al., 2003; Ebert, 2009; Frederiksen et al., 2008; Hinrichs et al., 2008; White et al., 2007). Zeng and colleagues (Zeng et al., 2005) demonstrated that granzyme B gene expression was higher in freshly isolated human T cells cultured in IL-15 plus IL-21, than in T cells cultured with either cytokine alone. To our knowledge, our report is the first to demonstrate up-regulated gene expression of three cytotoxic molecules (granzyme A, granzyme B and perforin 1) after antigen-specific stimulation of T cells cultured in IL-15 plus IL-21.

In addition, T cells cultured in the presence of both IL-15 and IL-21 showed the highest levels and accelerated kinetics of secreted IFNγ in response to B16gp100/A2 melanoma cells (Figures 4A and 5A). The effect of IL-15+IL-21 on IFNγ secretion is of a synergistic nature and is most likely explained by enhanced *Ifng* gene expression (Figure 6A). Interestingly, T cells cultured in the presence of IL-21 only, also showed increased *Ifng* gene expression, yet the gene expression level appeared to have been insufficient to result in significantly enhanced IFNγ protein production or release (*Supplementary Figure S1A* and Figure 4A, respectively). Since IL-15 and IL-21 affect T cell differentiation, we studied the effect of IL-15+IL-21 on IFNγ production via triple flow cytometry stainings, and identified CD62L-/CD44+ effector memory T cells as the major source for IFNγ (Figure 7B). Currently, we cannot exclude the contribution of NK and NKT cells towards the observed IFNγ production. In fact, IL-15 increased percentages of NK1.1 cells and CD8 T cells and decreased those of CD4 T and CD19 B cells, whereas IL-21 minimally but significantly decreased percentages of CD3 T cells (*Supplementary Figure S3*, and in agreement with (Geginat et al., 2001; Gill et al., 2009; Nakajima et al., 2008; Schluns and Lefrancois, 2003; Tan et al., 2002; Zhang et al., 1998)). The IL-15-induced increase in NK1.1+ cells was found in both the CD3- and CD3+ subsets, indicating an increase in both NK and NKT cells. Notably, both these cell types express CD44, and IL-15 has been shown to up-regulate expression and activation of CD44 on NK cells, which in turn regulates the expression of IFNγ (Sague et al., 2004).

With respect to IL-2 and IL-10 production, the IL-21-induced IL-2 secretion that we observed is in line with previous reports related to IL-21's suppressive effect on T cell differentiation (Gattinoni et al., 2005b; Hinrichs et al., 2008; Li et al., 2005a). IL-15+IL-21 also increased levels and accelerated kinetics of T cell secreted IL-2, but not IL-10, following tumour cell stimulation (Figures 4 and 5). Enhanced antigen-specific IL-2 secretion did not correlate with increased levels of IL-2 mRNA and cytosolic protein, suggesting that enhanced secretion was not caused by enhanced gene transcription. Antigen-specific IL-10 gene expression, intracellular protein and secretion were not differentially regulated in the different cytokine cultures (Figures 4, 5 and 6).

IL-15 and IL-21, when combined, resulted in predominantly CD8 T cells (*Supplementary Figure S3*) with potent T cell effector functions. The cooperative effects of IL-15 and IL-21 with respect to cytotoxicity, IFNγ and IL-2 secretion may be explained as follows. First and with respect to cytokine production, activation of distinct downstream mediators of IL-15 and IL-

21 may amplify production of IL-2 and IFNγ. In example, IL-21 signals predominantly through Jak3, STAT1 and STAT3, whereas IL-15 signals predominantly through STAT5 and ERK1/2 (de Totero et al., 2008). In fact, regulatory sites in the IFNγ promoter are responsive to both STAT- and ERK-dependent transcription factors (Egerton et al., 1998; Strengell et al., 2003). Second and with respect to tumour cell killing, expression of perforin in memory CD8+ T cells may be induced by IL-21, whereas degranulation of CD8+ T cells and release of perforin is triggered by IL-15 (Figure 3C, and reference (White et al., 2007)).

We postulate that enhanced IL-2 production of T cells cultured with IL-15+IL-21 may support *in vivo* T cell expansion, and that enhanced IFNγ production and cytotoxicity may contribute to *in vivo* anti-melanoma activities. This would provide a molecular basis to explain the findings by Zeng and colleagues, who reported enhanced CD8+ T cell expansion and anti-melanoma responses upon *in vivo* administration of both IL-15 and IL-21 (Zeng et al., 2005). Although differences between murine and human IL-15 and IL-21 cannot be excluded, we argue that our findings may be well translated to the human setting. First, the combination of IL-15 and IL-21 has been reported to improve the proliferation of human effector T cells (Huarte et al., 2009), which may be of importance to adoptive T cell therapy trials. In fact, this combination of cytokines may promote proliferation of human T cells better than murine T cells since we observed no beneficial effect of IL-15+IL-21 on murine T cell growth when compared to IL-2 (data not shown). Second, IL-15 combined with IL-21 reverses IL-15-induced down-regulation of CD28 and results in enhanced IL-2 and IFNγ production upon TCR and CD28 triggering (Marks-Konczalik et al., 2000). Moreover, a combination of IL-15 and IL-21 has recently been shown to enhance cytotoxicity and IFNγ production by PBMC of melanoma patients (Huarte et al., 2009), which is in strict accordance with our findings using mouse TCR-engineered T cells.

Taken together, we conclude that a combination of IL-15 and IL-21 provides T cells with two distinct properties, both having a potential advantage in clinical T cell therapy. On the one hand, the combination of IL-15 and IL-21 results in enrichment of less differentiated T cells (Gattinoni et al., 2005b; Hinrichs et al., 2008), which may improve persistence and anti-tumour activities of adoptively transferred T cells (Ciceri et al., 2007; Huang et al., 2005; Lamers et al., 2006a; Robbins et al., 2004). And on the other hand, this combination of cytokines results in functional potentiation of effector T cells, which enables T cells to directly act against a tumour. Our findings argue for the combined *ex vivo* treatment of TCR-engineered T cells prior to adoptive therapy with IL-15 and IL-21.

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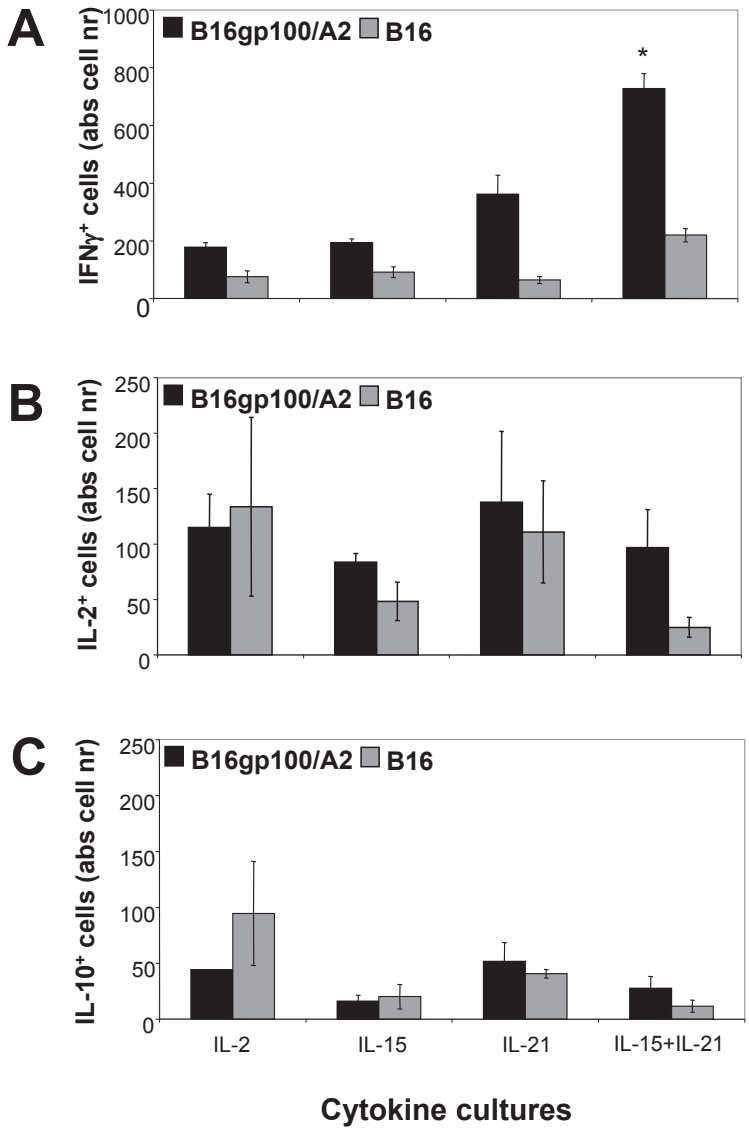
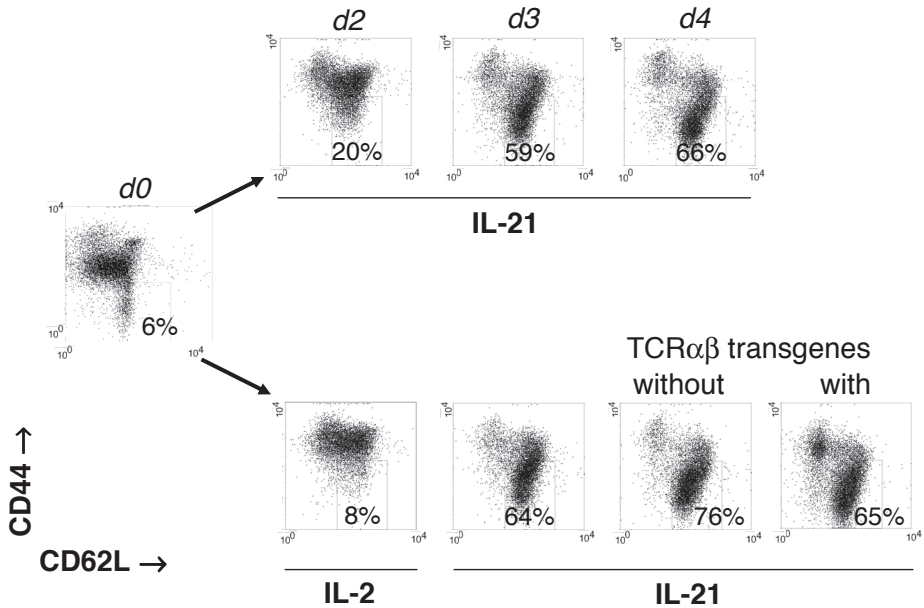
SUPPLEMENTARY FIGURES



