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Chapter 3

Prospects and limitations of T cell receptor gene therapy

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Prospects and Limitations of T Cell Receptor Gene Therapy

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Abstract: Adoptive transfer of antigen-specific T cells is an attractive means to provide cancer patients with immune cells of a desired specificity and the efficacy of such adoptive transfers has been demonstrated in several clinical trials. Because the T cell receptor is the single specificity-determining molecule in T cell function, adoptive transfer of TCR genes into patient T cells may be used as an alternative approach for the transfer of tumor-specific T cell immunity. On theoretical grounds, TCR gene therapy has two substantial advantages over conventional cellular transfer, as it can circumvent the demanding process of *in vitro* generation of large numbers of specific immune cells and it allows the use of a set of particularly effective TCR genes in large patient groups. Conversely, TCR gene therapy may be associated with a number of specific problems that are not confronted during classical cellular therapy. Here we review our current understanding of the potential and possible problems of TCR gene therapy, as based on *in vitro* experiments and mouse model systems. Furthermore, we discuss the prospects of clinical application of this gene therapy approach, and the possible barriers on the route towards clinical use.

Keywords: T cell receptor (TCR), adoptive therapy, gene therapy, T lymphocytes, major histocompatibility complex (MHC).

INTRODUCTION

Antigen Recognition by T Cells

The key event in T lymphocyte-mediated immune recognition is the ligation of the T cell receptor (TCR) on the T cell surface to a peptide-major histocompatibility complex molecule (pMHC) on the surface of a target cell. Classical major histocompatibility complex (MHC) molecules come into two flavors, named MHC class I and MHC class II. Virtually every nucleated cell in the body expresses MHC class I molecules, whereas MHC class II molecules display a more restricted tissue distribution, and are mostly found on so-called "professional antigen presenting cells" (pAPC). MHC class I molecules present a sampling of peptides that are derived from proteins expressed within the cell, including pathogen-derived peptides when a cell has been infected. When such foreign peptides are present in this mixture of pMHC complexes, cytotoxic ($CD8^+$) T cells that express a T cell receptor that recognizes the foreign peptide bound to MHC will become activated. Activation of T cells is followed by a strong proliferative burst and acquisition of effector functions, most notably the capacity to kill the offending cells. Foreign antigens complexed with MHC class II molecules are recognized by helper T cells, characterized by expression of the CD4 co-receptor. In this case, recognition also leads to T cell activation and proliferation. However, activated CD4+ T cells generally do not kill antigen-expressing cells, but stimulate the activity of other immune cells, such as dendritic cells, CD8+ T cells and B cells. During thymic development T cells acquire their clone-specific T cell receptor through recombination of the T cell receptor loci. As a consequence of these recombination events, each T cell clone is endowed with a unique T cell receptor, with an undefined specificity. To ensure that these randomly formed T cell receptors have the capacity to recognize MHC, but do not recognize MHC molecules complexed with peptides derived from endogenous proteins, newborn T cells are subjected to two selection processes in the thymus. First, T cells that fail to bind endogenous pMHC complexes altogether, such as T cells that express a T cell receptor that is not expressed at the cell surface, die by neglect. Second, and more important for this review, T cells that recognize MHC molecules containing self-peptides with high avidity are induced to undergo apoptosis, thereby preventing the egress of autoreactive T cells into the periphery. As a consequence of the process of random creation of T cell receptors followed by the selective removal of those receptors that recognize self-antigens, the peripheral T cell repertoire is largely ignorant of self, but can respond to foreign antigens.

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Passive and Active Immunization

Active immunization with antigens (often referred to as vaccination) has long been used to boost the size and activity of antigen-specific T cell populations. Such active immunization works well for the induction of T cell immunity against foreign antigens in healthy individuals. However, if T cell responsiveness is impaired, as in patients with an acquired or congenital immunodeficiency, or in case of pharmacological immunosuppression in transplant recipients, the value of active immunization is limited. Likewise, most of the tumor-associated antigens that are the target of immunotherapy are non-mutated self-antigens. While T cell responses against such antigens can be detected for at least some tumor types, these responses are generally weak and the avidity of the interaction between TCR and pMHC is suboptimal [de Visser *et al.,* 2003]. As a consequence, the value of vaccination strategies that aim to enhance T cell reactivity against self-antigens is likely to be limited.

Both in the case of general immunodeficiency and in the case of specific self-tolerance, introduction of the desired T cell reactivity through adoptive transfer forms an alternative and arguably preferred strategy. Adoptive transfer strategies used to date come in two flavors. In the setting of allogeneic stem cell transplants (allo-SCT) for the treatment of leukemias, unselected donor lymphocytes are infused. Because of polymorphic differences between donor and recipient, infused T cells will encounter antigens that are perceived as foreign (i.e. that were absent during T cell selection in the thymus of the donor) and will react to these "minor histocompatibility antigens". Recognition of minor histocompatibility antigens expressed on the leukemic cells has been demonstrated to correlate with tumor regression, providing a very strong indication that circumventing T cell tolerance towards tumor-associated self antigens can result in tumor regression [Marijt *et al.,* 2003]. A major drawback of this strategy of donor lymphocyte infusion is formed by the lack of tumor cell-specificity in the infused T cell population. As a consequence, T cell reactivity is not limited to the tumor cells and the incidence of graft-versus-host-disease (GvHD) is high [Dazzi *et al.,* 1998].