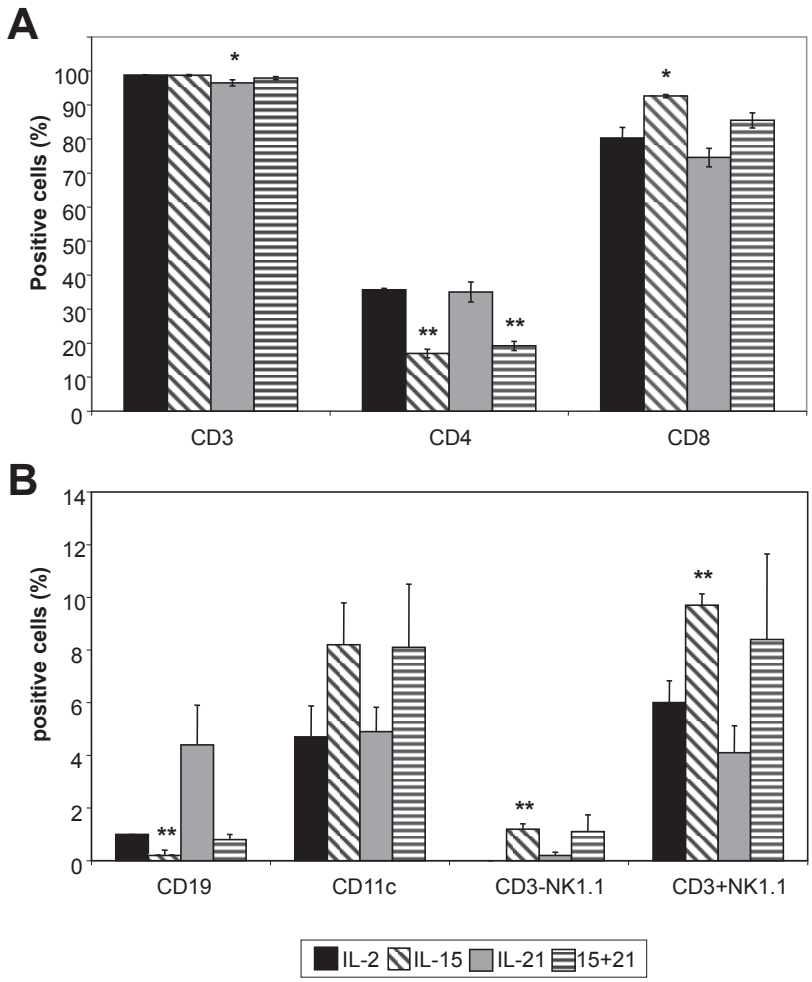
Supplementary Figure S1. A combination of IL-15 and IL-21 enhances antigen-specific production of T cell IFNγ

Murine splenocytes were gp100/A2 TCR-transduced, cultured with cytokines and stimulated with B16 target cells as described in legend to Figure 2. Stimulated T cells were analyzed by flow cytometry for the presence of intracellular IFNγ (A), IL-2 (B) or IL-10 (C). Intracellular cytokine stainings and analyses were performed as described in Materials and Methods. Mock-transduced T cells showed no cytokine production in any condition tested (data not shown). Absolute numbers of IFNγ+, IL-2+ or IL-10+ T cells are indicated at the Y-axis (mean±SEM, n=3, * = $p<0.05$ compared to IL-2).



Supplementary Figure S2. IL-21 induces a CD62L+/CD44- T cell phenotype independent of prior exposure to IL-2 and TCR gene transfer

Freshly isolated primary murine splenocytes were activated with ConA in the presence of either IL-2 (100 U/ml) or IL-21 (50ng/ml) with or without a 2-day IL-2 pre-treatment. Cytokines were refreshed every 2-3 days. Expression levels of CD62L and CD44 of viable T lymphocytes are shown on days 0, 2, 3 and 4 after start of culture. The results are from a representative experiment out of 3 independent experiments with similar results. A gene transfer control is shown at day 4 after start of culture. Freshly isolated splenocytes expressed on average 12% CD62L+/CD44- T cells (mice approximately 30 weeks of age). In addition, prior exposure to IL-2 and TCR gene transfer did not affect IL-21's ability to up-regulate expression of CCR7 (data not shown).



Supplementary Figure S3. IL-21 slightly decreases the percentages of total T cells, and IL-15 decreases the percentages of CD4 T and B cells yet increases the percentages of CD8 T cells, NK and NKT cells

Murine splenocytes were gp100/A2 TCR-transduced and cultured with IL-2, IL-15, IL-21 or IL-15+IL-21 as described in legend to Figure 1. Three days after addition of cytokines, T cells were analysed by flow cytometry for the expression of leucocyte and lymphocyte subset markers. T cell markers included: CD3, CD4 and CD8 (A) and B, DC, NK and NKT cell markers included: CD19, CD11c and NK1.1 (with or without CD3) (B). Percentages of positive cells are indicated at the Y-axis (mean±SEM, n=5, * = $p<0.05$ and ** = $p<0.005$ compared to IL-2).

CHAPTER 6

General Discussion

This chapter presents and discusses an overview of *in vitro* conditions advocated by the findings in this thesis to provide highly functionally avid yet not fully differentiated primary murine T cells following TCR gene transfer, conditions which may be of potential interest to clinical adoptive T cell therapy. Section 6.1 covers methods for transfer of TCR genes into primary murine T cells and different T cell activation stimuli prior to retroviral TCR gene transfer, whereas section 6.2 covers the use and effects of common-γ cytokines in short-term cultures of primary and TCR-transduced murine T cells. Finally, in section 6.3, I present and discuss future directions for clinical TCR gene therapy trials.

6.1 GENE TRANSFER METHODS

In chapters 2 and 3 of this thesis we have investigated the most optimal protocol for TCR gene transfer into primary murine T cells. Our findings can be summarized as follows (see Conclusions I).

CONCLUSIONS I: TCR gene transfer into primary murine T cells

	Major findings
Chapter 2	• A combination of amphotropic and ecotropic retroviral particles results in high TCR gene transfer into primary murine T cells • Transduction efficiency is improved by reducing T cell numbers during transduction • Transduction efficiencies are >90% for GFP and about 70% for both a murine and a human (i.e. murinized) TCR using the presented protocol • TCR-transduced T cells show antigen-specific function even after 2 weeks of culture (when approximately 14% of T cells is TCR+)
Chapter 3	• Activation of primary murine T cells with polystyrene CD3/CD28 results in most efficient TCR gene transfer • Activation with ConA results in similar TCR gene transduction efficiencies as activation with soluble or latex CD3/CD28; all less efficient than T cell activation with polystyrene CD3/CD28 • T cell numbers decline when cultured with IL-15+IL-21 when compared to IL-2 • Expansion in IL-15+IL-21 results in less-differentiated T cells, especially when combined with T cell activation with ConA or soluble CD3/CD28 • Activation of T cells with polystyrene CD3/CD28 results in antigen non-specific T cell functions

Several methods exist to gene modify T cells with the purpose to change their ability to recognize antigen. In our laboratory we have chosen to gene-modify T cells via retroviral gene transfer for several reasons. First, retroviral vectors transmit genes to recipient cells in a stable manner. Although retroviral oncogenesis may occur and has led to the development of leukemia in case of gene transfer into hematopoietic stem cells, no indications for transgene integration-dependent malignant transformation have been observed in case of gene transfer into differentiated T cells. Other advantages of oncoretroviral vectors include the ease to manipulate the retroviral genome, which is a well-characterized small genome, and the absence of viral replication in target cells due to the use of gene-engineered and

replication-deficient viruses (Engels and Uckert, 2007). A recent development in TCRαβ gene transfer through retroviral vectors has been the use of 2A peptide sequences that enable the introduction of the TCRα and TCRβ genes in a single vector (Leisegang et al., 2008). The 2A sequence is derived from a picorna virus, and is believed to result in equimolar expression of the TCRα and TCRβ chains (de Felipe et al., 1999; Klump et al., 2001; Szymczak et al., 2004). In addition, the 2A sequence, when compared to the IRES element which is approximately 500 bp longer, yields a smaller TCR genomic RNA that is expected to be more efficiently packaged into viral particles resulting in higher vector titres. An anticipated drawback of this system could be the immunogenicity of viral 2A sequences, either by residual 2A amino acids left at the N- and C-terminal proteins (Szymczak et al., 2004; Uckert and Schumacher, 2009) or parts of the virus-derived sequences. However, results obtained from different prediction analyses of epitope binding and proteasomal cleavage showed a very low probability for the presentation of P2A-derived epitopes in both mouse (H-2D^{b}) and human (HLA-A2.1) MHC molecules (Leisegang et al., 2008). In addition, adoptive transfer of T cells transduced with β-P2A-α TCR P14 retroviruses into immunocompetent C57BL/6 mice did neither affect the peripheral persistence nor the anti-tumour activity of the transferred β-P2A-α TCR-transduced T cells (Weinhold et al., 2007). Universal use of 2A sequences appears promising but needs further assessment in other systems.