As an alternative to the transfer of unfractionated T cells, tumor-specific T cells isolated from cancer patients, have been grown in large numbers, using *in vitro* expansion protocols. This strategy works well to enhance the tumor-selectivity of the transferred cell population. Furthermore, infusion of such tumor-specific T lymphocytes has now successfully been used to prevent and treat EBV-associated B cell lymphomas in transplant recipients [Gottschalk *et al.,* 2005] and for the treatment of metastatic melanoma [Dudley *et al.,* 2002a; Dudley *et al.,* 2005]. However, the widespread application of this more specific form of adoptive T cell therapy is limited by two factors. First, the *in vitro* expansion procedures that are used to produce large numbers of antigen-specific T cells are highly demanding. Second, and more important, in many patients it has proven difficult to isolate tumor-specific T cells that can be used for such *in vitro* expansion procedures.

In summary, adoptive transfer of antigen-specific T cells is likely to be a preferred form of T cell based immunotherapy in cases of general immunodeficiency and in the case of self tolerance. As detailed above, ligation of the T cell receptor to a major histocompatibility complex containing a specific peptide is the single specificity-determining event in T cell triggering. Consequently, introduction of T cell receptor genes into patient-derived T cells may be used as an alternative approach for the transfer of T cell immunity (Fig. **1**). In a clinical setting, this strategy would involve the introduction of TCR genes of interest into peripheral T cells of the patient followed by reinfusion of the genetically modified cells. The past 5 years has seen a steadily growing inter-

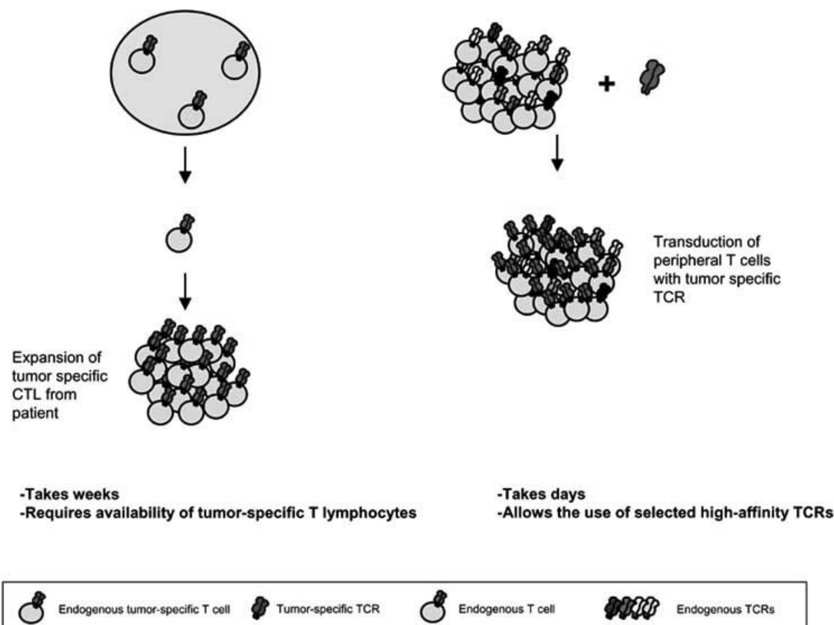

Fig. (I). Adoptive transfer of T cells and T cell receptors. Potential advantages of TCR gene transfer over conventional cellular transfer. Left: Immunotherapy *via* adoptive transfer of T cells. This approach has two major limitations. First, tumor specific T cells can only be isolated from a subset of patients and second, the *in vitro* expansion procedures that are used to produce large numbers of antigen-specific T cells are highly demanding. Right: TCR gene therapy. This approach has two potential advantages over conventional cellular transfer. First, collections of high-affinity tumor specific T cell receptors can be utilized for many patients, and second, TCR gene transfer circumvents the demanding process of *in vitro* generation of large numbers of specific immune cells.

est in this form of adoptive immunotherapy and at present, a number of groups are I). Defining strategies to acquire T cell receptor genes that are most suited for T cell receptor gene transfer and II). Testing the value of TCR gene therapy *in vitro* and in mouse models. In parallel, phase I clinical trials that test the feasibility of TCR gene therapy in patients with metastatic melanoma are ongoing and planned for the coming years. This parallel process of preclinical development and clinical testing will be useful to define under which conditions adoptive immunotherapy with T cell receptors can be of clinical value.

TCR GENE TRANSFER *IN VITRO* AND IN MICE

Several groups have created tumor-reactive or virus-reactive T cells by means of retrovirus-mediated TCR gene transfer, aiming to obtain proof of principle for TCR gene therapy in clinical trials [e.g. Calogero, *et al.,* 2000; Clay *et al.,* 1999; Cooper *et al.,* 2000; Fujio *et al.,* 2000; Heemskerk *et al.,* 2001; Orentas *et al.,* 2001]. When tested in *in vitro* assays, such TCR-modified cells behave as was both anticipated and hoped for, as revealed by cytokine secretion, proliferation and cytolysis upon encounter of antigen-laden cells. While transfer of TCR genes has now in many studies been shown to suffice to generate T cells with the desired antigen specificity, it is worth noting that the level of cell surface expression of the introduced T cell receptor is often low as compared to the endogenous T cell receptor. This low expression of the introduced T cell receptor may well reflect a lower activity of the promoter that drives transgene expression as compared to the endogenous TCR alpha and beta promoters. In addition, the introduced TCR chains will form heterodimeric complexes with endogenous TCR chains (creating so-called mixed dimers), thereby reducing the amount of the desired TCR alpha-beta dimer. For unexplained reasons, the low level expression of TCR genes upon TCR transfer is more prominent for human than for mouse TCRs, even when expressed in human cells and using the same promoter (Jorritsma *et al.,* unpublished observations), perhaps providing an experimental system to better define the underlying mechanism. Because the level of TCR expression has at least some effect on the antigen concentration required for T cell triggering [Labrecque *et al.,* 2001], ensuring a high level of TCR expression does remain an important issue.

In *in vitro* experiments, high TCR expression may be achieved by using high-titered retroviral stocks, resulting in multiple integrations per cell. However, in view of the increased risk of cellular transformation through insertional mutagenesis with increasing numbers of retroviral genomes per cell [Fehse *et al.,* 2004], this strategy for achieving high level transgene expression is clearly less attractive in the clinic, and the development of other strategies to enhance TCR transgene expression will be important (see below).

The first published *in vivo* data documenting T cell responses generated by TCR gene transfer made use of a TCR specific for an epitope derived from the influenza A nucleoprotein (NP) [Kessels *et al.,* 2001]. Results from these *in vivo* experiments demonstrated several points: After transfer of the TCR-modified T cells into wild-type mice, such “redirected T cells” can expand dramatically (i.e. 3-logs or more) upon *in vivo* antigen encounter (in casu infection with influenza A virus) and are able to home to effector sites in syngenic mice. Furthermore, in immunodeficient (RAG-1$^{-/-}$) mice, infusion of the TCR-modified T cells could promote regression of tumors that expressed the influenza A NP antigen.

The latter data suggest that TCR transfer can conceivably be used to restore virus-specific T cell immunity in immunodeficient individuals, analogous to the infusion of EBV-specific T cells in transplant recipients [Rooney *et al.,* 1995; Heslop *et al.,* 1997]. However, the applicability of TCR gene therapy would clearly be substantially greater when this approach can also be utilized to generate T cell responses against defined, tumor-associated self-antigens. To assess the feasibility of such targeting of self-antigens, we have recently evaluated whether TCR transfer can be used to induce immune responses against a defined self-antigen, to which the endogenous T cell repertoire is tolerant. Mice that transgenically express ovalbumin in Beta cells of the pancreas (RIP-OVA^{hi} mice) [Kurts *et al.,* 1998], are tolerant towards this tissue-specific neo-antigen and vaccination with ovalbumin-containing viral vaccines does not lead to Beta cell destruction (de Witte *et al.,* unpublished). However, when these mice receive small numbers of T cells modified with the OT-I TCR that recognizes an Ova-derived T cell epitope, the mice develop acute diabetes within two weeks following transfer. Furthermore, the onset of diabetes is preceded by a marked expansion of the OT-I-modified T cells in peripheral blood, and infiltration of these redirected T cells in the islets of Langerhans is apparent. These data demonstrate that I). TCR-modified cells can function *in vivo*, even in settings where the endogenous T cell repertoire is absent and II). Such cells can be used to target a defined self-tissue, in this case pancreatic Beta cells. While it should be apparent that there is little demand for improved strategies for inducing type I diabetes, these experiments provide proof of principle for the targeting of defined tumor-associated antigens through TCR gene therapy. Further studies should reveal the anti-tumor effects of such a strategy, preferably in spontaneous tumor models. In addition, such tumor models may be used to determine the effect of concurrent vaccination or adjuvant treatments such as blockade of regulatory T cells [Sutmuller *et al.,* 2001] or TGF-beta signaling [Gorelik *et al.,* 2001], administration of anti-CTLA-4 antibody [Egen *et al.,* 2002], or host conditioning [Goldrath *et al.,* 2000].

ACQUIRING HIGH AVIDITY TUMOR-SPECIFIC T CELL RECEPTORS.

The shared tumor-associated antigens that are suitable for tumor immunotherapy can be divided into three categories. I). Tumor-associated viral antigens, such as antigens derived from human papilloma virus (HPV) and Epstein-Barr virus (EBV). II). Minor histocompatibility antigens that are expressed in tumor cells, such as the HA-1 and HA-2 antigens that are expressed in many hematological malignancies. III). Tumor-associated self-antigens, such as the melanocyte differentiation antigens, the C/T antigens and antigens such MDM2 and p53 [Kawakami *et al.,* 1996]. It is relatively straightforward to isolate T cells expressing high affinity TCRs for the first category of viral antigens, as the relevant T cell repertoire has not been affected by tolerance. Likewise, the existence of individuals for which a given minor

histocompatibility antigen is non-self formed the basis for the discovery of these antigens, and such individuals are a reliable source of high avidity minor antigen-specific T cells. The isolation of high avidity T cells that are specific for non-polymorphic self-antigens is however less straightforward, because of self-tolerance. In view of the clear value of collections of T cell receptors that recognize such non-polymorphic self-antigens with high affinity, a host of strategies has been used or may be proposed to circumvent self-tolerance (Fig. **2**).