Technically, retroviral particles can be generated by transient transfection of three plasmids that encode either (1) TCR transgenes, (2) retroviral structural proteins and enzymes (*gag-pol*), and (3) envelope (*env*) proteins. Alternatively, viral particles can be obtained from stable packaging cell lines that are transduced with the TCR genes. The generation of stable producer cell clones ensures a continuous source of vector particles and facilitates extensive quality control. In addition, the genetic use of a selectable marker may enhance the identification and generation of vector-producing clones. However, the following reasons may argue against the making and use of stable vector-producing clones. First, the generation and characterization of multiple clones is laborious and time consuming. Second, permanent high titre expression of the transgene has been reported to adversely affect producer cell line growth (Pear et al., 1993). Third, the use of selection markers may enhance potential immunogenicity (Bonini et al., 1997; Verzeletti et al., 1998). Finally, testing of different transgenes in a preclinical setting would require the generation of multiple stable producer cell lines. Findings presented in this thesis would advocate transient transfection, which enables quick and easy evaluation of multiple TCR transgenes transferred into primary T cells. Therefore, we chose to use transient transduction for TCR gene transfer, and in **chapter 2** we explored the conditions most suitable for transient retroviral transduction of primary murine T lymphocytes.

To our knowledge our study is the first to use a TCR directed against a human tumour antigen to extensively compare *in vitro* conditions essential to the success of retroviral gene transduction into primary T cells, thereby facilitating preclinical assessment of TCR specificities that can potentially be used in clinical adoptive T cell therapy. Generally, systematic investigations into optimal conditions for retroviral TCR gene transfer into primary T cells were scarce (Hagani et al., 1999; Zhang et al., 2003). Some earlier studies generally reported low TCR gene transduction efficiencies and poor *in vitro* survival of transduced primary murine T cells (Abad et al., 2008; Hagani et al., 1999; Kessels et al., 2001; Kolen et al., 2002, and personal communication). Reported protocols that did result in high TCR gene expression include complicated experimental settings with or without enrichment of TCR-engineered T cells following gene transduction (Chamoto et al., 2004; Zhang et al., 2004). In chapter 2, we observed that (1) about 70 % of T cells expressed the TCR-Vβ transgene at day 5 following gene transfer, (2) T cells could be maintained for at least 2 weeks after gene transfer, (3) at which time they displayed functions directed by TCR transgenes (when only 14% of T cells expressed the introduced TCR-Vβ). In other words, levels of TCR transgene surface expression as a result of our transient transduction system allow immediate monitoring of T cell function without the need to enrich TCR-transduced T cells. The transient transduction protocol provided the versatility to test different transgenes, including anti-human melanoma-specific TCRs. Notably, experiments showed that TCR gene transfer into primary murine T cells was critically improved by the use of retroviruses pseudotyped with both MLV-A and MLV-E envelopes. The combination of two packaging cells is an extension of the 'ping-pong' system, which was described earlier for the transduction of human T cells (Eshhar et al., 2001). Previously, it was considered that murine T cells were transduced most efficiently by MLV-E pseudotyped retroviral particles (Engels et al., 2003; Hagani et al., 1999) and, although not the best viral system per se, it is this viral system that is recognized in reviews (Uckert and Schumacher, 2009). Additional observations made in chapter 2 include improvement of TCR gene transfer into primary murine T cells by the use of low T cell densities during transduction. Lower T cell densities result in higher ratios of number of virus particles per cell and as such may directly improve retroviral transduction. However, one cannot exclude that lower T cell densities facilitate cellular division and growth and thereby enhance the process of viral integration.

In light of clinical TCR gene therapy trials, where anti-CD3 (+/-anti-CD28) mAb stimulation is regularly used (Kershaw et al., 2006; Lamers et al., 2006a; Morgan et al., 2006), we have extensively tested T cell activation conditions in chapters 2 and 3. In chapter 2, we first demonstrated that T cell activation prior to TCR gene transduction was equally efficient when using a TCR-dependent (soluble anti-CD3/CD28 mAbs) stimulus or a TCR-independent stimulus (Concanavalin A, Con A). In **chapter 3**, we further tested T cell activation with ConA or anti-CD3/CD28 mAbs, including multiple formats of anti-CD3 and CD28 mAbs such as soluble mAbs or mAbs immobilized to polystyrene or latex beads (abbreviated as

soluble, latex or polystyrene CD3/CD28, respectively), with respect to *in vitro* phenotype and function of TCR-engineered T cells. Activation with ConA, soluble CD3/CD28 or latex CD3/CD28 resulted in TCR expression levels and T cell functions which were quite similar to each other. In contrast, T cells activated with polystyrene CD3/CD28 showed significantly higher TCR expression levels and anti-tumour T cell functions. The underlying mechanism for this observation, at least to some extent, remains elusive. Although formally we cannot exclude the contribution of different epitope specificities and/or ligand-binding affinities of the anti-CD3 and CD28 mAbs used in the different formats, we postulate that data presented in chapter 3 argue that strong T cell activation with beads, especially polystyrene beads, results in high-avidity TCR-engineered T cells. Subsequent important observations made with T cells activated with polystyrene CD3/CD28 include a phenotype that predominantly pointed to effector memory T cells, and T cell functions that appeared to be non-antigen specific. This was more prominent for cytotoxicity than for IFNγ production after target cell stimulation (see Table III, chapter 5).

Currently, the most appropriate definition of CD8 T cell phenotype for adoptive T cell therapy is under debate. Historically, T cells with strong *in vitro* tumour cell killing capacities were used, but Gattinoni and colleagues demonstrated that acquisition of full effector function *in vitro* impairs the *in vivo* anti-tumour efficacy of adoptively transferred CD8+ T cells (Gattinoni et al., 2005b). Interestingly, already in 1995 Evans and colleagues (Evans et al., 1995) showed that the therapeutic efficacy of freshly isolated murine anti-tumour T cells was superior to that of T cells expanded *in vitro*, but it took another decade to re-awaken the interest of the scientific community in the relationship between T cell differentiation state and ultimate *in vivo* (anti-tumour) functionality.

6.2 CYTOKINES IN ADOPTIVE T CELL THERAPY

In chapter 3 we have investigated TCR-redirected T cells that were expanded in the presence of IL-15+IL-21 versus IL-2 with respect to TCR transgene expression, T cell differentiation and T cell functions. In **chapters 4 and 5** we have extended these investigations and focussed on mechanisms behind the observed effects of IL-2, IL-15, IL-21 and IL-15+IL-21 on T cell differentiation and T cell function, respectively. Our main findings can be summarized as follows (see Conclusions II).

When analyzing our work we came across quite some discrepancies with findings by others. For example, previous studies using a combination of IL-2 and IL-21 for the expansion of anti-tumour T cells showed a similar up-regulation of CD62L as when IL-21 was used as single cytokine (Hinrichs et al., 2008). In our studies, however, we observed a significant difference