1. Ignore Tolerance

Tolerance to self-antigens is not absolute, as documented by the frequent occurrence of T cell –mediated autoimmune diseases. In fact, the development of skin depigmentation (vitiligo), due to autoimmune destruction of melanocytes has been taken as a paradigm for a self-specific immune response that could be beneficial when induced in melanoma patients. One relatively straightforward approach towards obtaining a collection of self antigen-specific T cell receptors is therefore to isolate T cells from individuals that can reasonably be expected to harbor a high avidity self antigen-specific T cell repertoire. Following this principle, several groups have isolated MDA-specific T cell receptors from melanoma patients, including patients displaying tumor regression following adoptive T cell therapy [Rosenberg *et al.,* 2004; Roszkowski *et al.,* 2005; Schaft *et al.,* 2003; Dudley *et al.,* 2001]. While such MDA-specific T cell receptors may well show tumor reactivity following TCR gene therapy, it is far from clear whether the affinity of these T cell receptors is optimal. Perhaps more importantly, while T cells specific for the melanocyte differentiation antigens are found with relative ease in melanoma patients, and in the case of MART-1 even in healthy individuals [Zippelius *et al.,* 2002], tolerance to other tumor-associated antigens appears to be more stringent, making isolation of T cell receptors recognizing such antigens a much more daunting task.

A possible improvement to this strategy of isolating the best of the remaining self antigen-specific T cell repertoire would be to make use of the fact that a number of autoimmune diseases has been described in recent years that result from mutations in genes that are essential for the induction of self tolerance. With respect to the possibility of isolating high avidity self-specific T cells, perhaps the most intriguing of these mutations is that of the AIRE gene. Mutations in AIRE lead to a multi-organ autoimmune syndrome (APS-I, APECED, [Villasenor *et al.,* 2005]) and studies in AIRE-deficient mice have provided evidence that the AIRE protein is required for the expression of at least some peripheral self-antigens within the thymic epithelium [Anderson *et al.,* 2002]. In the absence of AIRE, T cells that recognize such peripheral self-antigens do not undergo thymic deletion and can hence leave the thymus unharmed [Liston *et al.,* 2003]. Thus, peripheral blood lymphocytes of APS-I patients are likely to contain high avidity self-specific T cells that are absent in healthy individuals, and it may be useful to attempt to isolate desired high avidity self-specific T cells from these patients by *in vitro* stimulation.

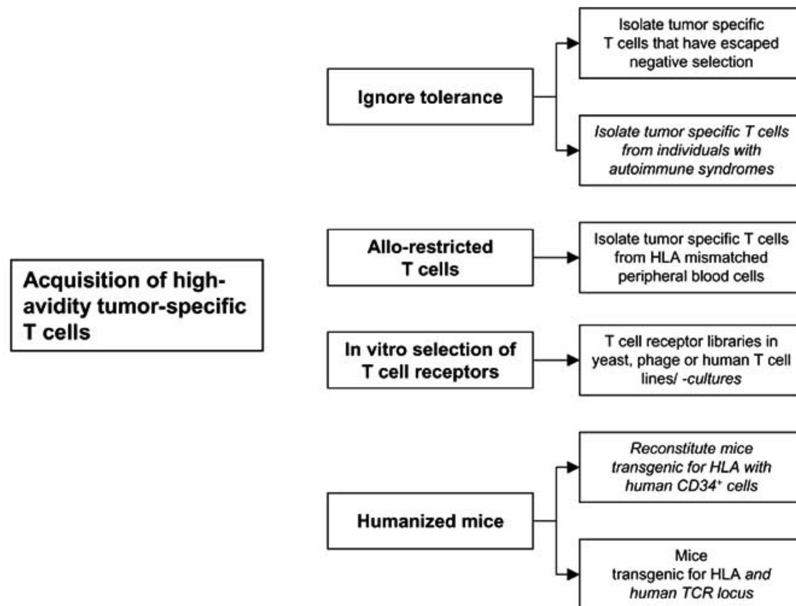

Fig. (2). Acquiring high avidity tumor-specific T cell receptors. To generate collections of T cell receptors that recognize self-antigens with high affinity, several strategies have been used (regular typing) or may be proposed (italic typing). References for the previously used approaches for obtaining tumor-specific T cell receptors are included within the section 'acquiring high avidity tumor-specific T cell receptors'.

2. Allo-Restricted T Cells

One of the earliest approaches to circumvent the negative effect of T cell tolerance on the T cell repertoire specific for tumor-associated self-antigens was developed by the group of Hans Stauss [Sadovnikova *et al.,* 1998]. In this approach, tumor-specific T cells are generated from peripheral blood lymphocytes of individuals that do not carry a given MHC allele (e.g. HLA-A2.1). Because self-tolerance is MHC-restricted (because different MHC alleles present different sets of peptides), these peripheral blood lymphocytes do include T cells with high avidity for self-antigens that can be presented by HLA-A2.1. Indeed, this approach, and new variations on this approach have been used successfully to isolate high avidity self-specific T cells for antigens such as WT-1 and Cyclin D1 [Sadovnikova *et al.,* 1998; Savage *et al.,* 2004]. There is now solid evidence that such allo-MHC-restricted T cells are diverse in their level of peptide specificity. Specifically, while certain allo-MHC-restricted T cells appear to primarily interact with the MHC alpha helices that line the peptide-binding groove, other allo-MHC-restricted T cells appear to display a level of peptide specificity that is similar to that of conventional antigen-specific T cells. Because a lack of cross-reactivity with other (non tumor-associated) self antigens is essential when using thus obtained TCRs in clinical trials, selection for and evaluation of a sufficient level of antigen specificity seems to be an aspect that requires particular attention for TCRs obtained by this approach [De Witte *et al.,* 2004].

3. *In Vitro* Selection of T Cell Receptors

Several approaches have been developed for the *in vitro* display of libraries of T cell receptors. TCRs have been expressed as fusion proteins both on yeast and on phage. Furthermore, full length TCRs have been expressed on a human T cell line by retroviral infection. All three of these TCR display systems have been used to select T cell receptors with either an altered fine specificity or an increased affinity [Holler *et al.,* 2000; Shusta *et al.,* 2000; Kessels *et al.,* 2000]. Furthermore, a possible addition to these *in vitro* strategies may come from the recent description of culture systems that allow the *in vitro* development of T lymphocytes from progenitor cells [La Motte-Mohs *et al.,* 2005]. At present, no data is available on the establishment of self-tolerance in such T cell populations and/ or possible ways to influence this process. However, it seems well possible that such cultures could contain T cells with desired specificities that would be purged during normal *in vivo* T cell development.

Analogous to the potential concern regarding the specificity of TCRs obtained by the allo-MHC approach (see above), it is presently highly unclear how *in vitro* selected TCRs can best be screened for unwanted cross-reactivity. T cell receptors are by nature highly cross-reactive and it has been estimated that a single TCR can recognize some 1 million structurally related peptide antigens [Mason, 1998]. Consequently, identification of TCRs solely based on their ability to recognize a tumor-associated self-antigen without screening against reactivity with other self-antigens may simply be insufficient. In this regard it is worth noting that the Kranz group has demonstrated that a high affinity TCR selected by their yeast display approach displayed cross-reactivity with other self-antigens [Holler *et al.,* 2003]. This study should thereby serve as an important warning for all attempts to create high affinity tumor-specific TCRs by *in vitro* selection: Selection of high affinity TCRs is clearly feasible, and selection for a sufficient level of specificity may be the more demanding step.

4. Humanized Mice

A final approach to obtain TCRs directed against self-antigens is to make use of mice transgenic for human HLA genes. The sequence of many of the self-antigens for which one might want to generate high avidity TCRs is only partially conserved between mice and men. For such epitopes, mice will be tolerant towards the murine sequence but not the human counterpart. For example, vaccination with a human MDM2 epitope has successfully been used to generate MDM2-specific T cells in HLA-A2.1 transgenic mice [Stanislawski *et al.,* 2001]. In these original experiments, HLA-transgenic mice were used in which the alpha3 domain of the MHC transgene was of murine origin, to allow interaction of the murine CD8 molecule with the transgene product. An interesting twist to this strategy is formed by the recent generation of human self antigen-specific T cells in mice transgenic for the wild type human HLA-A2.1 molecule [Kuball *et al.,* 2005]. Because the murine CD8 molecule does not efficiently recognize the human MHC alpha3 domain, HLA-restricted T cells that are induced in these mice are by necessity CD8-independent. In line with this, TCRs isolated from such cells can be used to generate MHC class I-restricted CD4+ helper T cells [Kuball *et al.,* 2005].

What are the potential problems of TCRs isolated from HLA-transgenic mice when used in the clinic? We consider a lack of sufficient specificity less likely to be a serious problem, because in this case (and contrary to the allo-MHC and *in vitro* selection approaches), the T cells have been selected against reactivity with hundreds or thousands of other HLA-peptide complexes during thymic development. A bigger concern is formed by the fact that TCRs obtained by this approach are of murine origin. Work in the antibody field has shown many years ago that infusion of unmodified murine antibodies leads to a rapid immune response against the infused antibodies and there is little reason to believe that T cells carrying murine TCRs will not undergo the same fate. Immunogenicity of antibodies can be lessened by generating chimeric molecules that, in the simplest design, contain constant domains of human origin and variable domains of murine origin [Bruggeman *et al.,* 1989]. The same approach is feasible for murine TCRs, but it is unclear whether the remaining murine segments are sufficiently small to avoid immune attack [Bruggeman *et al.,* 1989]. Analogous to the development of mouse strains that carry large parts of the human Ig loci [Bruggeman *et al.,* 1996], it seems reasonable to speculate that the development of mice transgenic for parts of the human TCR loci could form the ultimate solution to this problem. Secondly, recent studies have described the development of human T cells in immunodeficient mice [Traggiai *et al.,* 2004; Gimeno *et al.,* 2004] and such mice could potentially also form a source of human TCR sequences.

5. General Criteria for Clinically Useful T Cell Receptors

Irrespective of the strategy used for isolating T cell receptors specific for tumor-associated antigens, certain criteria should be met. As discussed above, isolated TCRs should be non-immunogenic and sufficiently specific, and depending on the technology used for TCR isolation, these issues are more or less likely to be a problem. In addition, the distribution of target antigen expression at a level detectable by the redirected T cells should be sufficiently restricted. As documented by the relatively mild side effects of infusion of T cells specific for melanocyte differentiation antigens [Dudley *et al.,* 2002b], antigen expression on non-malignant cells does not necessarily disqualify a potential target antigen. However, it is clear that the target antigen should not be ubiquitously expressed nor should it be expressed on vital organs. In this regard it seems particularly important to carefully consider the potential side effects of TCRs specific for antigens such as p53, MDM2, and telomerase. These antigens are overexpressed in tumor cells, but are also present in many normal tissues. These antigens are highly attractive targets because the function of these proteins is essential for tumor growth. However, it will be critical to ascertain that infused TCR-modified T cells are sufficiently selective for the transformed cells. The challenging task to manoeuvre between tumor-specificity and unwanted auto-immunity is well illustrated by the p53 oncogene product. While preferential killing of p53-overexpressing targets has been observed in a number of studies [Vierboom *et al.,* 1997], the molecular mechanism underlying this preferential recognition appears unclear. In general, oncoprotein over- expression can occur by two mechanisms, an increased rate of protein synthesis or a decreased rate of protein degradation. For the p53 protein, a decreased rate of protein degradation appears to be the dominant mechanism [Lohrum *et al.,* 1999]. Because MHC class I-restricted epitope presentation correlates with the rate of protein synthesis, and does not seem to be influenced by protein degradation [Yewdell *et al.,* 2003], a reduced rate of p53 degradation does not provide a satisfactory explanation for the observed tumor specificity. Potentially, the mechanism of p53 degradation differs between tumor cells and non-malignant cells [Offringa *et al.,* 2000], leading to a greater yield of class I epitopes in tumor cells, but pending a more detailed molecular explanation, it seems prudent to take extra care in using this antigen as a target in adoptive immunotherapy trials.