CONCLUSIONS II: Cytokine treatment of T cells for TCR gene therapy

	Major findings
Chapter 4	• Short-term exposure of primary T cells to IL-21, and to a lesser extent IL-21+IL-15, results in a significant enrichment of $CD62L^{hi}$, $CD44^{lo}$, $CD27^{int}$, $CCR7^{hi}$ naïve T cells • IL-21 reduces both the proliferation and apoptosis of naive T cells • IL-21 negatively affects T cell expansion but, when combined with IL-15, maintains T cell numbers for up to 2 weeks • IL-21 down-regulates expression of IL-2 receptor components, which is reversed by IL-15, and up-regulated the expression of IL-21R • Enrichment of $CD44^{lo}$ cells by IL-21 is at least partly caused by a phenotypic reversal of $CD44^{+}$ effector memory T cells into $CD44^{-}$ T cells • The combination of IL-15 and IL-21 significantly up-regulates the expression of genes related to T cell effector functions such as perforine, granzymes and IFNγ
Chapter 5	• IL-15, but not IL-21, significantly decreases surface expression of a genetically introduced TCR • IL-15+IL-21, and to a lesser extent IL-21, enhances antigen-specific T cell cytotoxicity, which is related to enhanced gene expression of granzymes A and B, and perforin 1 • IL-15+IL-21 synergistically enhances release levels and kinetics of T cell IFNγ and IL-2, but not IL-10 • Enhanced secretion of IFNγ by IL15+IL-21 is accompanied by increased gene expression and cytosolic protein content • IL-15+IL-21 induced IFNγ secretion is restricted to effector memory T cells

between IL-21 or IL-2+IL-21 cultures with respect to CD62L expression levels (measured by flow cytometry, see Figure 1 of chapter 4). Second, the observed lack of T cell expansion in IL-21 cultures in our studies is in contrast to reports on anti-CD3 mAb or antigen-stimulated mouse T cells in which IL-21 promoted T cell expansion (Hinrichs et al., 2008; Parrish-Novak et al., 2000). Third, targets of Wnt signalling such as *lef1* were previously reported to have an up-regulated expression upon exposure to antigen plus IL-21 (Hinrichs et al., 2008), but showed no differential expression upon exposure to IL-21 in our study. Finally, Huarte and colleagues found a significant reduction in FOXP3+ regulatory T cells (Treg's) in IL-15+IL-21 T cell cultures (Huarte et al., 2009), whereas our studies did not show any difference in foxp3 (gene) expression in any of the cytokine cultures tested (IL-2, IL-15, IL-21, and combinations thereof; data not shown). The observed discrepancies between our studies and other reports might be explained as follows. First, the experimental settings differed significantly. In example, we used common-γ cytokines to treat TCR gene-transduced primary T cells in the absence of antigenic stimulation, whereas a number of other studies used (isolated CD8+) TCR transgenic T cells stimulated with a combination of antigen and cytokine(s) *ex vivo* (Gattinoni et al., 2005b; Hinrichs et al., 2008). In yet other studies human primary T cells were stimulated with antigen-loaded dendritic cells (DC's) in combination with IL-15+IL-21 (Huarte et al., 2009). In our experiments we have deliberately chosen to stimulate TCR-engineered primary T cells with cytokines in the absence of cognate antigen because of the similarity to T cell expansions in clinical trials (Lamers et al., 2006a). Second, cytokines are pleiotropic molecules that exert actions that are highly context-dependent. For example, B cells exposed to IL-21 together with both antigen-specific B cell receptor stimulation and T cell help will undergo class switch and differentiate into antibody producing plasma cells, whereas B cells exposed to IL-21 together with Toll Like Receptor (TLR) stimulation in the absence of T cell

help will undergo apoptosis (Konforte et al., 2009). In another example, various T cell populations proliferate when exposed to IL-21 in the presence of anti-CD3 stimulation (Kasaian et al., 2002; Parrish-Novak et al., 2000), whereas T cells do not proliferate when exposed to IL-21 in the absence of a TCR signal (Kasaian et al., 2002; Parrish-Novak et al., 2000; van Leeuwen et al., 2002). Third, and with respect to the use of total splenocyte or human PBMC cultures, common-γ cytokines act on a large variety of cells, which may affect other cells present in the cellular suspension under investigation. For example, dendritic cells (DCs) are sensitive to both IL-15 and IL-21 (Dubois et al., 2005; Fukao et al., 2000; Mattei et al., 2001; Mnasria et al., 2008), and exposure to either one of these cytokines up-regulates the expression of different cytokine receptors (Dubois et al., 2005; Mattei et al., 2001). Eventually this may result in increased survival of mature DCs, the up-regulated expression of co-stimulatory molecules and the increased presentation of antigen by DCs to CD4+ and CD8+ T cells (Combe et al., 2006; Dubois et al., 2005; Mattei et al., 2001). The above example is in line with our observation of differences in distributions of leukocyte subsets (CD19+, CD11+, CD3+, CD4+, CD8+, NK1.1+) when culturing splenocytes with different common-γ cytokines (see *Supplementary Figure S3* of chapter 5). Finally, currently there is no defined phenotypical consensus with respect to CD8 T cell differentiation. The differentiation of naive T cells into effector and memory subsets is characterized by a complex and controversial classification of an increasing number of T cell subsets. Different T cell subsets are generally classified based on the expression of certain membrane proteins, mostly CD45RA/CD45RO, CCR7, CD27 and CD28 in human T cells, and CD62L, CD27, CD44 and CCR7 in murine cells (Appay et al., 2008; Gattinoni et al., 2005b; Sallusto et al., 2004; Sprent and Surh, 2002). However, one may find as many T cell subpopulations as there are combinations of the proposed markers (Appay et al., 2008). In addition, distinct T cell subpopulations may also be reflected by the expression of different intracellular molecules, such as IL-2, IFNγ, perforin and granzymes. Furthermore, phenotypical studies in T cells are often performed in models of viral disease (Alves et al., 2003; Gamadia et al., 2004; Papagno et al., 2004). These studies have shown that virus-specific CD4 and CD8 T cells display unique phenotypes and functions depending on their viral specificities. For example, whereas EBV-specific T cells express high levels of granzyme K but not B, CMV-specific T cells abundantly express granzyme B but little K (Appay et al., 2008). One could argue that in case T cell phenotype and function already differ between T cells of different viral specificities, significant heterogeneity of T cell subsets associated with other pathogens and cancer exists. Consequently, the value of TCR-transduced T cells generated according to the protocol described in this thesis will have to be tested in *in vivo* anti-tumour models.

In our experimental setting, the combination of IL-15 and IL-21 resulted in T cells that express high levels of introduced anti-melanoma TCR, contain a relatively high percentage of central memory T cells, produce both IL-2 and IFNγ, and show cytotoxicity upon melanoma cell recognition *in vitro*. We found that IL-15 and IL-21 halt T cell differentiation and promote

IL-2 secretion, findings that are in line with previous studies, yet profoundly enhance IFNγ production and cytotoxicity, findings that are novel when compared to previous studies. Such polyfunctional T cell responses (characterized by T cell production of both IL-2 and IFNγ) have been recently shown to be associated with improved protection against infection, when compared to monofunctional T cell responses (IFNγ release only) (Darrah et al., 2007). Preservation of naive/central memory T cells (a main activity of IL-21) on the one hand, and increased effector functions of more differentiated T cells (activities of IL-15+IL-21) on the other hand are considered most beneficial to generate anti-tumour T cells. Our current view with respect to the effects of IL-15+IL-21 on T cells is shown in Figure 1. In light of this view, I would propose the use of heterogeneous lymphocyte cultures, rather than sorted T cell populations, and expansion with IL-15 and IL-21 for the purpose of TCR gene therapy experiments. Although the *in vivo* anti-tumour efficacy of less differentiated T cells was improved when compared to that of effector T cells (Berger et al., 2008; Gattinoni et al., 2005b; Hinrichs et al., 2009; Hinrichs et al., 2008), the long-term efficacy of these anti-tumour populations are currently unknown (e.g. no recall experiments were performed). In addition, adoptive transfer of a combination of both naive and effector T cells has never been directly compared to adoptive transfer of naive or effector T cells only.

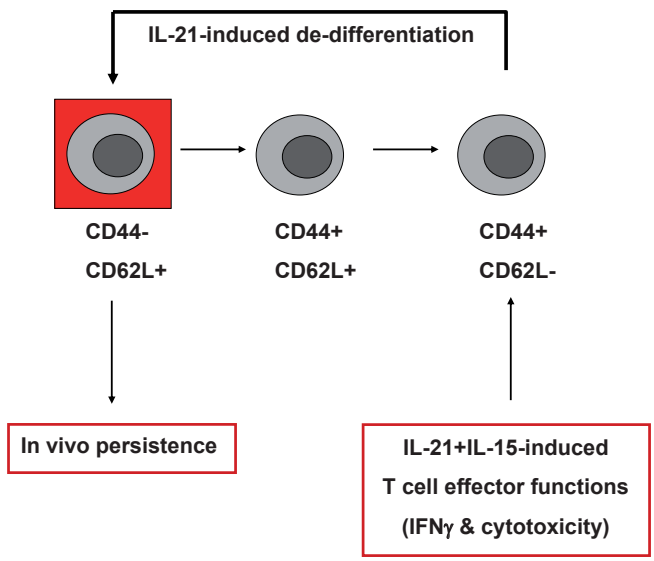


Figure 1. Current view with respect to the effects of IL-15+IL-21 on T cells
IL-21 on its own induces de-differentiation of T cells from CD44+ into CD44- T cells. CD44-/CD62L+ T cells are believed to have improved lymph node homing potential, and therefore increased *in vivo* persistence. When combined with IL-15, IL-21 also increases effector T cell functions such as cytotoxicity and IFNγ release. See chapter 4 and 5 for more details.

IL-15 and IL-21 have demonstrated potential for *in vivo* cytokine therapy in cancer patients. The use of IL-15 in human disease stays behind when compared to IL-21, and to date *in vivo* studies with IL-15 have been limited to animals. This is surprising considering that human IL-15 has already been cloned in 1994 (Anderson et al., 1995) and has shown strong therapeutic activity in multiple settings in animal models of human disease including cancer (Klebanoff et al., 2004). The current lack of human patient trials is partially due to difficulties in producing sufficient quantities of IL-15 protein and to restricted commercial licensing (Rosenberg, 1996). Recently, a step towards the clinical use of IL-15 protein has been made as its safety and immunological effects was tested in non-human primates (Berger et al., 2009). Yin and colleagues (Yin et al., 2009) demonstrated the importance of optimizing DNA-based IL-15 adjuvants in rhesus macaques as an alternative approach. In the latter study, low-doses of IL-15-encoding plasmid DNA in combination with influenza vaccines resulted in enhanced fractions of CD8+ central memory T cells, whereas high doses of IL-15-encoding plasmid DNA in combination with influenza vaccines resulted in decreased fractions of CD8+ as well as CD4+ central memory T cells. Although the underlying mechanisms of the observed differential effects of high versus low doses of IL-15 DNA on levels of central memory T cells still need to be determined, these findings do emphasize the importance of thorough testing prior to clinical application of cytokines. Therapeutic use of recombinant IL-21 protein has been proven effective in the treatment of various tumours in mouse models (Daga et al., 2007; Moroz et al., 2004; Sondergaard et al., 2007). In these models of lymphoma, melanoma, renal cell carcinoma and glioma, anti-tumour effects were mediated by CD8+ T cells (Moroz et al., 2004; Sondergaard et al., 2007), NK cell or B cells (Daga et al., 2007). In addition to having anti-tumour effects when given as monotherapy, IL-21 also shows significant additive effects together with a range of other biologicals modifying both innate and adaptive immune responses. These include IL-15 (Zeng et al., 2005), NKT cell agonist α-GC (Smyth et al., 2005) and a combination of antiDR5/anti-CD40/anti-CD137 (4-1BB) (Trimab) (Smyth et al., 2008). Finally, Skak and colleagues have recently demonstrated that IL-21 has additive anti-tumour effects in combination with certain chemotherapies, provided that IL-21 treatment is administered subsequent to chemotherapy (Skak et al., 2008). In phase I clinical trials, IL-21 proved to be safe and well tolerated in metastatic melanoma and renal cell carcinoma patients (Davis et al., 2007; Thompson et al., 2008). Currently, IL-21 is used in Phase II clinical trials for the treatment of humans with cancer (Andorsky and Timmerman, 2008; Spolski and Leonard, 2008a). In these trials mild lymphocytopenia is a frequently observed adverse effect of IL-21 (Andorsky and Timmerman, 2008; Spolski and Leonard, 2008a). Interestingly IL-21 increased the frequency of lymphocytes expressing CD62L and CCR7 (Davis et al., 2009), indicating that IL-21-induced lymphocytopenia might be caused by a redistribution of lymphocytes to secondary lymphoid compartments. Consistent with the phase I trials, NK cells and CD8+ T cells in phase II trials showed significant increases in perforin, granzyme B and IFN-γ expres-

sion following IL-21 (Davis et al., 2009). The up-regulation of CD62L and increase in perforin, granzyme B and IFNγ by IL-21 nicely agree with our *in vitro* findings.

In addition to IL-15 and IL-21, IL-7 may be of interest for the *ex vivo* expansion of anti-tumour T cells. IL-7 promotes the survival of both naive and memory T cells. Interestingly, IL-7Rα expression is similar among CD4 and CD8 T cells and among naive and memory T cells (Goldrath et al., 2002; Schluns et al., 2000; Swainson et al., 2006), making this cytokine broadly applicable in immunotherapy. However, use of IL-7 in anti-cancer AT might be limited to *ex vivo* applications rather than *in vivo* cytokine treatment. In a study by Rosenberg and colleagues, IL-7 administered to melanoma patients in combination with gp100 or MART-1 peptide vaccination, did not result in enhanced gp100 or MART-1 specific immunity (Rosenberg et al., 2006). *Ex vivo* use of a combination of IL-7 and IL-15, on the other hand, has been shown to generate T cells with a less differentiated CD8 T cell phenotype (i.e., central memory phenotype) that showed potent antigen reactivity and prolonged persistence in a mouse model (Kaneko et al., 2009). The latter report extends previous reports on IL-7's capacity to enhance survival and proliferation of memory T cell populations (Kieper et al., 2002; Ku et al., 2000; Schluns et al., 2000; Tan et al., 2002). In light of those results, it would be interesting to investigate how a combination of IL-7 plus IL-21 would relate to a combination of IL-15 plus IL-21 with respect to *ex vivo* expansion of anti-tumour T cells.

Clinical use of IL-15 and IL-21, or any cytokine for that matter, warrants close monitoring for any unwanted side effects, and long-term consequences of use of common-γ cytokines must be taken into account. Since, for example, IL-21 has been shown to stimulate NK- and T cell-mediated tumour immunity, play a central role in B cell differentiation and antibody production, and amplify the expansion of pro-inflammatory Th17 cells, it is not difficult to appreciate that IL-21 could be potentially involved in immunopathologies such as Rheumatoid Arthritis (Andersson et al., 2008; Daha et al., 2009; Jungel et al., 2004; Li et al., 2006), Inflammatory Bowel Disease (Caruso et al., 2007; Festen et al., 2009; Fina et al., 2008; Monteleone et al., 2005) and Multiple Sclerosis (Jones et al., 2009). In the light of possible safety issues, we highly favour the use of IL-15 and/or IL-21 during *ex vivo* expansion of T cells rather than the use of these cytokines for *in vivo* administration to support AT therapy. Future clinical trials will have to confirm whether the use of *ex vivo* IL-15 and IL-21 in cancer AT will result in objective and durable anti-cancer responses.

6.3 FUTURE DESIGN OF CLINICAL ADOPTIVE TCR GENE THERAPY TRIALS

Despite its potential, TCR gene therapy currently still faces a number of challenges, as summarized in Table I of chapter 1. In this last section of my thesis I would like to summarize some

strategies to tackle these challenges and deliver a more optimal design for future adoptive T cell therapy.

T cell activation

We have demonstrated that (sub)optimal T cell stimulation is preferred over supra-optimal stimulation. Strong T cell stimulation, especially when combined with IL-15+IL-21, resulted in significant antigen non-specific function of TCR-redirected T cells. In case of AT into cancer patients this antigen non-specific functionality could lead to toxicities. In addition to possible toxicities, the enhanced differentiation of T cells under the influence of (mainly polystyrene) CD3/CD28 stimulation argues against the use of this type of T cell activation prior to TCR gene transfer.

TCR gene transfer

We have shown that a combination of amphotropic and ecotropic virus particles results in efficient TCR gene transfer into primary murine T cells. Transient transfection and subsequent retroviral transduction of TCR constructs has proven efficient in primary human T cells as well (Schaft et al., 2003; Willemsen et al., 2000). However, stable retrovirus producer cell lines may have the advantage that they facilitate the vector production and quality controls at GMP level (Johnson et al., 2009; Lamers et al., 2006a). Retroviral transduction efficiency is further enhanced by low T cell densities during TCR gene transfer.

T cell expansion

In our studies we have focused on the use of common-γ cytokines for short-term *ex vivo* expansion of TCR-modified T cells. As discussed before, *ex vivo* manipulation of T cell characteristics may be preferred over *in vivo* T cell modification. Possible side effects of *in vivo* cytokine therapy are not only hypothetical, but also proven in literature, with the detrimental effects of high dose IL-2 administration being one of the most acknowledged examples (Rosenberg et al., 1986). In phase I clinical trials IL-21 proved to be safe and well tolerated in metastatic melanoma and renal cell carcinoma patients (Davis et al., 2007; Thompson et al., 2008), but long-term effects and effects in other patient populations have not yet been well established. The lack of clinical experience with *in vivo* administration of IL-15 further favours the *ex vivo* use of IL-15 and IL-21.

Choice of T cell target antigen and receptor format

Johnson and colleagues reported on serious melanocyte destruction, in skin, eyes and ears of melanoma patients treated with TCR-modified T cells (Johnson et al., 2009). In an earlier clinical trial with renal cell carcinoma patients using autologous T cells transduced with an antibody-based receptor, severe liver toxicity was observed, which coincided with the expression of the targeted antigen by larger bile duct epithelial cells (Lamers et al., 2006a). Whereas

these reports have already posed sufficient reason for concern, a recent article by Morgan and colleagues should be a wake-up call for all those involved in the design of TCR gene therapy trials. Morgan and colleagues sadly reported on the death of a metastatic colon carcinoma patient treated with T cells redirected with an ErbB2-specific chimeric antigen receptor (CAR) (Morgan et al.). The death of this patient was reported to be related to a cytokine storm, most probably induced by large numbers of infused T cells in the lungs that recognized low levels of ErbB2-positive lung epithelial cells. Notably, this study used a next generation anti-ErbB2 CAR containing both a humanized single-chain Fv fragment and optimized co-stimulatory signalling domains designed for increased cytokine secretion, lytic activity, and robust *in vivo* anti-tumour activity in a human breast cancer xenograft model (Zhao et al., 2009). The authors discussed that the antibody from which the anti-ErbB2 CAR was developed (Herceptin) has been used safely in thousands of patients. However, a supra-optimal receptor to gene-engineer highly avid T cells is different from antibody therapy. Indeed, while antibodies are subject to clearance by the body (e.g., the erbB2/FcγRIII bispecific mAb used in a clinical trial by Weiner and colleagues had a *t*1/2 of 20 hours (Weiner et al., 1995), T cells can expand in cell numbers following antigen stimulation and continuously produce effector cytokines. In addition, Morgan *et al.* state that no toxicity was observed in breast cancer patients treated with autologous anti-ErbB2 cytotoxic T lymphocyte clones (Bernhard et al., 2008). Again, the incorporation of a receptor selected for enhanced effector functions into patient T cells is different from a naturally selected non-modified TCR. The choice of target antigen and receptor format would have warranted thorough preclinical testing before proceeding to clinical applications. The report by Morgan et al (Morgan et al.) highlights two lessons: the importance of targeting safe (non-self) antigens for T cell therapy in combination with the careful use of co-stimulation-optimized receptors.