On a more general note, in the first TCR gene therapy trials it seems sensible to focus on target antigens for which it has been shown that substantial *in vivo* T cell responses are not associated with unmanageable side effects. Secondly, such trials should perhaps preferentially use naturally occurring T cell receptors rather than *in vitro*/ *in vivo* selected T cell receptors, awaiting reliable assessment of the risk of off-target recognition. Examples of candidate antigens that may be targets in such trials are the HA-1 and HA-2 minor histocompatibility antigens expressed exclusively in hematological cell types, and the melanocyte differentiation antigens, such as MART-1 and gp100.

DELIVERY OF TCR GENES TO PERIPHERAL T CELLS: VECTOR SYSTEMS

Having identified a high affinity T cell receptor that recognizes a suitable tumor-associated antigen, which strategy should be used to express this T cell receptor in T lymphocytes? Vector systems that are used for gene delivery into peripheral T cells should possess a few essential characteristics. They must be suitable for delivering genes to cells ex vivo, and the delivered DNA should become stably integrated in the host cell genome, or be maintained episomally to allow replication at an identical rate as the host cell. All TCR gene transfer studies published to date have made use of oncoretroviral vectors for transgene delivery. Analogous to other gene therapy approaches that utilize such vector systems, the genomic damage that arises as a consequence of retroviral integration may lead or contribute to malignant transformation. Lentiviral vectors could form a safer alternative, because the commonly used self-inactivating lentiviral vectors drive transgene expression from an internal promoter in stead of the viral LTR. Such internal promoters may be less likely to disturb expression of endogenous genes in the vicinity of integration sites, although experimental data on this issue are still scant. Secondly, lentiviral integration appears to be less biased towards transcription start sites as compared to oncoretroviral vectors [Mitchell *et al.,* 2004]. Nevertheless, lentiviral vectors do also preferentially integrate in transcription units and gene-dense regions of the genome, suggesting that deregulation of local gene activity may occur as well, and that the increase in safety is likely to be far from absolute.

The adverse effects of the genomic damage caused by current integrating vector systems has become a focus of attention following the occurrence of therapy-related leukemias in a gene therapy trial for patients with severe combined immunodeficiency-X1 (SCID-X1). To value the consequences of these adverse events for TCR gene therapy approaches it is important to realize that a number of specific aspects of the SCID-X1 trial are likely to have formed contributing factors. SCID-X1 is a genetic disorder that is caused by mutation of the gene that encodes the common γ-chain (cγ) cytokine-receptor subunit. cγ-deficiency leads to a block in T cell and NK cell differentiation and the resulting immunodeficiency is fatal if untreated. In this clinical trial [Cavazzana-Calvo *et al.,* 2000], haematopoietic stem cells (HSC) were transduced with a retrovirus that encoded the cγ gene. As a result, both the number and activity of immune cells reached normal values within months of treatment, making this trial the first unambiguous success of gene therapy. However, three of twelve treated patients developed T cell leukemias within several years after treatment. Several factors are likely to have contributed to the frequent occurrence of T cell leukemias in this patient group. First, the massive proliferation of the HSC that led to reconstitution of normal immune function may have selected for cells with increased proliferative potential. Second, genes that are essential for the capacity to self renew are active in hematopoietic stem cells, and because oncoretroviral vectors preferentially integrate in active loci, the frequency of integration

events that resulted in or contributed to a transformed phenotype may have been high. Finally, recent data suggest that the T cell leukemias that occurred in two of the three X-SCID patients that developed T cell leukemias following therapy may in part be due to oncogenic activity of the introduced transgene [Hacein-Bey-Abina *et al.,* 2003; Dave *et al.,* 2004].

Because the proliferation of TCR-modified T cells will be substantially less than that of the hematopoietic stem cells in the SCID-X1 trial, and because the TCR transgene is not known to have any oncogenic characteristics, the risk of this type of adverse events upon introduction of TCR genes does not necessarily equal the risk observed in the SCID-X1 trial. Additionally, phase I clinical studies of TCR gene therapy will likely be restricted to patient groups that have failed standard treatment, making the occurrence of possible side-effects more acceptable. Nonetheless, an important area of research for the coming years will be to define strategies to achieve TCR transgene expression with a minimal number of integration sites.

Gene therapy trials to date have generally tried to maximize transgene expression by using high-titered retroviral stocks or by identifying retroviral envelopes/ cell growth conditions that lead to higher numbers of integration sites. As discussed above, high-level expression of TCR transgenes is generally more difficult to achieve as compared to other transgenes, and all preclinical evaluations of TCR gene therapy have used conditions in which the number of retroviral integrations per cell is likely to be high. However, in view of the growing concern regarding the genotoxic effects of retroviral integrations, efforts should be put into maximizing transgene expression per integration site instead of maximizing the number of integrations. A straightforward approach towards this goal would seem the identification of promoter and enhancer elements that lead to maximal transgene expression. However, maximizing promoter/ enhancer activity will likely also result in an increased effect on the expression of genes in the vicinity of the integration site. It has been argued that deregulation of neighboring genes through the action of retroviral promoter/ enhancer elements may be minimized through the use of chromatin insulator elements, but the value of this approach needs further evaluation [Anson, 2004]. A different approach towards the goal of maximizing transgene expression without increasing the number of integration sites would be to enhance transgene expression at the post-transcriptional level. Expression may for instance be maximized by promoting RNA stability, splicing and/ or transport. A recent example of an element that has been included in retroviral vectors with this purpose is the Woodchuck Hepatitis post-transcriptional regulatory element (WPRE) [Schambach *et al.,* 2000]. There are some indications that the original form of the WPRE may have oncogenic activity and a modified WPRE in which this potential oncogenic activity is ablated has been described [Kingsman *et al.,* 2005].

Transgene expression may also be enhanced by creating synthetic genes in which the codon usage has been altered to generate a maximal amount of transgene product, The genetic code is redundant in that many of the 20 amino acids are encoded by multiple codons. It has long been known that highly expressed mammalian genes share a similar codon usage, suggesting that codon usage can affect protein production. In line with this, there are many examples of non-mammalian genes of which the expression can be substantially increased when the codon usage is altered to resemble that of highly expressed mammalian genes [Haas *et al.,* 1996]. Furthermore, because mammalian genes that are expressed at lower levels have a codon usage that diverges from that of highly expressed mammalian genes, codon optimization can also affect the expression of such genes, although probably to a lesser extent [Haas *et al.,* 1996]. It is noted that other aspects, such as the presence of secondary structures within the codon region of genes can also affect gene expression and such factors may also be taken into account when designing synthetic genes for (TCR) gene transfer [Ross, 1995].

With the aim of generating high level TCR expression with a minimal number of integration sites, we have created fully synthetic TCR genes in which codon usage and RNA structure have been optimized. For the two TCRs tested to date, this optimization leads to an approximately 2 and 10 fold increase in expression of a MART-1- and GP100-specific TCR respectively (Jorritsma *et al.,* unpublished). Based on these data, we suggest that optimization of aspects such as codon usage is likely to be of benefit for TCR gene therapy trials in general.

DELIVERY OF TCR GENES TO PERIPHERAL T CELLS: RECIPIENT CELLS

The most straightforward approach to generate TCR-modified T cells is the genetic modification of unfractionated peripheral blood cells. In this approach non-T cells, $CD4^+$ and $CD8^+$ T cells (including both αβ-T cells and γδ-T cells) are all genetically modified. Moreover, the genetically modified T cells have a wide diversity of endogenous TCRs and consequently antigen specificities. With respect to the infusion of TCR-modified non-T cells, there is no evidence or even suggestion that such cells could contribute to tumor control, and in view of the correlation between the number of infused integration events and the risk of cellular transformation, co-transfer of TCR-modified non-T cells seems ill-advised. With respect to the infusion of both genetically modified $CD4^+$ and $CD8^+$ T cells, $CD4^+$ T cell help can promote both primary and memory $CD8^+$ T cell responses [Bevan, 2004] and co-transfer of TCR-modified $CD4^+$ T cells might therefore be of benefit. However, most of the T cell receptors that have been isolated for TCR gene transfer purposes to date are derived from $CD8^+$ T cells and require the presence of the CD8 co-receptor for efficient antigen recognition. For such receptors, co-infusion of genetically modified $CD4^+$ is unlikely to have any immunological effect and again, with the purpose of minimizing the number of infused integration events, should be avoided. Co-transfer of $CD4^+$ T cells modified with MHC class I-restricted TCRs might be considered in cases where the introduced TCR can function in a CD8-independent fashion [Kuball *et al.,* 2005], or when the CD8 co-receptor is also introduced. In support of the co-transfer of TCR-modified $CD4^+$ T cells, recent data demonstrate that $CD4^+$ T cells that are redirected towards MHC class I ligands can provide help to cytotoxic T cells, both *in vitro* and *in vivo* [Morris *et al.,* 2005].

As a final refinement it is worth considering the use of oligoclonal pathogen-specific T cells as recipient T cells, rather than polyclonal T cells with undefined specificities. In an elegant series of experiments, Heemskerk and colleagues have demonstrated that CMV-specific T cells can be reprogrammed (or perhaps more accurately "co-programmed") into tumor-reactive T cells by TCR gene transfer [Heemskerk *et al.,* 2004]. The use of such oligoclonal antigen-specific T cells as recipient cells has a number of potential advantages. First, because the number of different endogenous TCRs that is present within the gene-modified T cell population is small, the risk of autoimmune manifestations may be more limited [Schumacher, 2002]. Additionally, because the gene-modified T cells also (continue to) recognize CMV antigens (CMV and other herpesviridae cause persistent infections in man), the pool of TCR-modified cells may remain of a substantial size and in an activated state, due to the triggering of the endogenous TCR. A downside of this approach is formed by the fact that the use of antigen-specific T cells as recipient cells requires the purification of such cells from peripheral blood lymphocytes, and that an *in vitro* expansion of gene-modified cells may be required to generate substantial numbers. Whether the advantages of this approach outweigh these disadvantages should be determined in clinical trials.