Patient pre-conditioning

Although not studied in the experiments described in the current thesis, patient pre-conditioning has been shown to substantially increase the efficacy of TCR gene therapy trials (see also chapter 1, section 1.2.4). AT in melanoma patients combined with a non-myeloablative regimen (cyclophosphamide plus fludarabine) is believed to enhance the efficacy of AT in a number of ways. First, solid tumours are rendered accessible to transferred T cells by change of the vascular tumour endothelium (Ganss et al., 2002), and induction of tumour cell death (Nowak et al., 2003). Second, depletion of endogenous T cells is believed to increase availability of (common-γ) cytokines for adoptively transferred T cells, resulting in their improved anti-tumour T cell responses (Gattinoni et al., 2005a; Muranski et al., 2006). Third, removal of regulatory T cells (Tregs) and other suppressor cells such as myeloid derived suppressor cells (MDSC) strengthens the effects of therapeutic effector T cells (Ghiringhelli et al., 2004; Polak and Turk, 1974). And finally, activation of endogenous APC, especially dendritic cells (DCs), further enhances T cell responses (Paulos et al., 2007; Saini et al., 2009; Salem et al.; Salem et al., 2009).

To summarize, I would propose the following steps in designing a future TCR gene therapy trial in metastatic melanoma patients (steps 2-8 are schematically represented in Figure 2):

1. Use TCRs directed against non-self antigens, such as MAGE antigens.
2. Genetically optimize the TCR format to ensure preferential pairing of introduced TCRα and -β
3. Incorporate the TCRα and TCRβ chains into a retroviral vector, separated by a 2A peptide sequence in the following order: TCRβ-2A-TCRα
4. Isolate patient PBMC and shortly activate these in a (sub)optimal manner (i.e., soluble CD3/CD28 mAbs) in the presence of IL-2
5. Transduce activated PBMC with the TCR encoding retroviral particles in the presence of IL-2
6. Pre-condition metastatic melanoma patients with a non-myeloablative regimen (i.e., cyclophosphamide plus fludarabine)
7. Expand TCR-transduced patient T cells with a combination of IL-15 plus IL-21
8. Infuse expanded TCR-redirected T cells back into metastatic melanoma patients together with low-dose IL-2
9. Monitor TCR gene-modified T cell-treated patients for side effects and anti-tumour effects

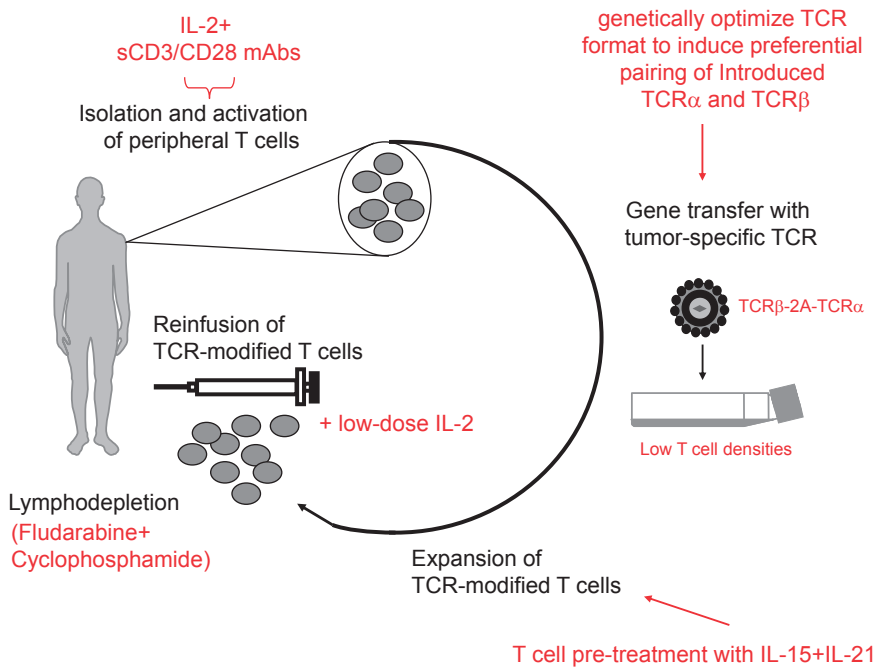


Figure 2. Proposed design of a future adoptive TCR gene therapy trial in metastatic melanoma
Peripheral blood T cells are obtained from melanoma patients and activated w/ sCD3/CD28 mAbs. Activated T cells are retrovirally tranduced with a gene-modified TCR format that is specific for a non-self tumour antigen, and expanded in short-term cultures in the presence of IL-15 plus IL-21. Prior to re-infusion of TCR-redirected T cells, melanoma patients are pre-conditioned with non-myeloablative chemotherapeutics. T cells are infused together with low-dose IL-2.

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Summary

Samenvatting

SUMMARY

Adoptive transfer of tumour-specific T cells is a successful clinical treatment option for metastatic melanoma, with anti-tumour responses that exceed those of chemotherapy and radiation. The introduction of genes encoding tumour-specific T cell receptors (TCR) in T cells, and the therapeutic use of these T cells (TCR gene therapy), has been developed as a treatment strategy to increase the success rate and applicability of adoptive T cell therapy. The aim of the research presented in the current thesis was to determine optimal *in vitro* conditions to obtain highly functional yet minimally differentiated primary T cells following TCR gene transfer.

Chapter 1 provides an overview of the development and current status of TCR gene therapy, and covers the following 4 parts. First, a summary is given of the current literature on TCR gene therapy of solid tumours. Second, a number of challenges of TCR gene therapy are presented, including therapy-related toxicities, suboptimal functional T cell avidity and compromised peripheral persistence of anti-tumour T cells. In addition, potential solutions to these challenges are proposed. Thirdly, gene transfer methodologies, both non-viral and viral, and protocols for retroviral gene transfer are presented. And finally, the use of common-γ cytokines in the *ex vivo* expansion of (TCR modified) T cells is discussed, with emphasis on their potential in anti-tumour therapy.

In **chapter 2**, we have developed a straightforward and versatile procedure to transfer TCRαβ genes into primary murine T cells, which is based on transiently produced retroviruses. To this end, we have compared retroviruses expressing different retroviral envelopes that have been generated by a (co-) culture of different packaging cell lines. Our results demonstrated that MLV-A and MLV-E pseudotyped viruses resulted in the highest T cell transduction efficiencies. In addition, T cell densities during transduction were inversely related to transduction efficiencies. Further optimization of transduction parameters, such as concentration and duration of Conconavalin A stimulation of T cells prior to gene transfer; rIL-2 concentration; the use of Retronectin; and the number of infection cycles, resulted in transduction efficiencies of > 90% for GFP. Using similar conditions, we observed about 70% transduction efficiencies for a human TCR, which required the genetic incorporation of murine TCR-C domains (i.e., TCR murinization) to allow functional surface expression in murine T cells.

In **chapter 3**, we have tested T cell activation using a lectin or various formats of anti-CD3/CD28 mAbs since it is well documented that the mode of T cell activation prior to gene transfer affects gene transduction efficiencies. To explore *in vitro* conditions responsible for an optimal T cell phenotype and function in more depth, we have combined T cell activation methods with treatment of TCR-engineered T cells either with IL-2 or a combination of IL-15

and IL-21 (the latter combination of cytokines was studied more extensively in chapters 4 and 5). We observed that the lectin Concanavalin A, and to a lesser extent soluble anti-CD3 and CD28 mAbs, resulted in functional surface expression of the TCRαβ transgenes and enhanced fractions of naive T cells. T cell functions and limited T cell differentiation were most significant when T cells were treated with a combination of IL-15 and IL-21 rather than IL-2. In comparison, anti-CD3 and CD28 mAbs coated to beads resulted in improved functional TCR expression levels yet enhanced T cell differentiation as well as significant non-specific T cell responses (not observed with lectin or soluble mAbs).

In **chapter 4**, we have set out to elucidate the mechanisms behind IL-15 and/or IL-21's effect on T cell differentiation and activation. IL-21, and to a lesser extent IL-15, has previously been shown to suppress differentiation of naive T cells into effector T cells. The rationale of the present and other studies into the effects of cytokines on T cell differentiation is provided by the direct relationship between clinical anti-tumour efficacy of T cells and T cell persistence, with the latter being related to the differentiation state of transferred T cells. T cells cultured in the presence of various cytokines, as well as their subsets based on T cell differentiation markers, were evaluated for proliferation and expansion, apoptosis, cytokine receptor expression and gene expression profile. We demonstrated that short-term exposure of primary T cells to IL-21, and to a lesser extent the combination of IL-21 and IL-15, resulted in a significant enrichment of naive T cells, which was paralleled by differential expression of genes involved in T cell differentiation. Although IL-21 reduced both proliferation and apoptosis of naive T cells, our data suggested that a phenotypic reversal of effector memory T cells into naive T cells contributed most significantly to the observed enrichment of naive T cells. IL-21 induced a down-regulated expression of IL-2R chains and anti-apoptotic Bcl molecules, which was restored by IL-15, thereby potentially contributing to the observed increase in overall T cell proliferation in the presence of IL-21 and IL-15. Interestingly, IL-21 plus IL-15 resulted in a gene expression profile that corresponds to CD8 T cell effectors.

In **chapter 5**, we have extended findings from chapter 4 by investigating the use of common-γ cytokines in the setting of TCR-engineered T cells. We demonstrated that the combination of IL-15 and IL-21, and to a lesser extent IL-21, enhanced antigen-specific T cell cytotoxicity, which was related to enhanced expression of granzymes A and B, and perforin 1. Furthermore, IL-15 and IL-21 synergistically enhanced release levels and kinetics of IFNγ by effector memory T cells. Putting the data from chapters 4 and 5 together, we hypothesize that starting from a heterogeneous T cell population, IL-21 de-differentiates effector memory T cells into naive T cells, whereas the combination of IL-15 and IL-21 induces a strong T cell effector phenotype in remaining differentiated T cells.

In **chapter 6**, we have placed findings from chapters 2 to 5 in a broader context of TCR gene therapy. Emphasis is given to gene transfer methods, including different T cell activation stimuli, and the *ex vivo* use of common-γ cytokines in adoptive T cell therapy. Strategies to tackle current challenges of TCR gene therapy, including the ones discussed in the chapters 2 to 5, are presented and combined into a proposed design for a future TCR gene therapy trial.

In my opinion, we have succeeded in determining some factors important to efficient TCR gene transfer into primary T lymphocytes and expansion into T cells with high transgenic TCR expression, a central memory-like phenotype and potent anti-tumour functions *in vitro*. Factors identified in our studies are not the sole factors improving TCR gene therapy, and future experiments will have to identify other important factors as well as to confirm the *in vivo* functionality of T cells generated under the conditions described in the current thesis. Future clinical trials should, in my opinion, focus on the following points: choice of T cell target antigen; molecular receptor format; combination with other forms of therapy; monitoring anti-tumour and side effects; and ultimately the inclusion of non-melanoma cancers. With regard to a near future TCR gene therapy trial for metastatic melanoma, one could consider the following steps. A TCR directed against MAGE antigens could be considered, with a molecular TCR format to ensure preferential pairing of the TCRα and -β chains, and incorporation into a retroviral vector in which both TCR chains are separated by a 2A peptide sequence. Patient T cells should preferably be activated with soluble anti-CD3/CD28 mAbs, and TCR-engineered T cells should be expanded with a combination of IL-15 and IL-21 prior to T cell infusions. Patients in such a trial should be pre-conditioned with a non-myeloablative regimen to further facilitate engraftment and persistence of adoptively transferred T cells. Long-term follow up of patients after transfer of TCR gene-modified T cells is essential, both in terms of anti-tumour efficacy as well as safety. I am confident that such a trial will demonstrate the full potential of anti-tumour TCR gene therapy.

SAMENVATTING

Klinische behandeling van gemetastaseerd melanoom met witte bloedlichamen, de zogenaamde T lymfocyten, die de tumour herkennen (ook wel: adoptieve T cel therapie) is succesvoller gebleken dan chemotherapie of bestraling. De introductie van genen coderend voor een tumourspecifieke T cel receptor (TCR) in patiënt T cellen, en het klinische gebruik van deze T cellen (TCR gen therapie), is ontwikkeld als strategie om het succes en de toepasbaarheid van adoptieve T cel therapie te vergroten. Het doel van het onderzoek dat in dit proefschrift beschreven wordt is om de meest optimale laboratorium-omstandigheden te bepalen voor het creëren van TCR-gemodificeerde T cellen die tumour cellen goed herkennen en niet volledig uitgerijpt en daardoor waarschijnlijk uitgeput zijn voordat ze klinisch gebruikt zouden worden.

Hoofdstuk 1 geeft een overzicht van de ontwikkeling en huidige status van TCR gen therapie, en omvat de volgende 4 onderdelen. Ten eerste word een samenvatting gegeven van de huidige literatuur over TCR gen therapie ter behandeling van solide tumouren. Ten tweede wordt een aantal barrières ten aanzien van het klinisch succes van TCR gen therapie beschreven, waaronder toxiciteit, suboptimale functionele T cel aviditeit en verminderde overleving van T cellen na infusie. Daarnaast worden ook mogelijke oplossingen voor deze problemen besproken. Ten derde worden verschillende methoden voor gen transfer, zowel viraal als niet-viraal, alsook verschillende protocollen voor retrovirale gen transfer gepresenteerd. Tenslotte wordt het gebruik van zogenaamde "common-γ" cytokinen in de *ex vivo* kweek van (TCR-gemodificeerde) T cellen besproken, waarbij de nadruk ligt op mogelijkheden van dergelijke cytokinen in antitumour therapie.

In **hoofdstuk 2** hebben we een snel, effectief en flexibel protocol opgezet om TCRαβ genen te introduceren in primaire muis T cellen, welke is gebaseerd op transiënte produktie van retrovirussen. We hebben dit gedaan door retrovirussen met verschillende retrovirale envelop eiwitten te vergelijken, waarbij de retrovirussen gegenereerd werden door verschillende combinaties van virus producerende cellen. Onze resultaten laten zien dat retrovirussen met de zogenaamde MLV-A en MLV-E envelop eiwitten leiden tot de hoogste transductie efficiëntie. Hiernaast blijkt de dichtheid van T cellen tijdens transductie omgekeerd evenredig te zijn met de transductie efficiëntie. Verdere optimalisatie van het transductie protocol, wat betreft de concentratie en duur van Concanavalin A-stimulatie van T cellen voorafgaand aan gen transfer; rIL-2 concentratie; gebruik van Retronectine; and het aantal retrovirale infecties, resulteerde in transductie efficienties van > 90% voor het fluorescerende reporter transgen GFP. Hetzelfde protocol leidde tot een transductie efficiëntie van ongeveer 70% voor een humaan TCR transgen, waarvoor het gebruik van muis TCR-C domeinen noodzakelijk bleek (i.e. TCR murinizatie) om functionele expressie in muis T cellen mogelijk te maken.

In **hoofdstuk 3** hebben we T cel activatie met ofwel een lectine ofwel verschillende vormen van anti-CD3/CD28 antistoffen uitgevoerd, omdat literatuur laat zien dat de manier van T cel activatie voorafgaand aan gen transfer de transductie efficiëntie beïnvloedt. Om meer in detail te onderzoeken welke laboratorium-omstandigheden resulteren in optimale T cellen (zowel qua oppervlakte-merkers als T cel funkties), hebben we T cel activatie methoden gecombineerd met behandeling van TCR-gemodificeerde T cellen met ofwel IL-2 ofwel een combinatie van IL-15 en IL-21 (de laatste twee cytokinen zijn in meer detail bestudeerd in hoofdstukken 4 en 5). We zagen dat het lectine Concanavalin A, en in mindere mate anti-CD3/CD28 antistoffen in oplossing, resulteerde in functionele expressie van TCRαβ transgenen op het celoppervlak, en verhoogde fracties van zeer jonge, zogenaamde naïeve T cellen. T cel functionaliteit en beperkte T cel differentiatie waren het duidelijkst wanneer T cellen werden behandeld met een combinatie van IL-15 en IL-21, in plaats van IL-2. Daarentegen zorgen anti-CD3/CD28 antistoffen welke gekoppeld waren aan bolletjes weliswaar voor verhoogde TCR expressie, maar ook voor een sterke T cel differentiatie en non-specifieke T cel reactiviteit (welke niet het geval was bij gebruik van lectine of antistoffen in oplossing).

In **hoofdstuk 4** hebben we geprobeerd om de mechanismen verantwoordelijk voor de effecten van IL-15 en/of IL-21 op T cel differentiatie en activatie te achterhalen. Het was eerder aangetoond dat IL-21, en in mindere mate IL-15, het 'ouder' worden van naïeve T cellen in effector T cellen (de eerder genoemde T cel differentiatie) onderdrukt. De rationale achter deze en andere studies naar de effecten van cytokinen op T cel differentiatie is te vinden in de directe relatie tussen klinische antitumour effectiviteit en T cel persistentie, waarbij de T cel persistentie zelf weer gerelateerd is aan de mate van T cel differentiatie. T cellen gekweekt in aanwezigheid van verschillende cytokinen, alsmede de verschillende T cel subsets, werden geanalyseerd wat betreft proliferatie, expansie, celdood, cytokine receptor expressie en gen expressie profiel. We hebben laten zien dat kortdurende kweek van T cellen met IL-21, en in mindere mate IL-21 en IL-15, resulteert in een significante verrijking van naïeve T cellen, iets dat samengaat met een specifieke expressie van genen betrokken bij T cel differentiatie. Hoewel IL-21 zowel de proliferatie als de celdood van naïeve T cellen verminderde, suggereert onze data dat een veranderde expressie van bepaalde oppervlakte-merkers (kenmerkend voor naïeve T cellen) het meest bijdraagt aan de waargenomen verrijking van naïeve T cellen. IL-21 induceerde een verlaging van de expressie van IL-2 receptorketens en anti-apoptotische Bcl moleculen, hetgeen voorkòmen kan worden door IL-15 en kan leiden tot de waargenomen toename in totale T cel proliferatie in aanwezigheid van zowel IL-21 als IL-15. Een interessante bevinding was dat de combinatie van IL-21 en IL-15 resulteert in een genexpressie profiel dat kenmerkend is voor CD8-positieve effector T cellen.