The greatest departure from the genetic modification of unfractionated peripheral blood lymphocytes was described in a recent paper by Yang and Baltimore, in which antigen-specific T cells were generated by genetic modification and reinfusion of hematopoietic stem cells (HSC) [Yang *et al.,* 2005]. An advantage of this approach is that (at least in mice) there is a steady output of genetically modified T cells, and it may be possible to utilize this approach to enhance the graft-versus-Leukemia effect in allo-SCT settings. Disadvantages of this approach are that reconstitution of the peripheral blood T cell compartment in recipients of HSC is rather slow, thereby delaying the anti-tumor effect of the thus-generated tumor-specific T cells. Furthermore, this approach only appears feasible for TCRs that are not subject to negative selection during thymic development, whereas modification of peripheral blood lymphocytes also allows the introduction of T cell specificities that are normally absent. Finally, the occurrence of gene therapy-induced T cell leukemias following the genetic modification of HSC in the SCID-X1 trial may also make HSC a less attractive cell compartment for genetic manipulation.

CLINICAL IMPLEMENTATION OF TCR GENE THERAPY

What would the introduction of TCR gene transfer for clinical purposes imply? With a set of well-characterized tumor specific TCRs present, a patient would first be HLA-typed to establish whether this collection contains one or multiple TCRs that are restricted by one of the HLA alleles carried by the patient. Subsequently it is determined whether the selected TCRs do not display allo-reactivity with any of the other HLA alleles present in this recipient. Even though mouse experiments suggest that the risk of allo-reactivity of the transduced cells *in vivo* is limited, the possibility of allo-reactivity should -at least for now- also be assessed on a per patient basis. To this purpose, a small peripheral blood sample is taken of which T cells are transduced with the selected TCR(s). Following transduction, lack of reactivity against autologous non-transformed cells can be tested. This should offer a reasonable indication of the risk of side effects due to off-target recognition. However, it is noted that this *in vitro* analysis cannot exclude undesirable reactivity towards allogeneic MHC molecules complexed with tissue-specific peptides. Evaluation of toxicity following infusion of TCR-modified cells should therefore be used to compile listings of permissible HLA alleles for each T cell receptor, which may eventually allow reliable assessment of potential side-effects of TCR gene transfer without a requirement for *in vitro* testing on a per patient basis.

Leukapheresis material of the patient is subsequently obtained to introduce the selected TCR genes. To avoid introduction of retrovirally-modified non-T cells, total T cells or CD8+ T cells may be purified. Because the oncoretroviral vector systems that are currently used for TCR gene transfer require cell division for retroviral integration, *in vitro* activation of the isolated T cells is essential. This can be achieved either by TCR triggering alone, or preferably by combining this with engagement of co-stimulatory molecules such as CD28 [Kalamasz *et al.,* 2004]. After retroviral transduction, the T cells may be expanded *ex vivo*, or alternatively, the cells can be re-infused shortly after transduction. We favor the second possibility for two reasons. First of all, murine data indicate that TCR-transduced cells can expand dramatically *in vivo* and may therefore not necessitate extensive *in vitro* culturing to achieve the required number of antigen-specific T cells [Kessels *et al.,* 2001] Secondly, the *in vivo* survival of T cells that have been cultured for prolonged periods *in vitro* appears to be limited [Kolen *et al.,* 2002].

To allow *in vivo* proliferation and differentiation of the TCR-transduced T cells following infusion, lymphodepletion of the host prior to transfer is likely to be beneficial. This assumption is based on a number of studies, that have shown that T cells infused in lymphopenic mice or patients undergo a proliferative burst and acquire T cell effector functions [Dudley *et al.,* 2002b; Goldrath *et al.,* 2000]. The beneficial effect of chemotherapy-induced lymphodepletion may be due to three factors. First, this conditioning regimen can increase access of the infused cells to cytokines that promote cell survival/ expansion. Second, the chemotherapy that is used to achieve lymphodepletion may lead to increased presentation of tumor-derived antigens, by induction of tumor cell apoptosis. Third, lymphodepletion will lead to the removal of $CD4^+CD25^+$ regulatory T cells that have been shown to suppress antigen-specific T cell responses in a variety of systems [Klebanoff *et al.,* 2005]. Should the beneficial effect of lymphodepletion turn out to be mostly be due to the removal of $CD4^+CD25^+$ regulatory T cells, the use of conditioning regimens that selectively remove this cell subset may in fact be preferable over the currently used chemotherapy-induced lymphodepletion. In addition to the possible positive effect of lymphodepletion on the *in vivo* expansion/ survival of TCR-modified T cells, lymphodepletion will likely also limit the risk of immune rejection of the TCR-modified cells, which may be a concern in particular for TCRs of murine origin.

It seems well possible that *in vivo* activity of the TCR-modified cells can be further optimized by adjuvant treatment such as anti-CTLA4 treatment [Phan *et al.,* 2003; Sanderson *et al.,* 2005]. Before implementing such adjuvant treatments, which at least for anti-CTLA4 treatment is associated with substantial toxicity [Phan *et al.,* 2003; Sanderson *et al.,* 2005], the added value of such combined treatments should first be evaluated in preclinical research.

Can we expect a monoclonal T cell response, induced by transfer of a single TCR, to result in complete tumor regression? Perhaps not. Many of the antigens that are currently under consideration for TCR gene transfer trials are not essential for tumor growth and expression of these antigens is in fact sometimes heterogeneous. What we can expect from a successful phase I clinical trial is that the TCR-modified T cells exert a substantial selective pressure, resulting in a partial/ transient tumor regression, perhaps accompanied by a loss of expression of the targeted tumor antigen. If initial results do fit this scenario, the incentive for infusion of TCR-modified cells targeting multiple tumor-associated antigens will be substantial.

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