In **hoofdstuk 5** hebben we voortgebouwd op onze bevindingen uit hoofdstuk 4, door het gebruik van "common-γ" cytokinen in de setting van TCR-gemodificeerde T cellen te onder-

zoeken. We hebben laten zien dat de combinatie van IL-15 en IL-21, en in mindere mate IL-21, T cellen beter in staat stelt tumourcellen te doden, hetgeen gerelateerd is aan expressie van cytotoxische moleculen zoals granzymes A en B, en perforine 1. Daarnaast zorgt een combinatie van IL-15 en IL-21 voor een synergistische toename in de hoeveelheid uitgescheiden IFNγ, en een versnelde uitscheiding van IFNγ, door gedifferentieerde effector memory T cellen. Wanneer de data uit hoofdstuk 4 en 5 gecombineerd worden, veronderstellen we dat uitgaande van een heterogene T cel populatie, IL-21 resulteert in de-differentiatie van de effector memory T cellen in naïeve T cellen, terwijl de combinatie van IL-15 en IL-21 resulteert in een sterk T cel effector profiel in de overblijvende gedifferentieerde T cellen.

In **hoofdstuk 6** hebben we de bevindingen van hoofdstukken 2 tot en met 5 in een bredere context van TCR gen therapie geplaatst. Nadruk is hierbij gelegd op gen transfer methoden, inclusief verschillende T cel activatie stimuli, en het gebruik van "common-γ" cytokinen in adoptieve T cel therapie. Strategieën om de huidige barrières van TCR gen therapie, inclusief de besproken strategieën in hoofdstukken 2 tot en met 5, worden bediscussieerd en gecombineerd tot een voorstel voor een toekomstige klinische TCR gen therapie studie.

Naar mijn mening zij we erin geslaagd om factoren te bepalen die van belang zijn voor efficiënte TCR gen transfer en expansie van primaire T lymfocyten met een hoge transgene TCR expressie, een jong fenotype (een zogenaamd "central memory" fenotype) en potente anti-tumour functies in het laboratorium ("*in vitro*"). De door ons bepaalde factoren zijn niet de enige factoren die succesvolle toepassing van TCR gen therapie positief beïnvloeden, en toekomstige experimenten zullen andere belangrijke factoren dienen te identificeren, alsook de *in vivo* functionaliteit (funktionaliteit in proefdieren) dienen te bevestigen van T cellen gegenereerd onder condities zoals beschreven in het huidige proefschrift. Toekomstige klinische studies moeten, naar mijn mening, nadruk leggen op de volgende punten: keuze van T cel target antigen; moleculaire vorm van de receptor; combinatie met andere vormen van therapie; monitoren van antitumour effecten en bijwerkingen; en uiteindelijk de inclusie van andere kankersoorten. Op korte termijn zou een TCR gen therapie studie ter behandeling van gemetastaseerd melanoom als volgt kunnen worden uitgewerkt. Een TCR specifiek voor MAGE antigenen zou kunnen worden gebruikt, waarbij de receptor moleculair zo moet worden aangepast dat preferentiële paring tussen de geïntroduceerde TCRα en -β ketens wordt verzekerd, en de receptor dusdanig in een retrovirale vector moeten worden geplaatst dat beide TCR ketens door een 2A peptide sequentie gescheiden worden. T cellen van de patiënten moeten bij voorkeur worden geactiveerd met anti-CD3/CD28 antistoffen in oplossing en vervolgens, na TCR gen transfer, kortdurend worden gekweekt met een combinatie van IL-15 en Il-21 voorafgaand aan infusie terug in de patiënt. De patiënten zouden in een dergelijke klinische studie een voorbehandeling moeten doorlopen met een zogenaamd niet-myeloablatief schema zodat de therapeutische T cellen in de patiënten beter overleven.

Het vervolgen van behandelde patiënten op lange termijn is essentieel, zowel wat betreft antitumour effectiviteit als veiligheid. Ik heb er vertrouwen in dat een dergelijke klinische studie het volle potentieel van antitumour TCR gen therapie zal aantonen.

CURRICULUM VITAE

Nadine Pouw was born on the 13th of February 1978 in Naarden. She attended secondary school at the Visser het Hooft Lyceum in Leiden, where she graduated in 1997. She studied Fundamental Biological Sciences at the Utrecht University. Her first graduation project, entitled "Secretory and intracisternal granules in AKH producing cells of *Locusta Migratoria*", was performed at the Department of Biochemical Physiology at Utrecht University, under the supervision of Dr. L.F. Harthoorn and Prof. D.J. van der Horst. Her second graduation project, entitled "Establishment of a dynamic *in vitro* model to investigate cellular immunotherapy of human solid tumours", was performed at the Department of Immunohematology and Blood Transfusion at the Leiden University Medical Center, under the supervision of Dr. L.W.H. Hambach and Prof. E.A.J.M. Goulmy. After a literature study to investigate "Emotional Stress and Alopecia Areata", under the supervision of Dr. M.M.H.M. Meindardi (department of Dermatology AMC) and Dr. J.C.C.A. Lambers (department of Dermatology, AtriumMC), Nadine graduated in 2003. In September 2003 she started a PhD project at the Laboratory of Experimental Tumour Immunology (Department of Medical Oncology) at the Erasmus MC in Rotterdam, under the supervision of Dr. J.E.M.A. Debets, Prof. G. Stoter and Prof. J. Verweij. The results of that project are described in this thesis. In September 2008 Nadine started working as a Clinical Research Associate at the HOVON Data Center. As of September 2009, she works as a Medical Information Associate at Genzyme.

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PhD PORTFOLIO

Name:	Nadine Pouw
Research School:	Molecular Medicine
Period:	2003-2010
Supervisor/co-promotor:	Dr. J.E.M.A. Debets
Promotor:	Prof. J. Verweij

1. PhD training 1 ECTS = 28h workload	Year	Credits
1.1 General academic skills		
1.2 Research skills		
1.3 In-depth courses		
• Molmed course "Oncogenesis and Tumour Biology"	2003	1
• Molmed course "Biomedical Research Techniques"	2003	1
• Molmed course "In vivo imaging, from cell to organism"	2004	1
• Molmed course "Gene Therapy"	2006	1
• Course Molecular Immunology	2008	3
1.4.1 Oral Presentations		
• Annual Tumour Immunology meeting, Breukelen, The Netherlands	2005	1
• Young Investigator Meeting Melanoma Group, EORTC, Berlin, Germany	2006	1
• Symposium on Cellular Therapy of Cancer, Manchester, UK	2006	1
• Annual meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2006	1
• European Society for Cellular and Gene Therapy, Rotterdam, The Netherlands	2007	1
1.4.2 Poster Presentations		
• Molecular Medicine Day, Erasmus MC, Rotterdam, The Netherlands	2004	1
• Molecular Medicine Day (Best Poster Award)	2005	1
• Annual meeting of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2005	1
• Molecular Medicine Day, Erasmus MC, Rotterdam, The Netherlands	2006	1
• European Congress of Immunology, Paris, France	2006	1
1.5 International conferences		
• Joint Annual meeting of the Dutch and German Societies of Immunology (JAMI, NVVI), Maastricht, The Netherlands	2004	1
• Young Investigator Meeting Melanoma Group EORTC, Berlin, Germany	2006	1
• European Congress of Immunology, Paris, France	2006	1
• Symposium on Cellular Therapy of Cancer, Manchester, UK	2006	1
• European Society for Cellular and Gene Therapy, Rotterdam, The Netherlands	2007	1
1.6 National conferences		
• Immunogene therapy symposium, VUMC, Amsterdam	2004	1
• Spring Symposium, Dutch Society on Gene Therapy, Utrecht, The Netherlands	2004	1
• Fall Symposium, Dutch Society on Gene Therapy, Utrecht, The Netherlands	2004	1
• Annual Dutch Tumour Immunology meeting, Breukelen, The Netherlands	2005	1
• Annual meetings of the Dutch Society of Immunology (NVVI), Noordwijkerhout, The Netherlands	2006	1
1.7 Seminars and Workshops		
• Workshop on Adoptive T cell therapy of Cancer (EU FP6 ATTACK), Montpellier, France	2006	1
1.8 Didactic skills (Lecturing)		

1.9 Other		
• Medical Oncology research meetings, 6x yearly	2003-2007	1
• JNI Scientific research Meetings, 6x yearly	2003-2007	1
• Molecular Medicine PhD retreat	2005-2006	1
2. Teaching activities		
	Year	Credits
2.1 Lecturing		
• Molecular Medicine Course on Gene Therapy	2006	0.5
• Molecular Medicine Course on Biomedical Research Techniques	2007	0.5
2.2 Supervising practicals and excursions		
2.3 Supervising thesis		
2.4 Other		
• Co-supervision lab technician	2006-2008	4
Total Credits		**34**

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